THE MURINE AMNIOTIC FLUID MACROPHAGE: UPREGULATION OF CLASSICAL AND ALTERNATIVE ACTIVATION MARKERS PRIOR TO LABOR AT TERM

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DEDICATION

To my husband, Dan.

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The beauty of science lies not *only* in discovery but in the invaluable interactions with those who give so generously of their time and expertise. The work presented here would not have been possible without the unwavering support of family, friends, colleagues and mentors. I am tremendously thankful to all those who have inspired and challenged me throughout this journey.

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When in silence I sit and think,
Of all the things that could have been,
I realize that there is none so great
An accomplishment than to have the will to
Strive in pursuit of a dream.

THE MURINE AMNIOTIC FLUID MACROPHAGE: UPREGULATION OF CLASSICAL AND ALTERNATIVE ACTIVATION MARKERS PRIOR TO LABOR AT TERM

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The initiation of labor at term and preterm is associated with an inflammatory response, with increased interleukins in amniotic fluid (AF) and infiltration of the myometrium by neutrophils and macrophages (Mϕ). Whereas, in preterm labor, intra-amniotic infection may provide the inflammatory stimulus for increased AF interleukins and inflammatory cell migration, the stimulus for these events at term has remained unclear. In studies using pregnant mice, we observed that the Mϕ that invade the maternal uterus near term originate from the fetus. Furthermore, we obtained compelling evidence that surfactant protein-A (SP-A), a developmentally regulated C-type lectin secreted by

the fetal lung into AF near term, activates AF Mϕ, which migrate into the pregnant uterus where their local release of interleukin-1 serves to activate nuclear factor κB (NF-κB) pathways. Activation of the NF-κB pathway results in increased expression of genes that promote uterine contractility and negatively impacts the capacity of progesterone receptors to maintain uterine quiescence, culminating in the onset of labor [1]. We propose that interactions of Mϕ surface receptors with SP-A, at term, or bacterial lipopolysaccharide at preterm, initiate changes in Mϕ phenotypic properties, resulting in the enhanced expression of genes that promote Mϕ migration to the uterus. The objectives of this study are: (1) to analyze the phenotypic changes of mouse AF Mϕ associated with the developmental induction of *SP-A* synthesis and secretion by the fetal lung into AF: (2) to determine if *SP-A*, *SP-D*, and *SP-A/D* double deficiencies delay labor at term and to (3) to analyze trafficking patterns of fetal Mϕ leading to the induction of labor. The findings presented herein suggest that late gestation AF Mϕ upregulate classical and alternative activation markers in tandem as term approaches. Moreover, their phenotypic profile implies that they may modulate both pro- and anti-inflammatory functions simultaneously near term. Trafficking studies using heterozygous fetal-derived amniotic fluid macrophages expressing EGFP under control of the Mϕ-specific CSF-1 receptor, demonstrate the absence of this subpopulation in the maternal uterus. Parturition studies in surfactant protein A and -D deficient mice reveal that deficiency in *SP-A* and –D does not affect the timing of labor. Intriguingly, Toll-like receptor 2 deficient mice demonstrate a delay in the time to parturition pointing to its potential role in mediating a signal for labor at term.

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LIST OF ABBREVIATIONS

α– anti-

Ab – antibody

ACAID – anterior chamber-associated immune deviation

AF– amniotic fluid

APC – allophycocyan (fluorescent tag)

APC – antigen presenting cell

Arg 1 – arginase 1

AMϕ – alveolar macrophages

AF Mϕ – amniotic fluid macrophages

BSA – bovine serum albumin

CCL – chemokine (C-C motif) ligand

CCR– chemokine (C-C motif) receptor

$CD4^+$ – T cells

$CD8^+$ – cytotoxic T cells

CD45 – pan leukocyte antigen

CD54 – ICAM-1

CD62L – L-selectin

cDNA – complementary DNA

COX – cyclooxygenase

$cPLA_2$ – cytosolic phopholipase A_2

CRH –corticotrophin-releasing hormone

Crry – complement receptor-related gene Y

cRNA – complimentary RNA

CSF-1 – colony stimulating factor-1

CTL – cytotoxic T cell

CXCL – chemokine (C-X-C motif) ligand

CXCR – chemokine (C-X-C motif) receptor

CX_3CR1 –fractalkine receptor

DC – dendritic cell

DNA-deoxyribonucleic acid

ds – double-stranded

EGFP – enhanced green fluorescent protein

FACS – fluorescence activated cell sorting

FasL – Fas ligand

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

FIZZ1 – found in inflammatory zone 1

GM-CSF – granulocyte macrophage colony stimulating factor

GFP – green fluorescent protein

h – human

HLA-G – human leukocyte antigen G

HLA-E – human leukocyte antigen E

HPA axis – hypothalamic-pituitary-adrenal axis

ICAM-1 – intracellular adhesion molecule 1

IDO – indoleamine 2,3-dioxygenase

IFN – interferon

Ig – immunoglobulin

LH – luteinizing hormone

IKK– IκB kinase

IL – interleukin

IL-1RII – IL-1 receptor type II, IL-1 receptor decoy

LPS – lipopolysaccharide

LT A– lymphotoxin alpha

M1 – classical activation state

M2 – alternative activation state

MAC-1 –macrophage-1 antigen

MD-2 –myeloid differentiation 2

M-CSF–colony stimulating factor 1

Mϕ – macrophage

Mo – monocyte

MGL1 – macrophage galactose N-acetyl-galatosamine (GalNac), CD301a

MGL2 – macrophage galactose N-acetyl-galatosamine (GalNac), CD301b

MHC – major histocompatibility complex

MMP – matrix metalloproteinase

MR – mannose receptor

Mrc 2– mannose receptor, C type 2

MyD88 – myeloid differentiation factor 88

NF-κB – nuclear factor κB

NO – nitric oxide

NOD – nucleotide oligomerization domain

NOS2– inducer of nitric oxide synthase

iNOS –inducible nitric oxide synthase

P_4 – progesterone

PAMP –pathogen-associated molecular patterns

PBMC – peripheral blood mononuclear cell

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PE – phycoerythrin

PFA – paraformaldehyde

PG – prostaglandin

PRR – pattern recognition receptor

PRROM – preterm rupture of membranes

PR – progesterone receptor

PTB – preterm birth

PTL – preterm labor

qRT-PCR –quantitative reverse transcription polymerase chain reaction

R – receptor

RT – room temperature

RDS – respiratory distress syndrome

SIRP-α – signal regulatory protein alpha

SP – surfactant protein

Sftpa – surfactant protein A gene

Sftpb – surfactant protein B gene

Sftpc – surfactant protein C gene

Sftpd – surfactant protein D gene

SP-A – surfactant protein A

SP-B – surfactant protein B

SP-C – surfactant protein C

SP-D – surfactant protein D

TAM –tumor-associated macrophages

Tg – transgenic

TGF-β – transforming growth factor-β

Th –T helper cell

Th1 –pro-inflammatory immune response

Th2 –anti-inflammatory immune response

TLR – toll-like receptor

TLR2 – toll-like receptor 2

TLR4 – toll-like receptor 4

TNF – tumor necrosis factor

TRAIL – tumor necrosis factor-related apoptosis-inducing ligand

YS – yolk-sac

Ym1 – chitinase 3-like 3

Ym2 – chitinase 3-like 4

20α-HSD –20 alpha hydroxysteroid dehydrogenase

CHAPTER I: INTRODUCTION

Literature Review

Overview

Childbirth (parturition) is a critical biological process, yet the fundamental molecular pathways that trigger this event remain unknown. Decades of research have made it clear that the mechanisms for signaling labor at term are complex and multifactorial. Escalating preterm birth (PTB) rates mandate a more thorough understanding of these signals in order to better understand the underlying pathophysiology of PTB.

It is reasonable to posit, that a successful strategy for ensuring reproductive efficacy would integrate both a fetal signal(s) indicative of fetal maturity, as well as a maternal signal(s) representative of uterine and cervical receptivity to uterotonic (contractile stimuli) factors. Convergence of such a bimodal signaling cascade is a reasonable supposition as it would ensure successful birth and species propagation. Indeed, the diversity of this signaling cascade has been postulated to be a result of maternal-paternal genetic conflict [2] for resources. Nonetheless, survival of the semi-allogenic allograft requires additional precision in balancing maternal immunity for the protection of the mother from pathogen insult as well as protection of the fetus by suppression of overt maternal inflammatory responses. Hence, the mechanisms of parturition have proven to be challenging and are further complicated by physiological

intra-species differences among mammals; imparting an additional level of complexity in deciphering the fundamental cascades required for signaling labor at term.

Many studies have established that both term and preterm labor are associated with an inflammatory response [3]. This is exemplified by findings that both term and preterm labor are associated with increased levels of interleukins and inflammatory mediators (*e.g.* IL-1β, IL-6, TNF-α) in amniotic fluid and with infiltration of the myometrium and cervix by neutrophils and macrophages [3-4]. These inflammatory signals and mechanisms have been demonstrated to play a part in the labor-associated cascades that result in upregulation of pro-inflammatory cytokines that exert their effects on the synthesis of prostaglandins and tissue proteases [5]. It is important to note that these cascades may also serve to disrupt the delicate balance of fetal-maternal tolerance required for the maintenance of pregnancy [6-7].

Despite collective efforts to define the mechanisms responsible for term and preterm birth, we are no closer to predicting or preventing preterm labor (PTL). This is due, in part, to our incomplete understanding of the molecular pathways that mediate the onset of normal labor at term. Insights into the mechanisms involved in the timing of normal parturition at term will shed light on the intricate roles and cellular and molecular cross-talk between endocrine and immunological systems. More importantly, it will provide improved therapeutic strategies for the prevention and intervention of preterm birth (PTB).

The Incidence of Prematurity in the United States

Preterm birth, defined as less than 37 weeks of gestation, is the leading cause of neonatal morbidity and accounts 75% of neonatal mortality in developed countries [8-10]. In the United States, the incidence of preterm birth (PTB) has risen to 12-13% within the last two decades [8, 10-12]. Despite our advancing knowledge and awareness of predisposing risk factors, accurate prediction and prevention of PTB remains elusive [10, 13-17]. Disparities in the incidence of PTB among black African-Americans (18.3%) are significantly increased compared to white Americans (11.4%). Moreover, African-Americans are approximately four times more likely to have very early preterm birth (< 32 wks) compared to women of other racial or ethnic groups [8, 10, 18]. This is believed to contribute directly to the high rate of preterm birth in the USA compared to other countries.

The severity and incidence of health risks associated with PTB are inversely correlated with the gestational age and the time of birth [19]. During the last trimester of pregnancy, vital organs undergo maturation in preparation of life outside the womb. Disruption during this critical period can result in long-term sequelae in surviving infants due to multiple complications in organs, including the eyes, lungs, gastrointestinal track and brain leading to significant morbidity [19-23].

In addition, of the 500,000 babies born prematurely annually [8], 25,000 will develop respiratory distress syndrome (RDS) due to the inability of the premature neonatal lungs to produce adequate amounts of pulmonary surfactant, a developmentally regulated, glycerophospholipid-rich lipoprotein necessary for alveolar expansion and

required for normal air breathing [24]. In the absence of surfactant increased surface tension results in collapse of lung alveoli and the inability to breath. Treatment of preterm infants with exogenous surfactant improves fetal respiratory function but does little to mitigate long term complications associated with PTB. Astoundingly, the costs associated with PTB and its complications were ~30 billion dollars in 2005. This estimate accounts for the cost of intervention at the time of birth, long term medical care, and lost household income as well as market productivity [25]. In short, prematurity is an escalating medical crisis requiring immediate attention.

Idiopathic Preterm Labor is the Predominant Cause of Preterm Birth

Studies point to genetic, social, and environmental influences as key contributors to the incidence of PTB [26]. It is estimated that 20-30% of PTB cases are due to underlying infection, 25-30% are a result of premature rupture of membranes (PPROM) and 40-45% are due to spontaneous (idiopathic or of unknown cause) labor. Thus, idiopathic preterm delivery accounts for the largest number of preterm births [3, 8, 10, 27]. It has been proposed that within this cohort, there is underlying ‘silent’ infection [28] but this has not been unequivocally demonstrated. Irrespective of the etiologies of preterm delivery, it is postulated that the mechanisms involved in initiating normal labor at term and those that incite PTB during infection are similar.

Although care and survival of premature infants has improved significantly, prevention will require development of tocolytic agents designed to inhibit the earliest

stages of the labor initiation cascade. Further research into the mechanisms governing this process will provide insight into the pathogenesis of preterm labor (PTL).

Mechanisms of labor

The mammalian embryo cannot survive outside the womb until a number of organ systems are highly developed. Thus, it is imperative that fetal development and the timing of labor be precisely coordinated to ensure species propagation. To date, many studies have focused on the physiological processes involved in uterine smooth muscle (myometrial) activation and cervical softening, specifically on those mechanisms that control uterine contractility and cervical ripening.

It is well established that during mammalian parturition, the uterus and cervix work in unison to facilitate birth. During contraction of the uterus, the upper dome-shaped region, known as the fundus, is believed to generate the majority of the contractile force while the lower uterine segment, or corpus, relaxes. This process transmits gradient force to the cervix by producing oscillating rhythmic contractions of increasing amplitude and frequency which facilitate fetal and placental expulsion. Once labor is initiated, the process is irreversible. Thus, discordance in the timing of the uterine-cervical compartment activation and fetal viability often result in adverse consequences to the fetus and its survival.

Accumulating evidence now demonstrates that uterine smooth muscle (myometrium) develops a contractile phenotype as pregnancy approaches term and that the cervix undergoes remodeling, softening and dilatation (ripening) in preparation for

labor [29-31]. In the uterus, transformation from quiescence to an activated state includes induction of several families of proteins, including a cassette of contraction-associated protein's (CAPs) that convert the quiescent myometrium to a contractile state. CAPs include gap junction proteins (connexins), oxytocin, ion channels and prostanoid receptors as well as enzymes involved in prostaglandin synthesis [32] Although changes in the uterine and cervical tissues are distinct, it is clear that hormonal signals initiate these changes [29-30]. It is probable that these signals are initiated at various stages of the parturition process and become integrated in the myometrium where they culminate in synchronized contraction of the uterus.

Although we have gleaned much information through extensive study, several major questions remain unanswered: *What initiates this cascade? What are the biochemical processes that precede the earliest changes in the uterus and cervix in preparation for labor at term? And, how are these mechanisms triggered in cases of PTB?* So far, several theories have provided partial answers.

Corticotrophin-Releasing Hormone and the Timing of Birth: The Hypothalamic-Pituitary-Adrenal Axis (HPA)

Early studies in sheep provided insight into the critical role of steroid hormones in labor. In these studies, researchers revealed that the maturating fetal hypothalamus releases corticotrophin-releasing hormone (CRH). CRH production stimulates production and release of adrenocorticotropic hormone (ACTH) from the fetal pituitary gland which, in turn, induces cortisol secretion by the fetal adrenal gland. The increase in cortisol

enhances the expression of cyclooxygenase 2 (COX-2) which promotes increased production of prostaglandins (PGs) [33-34]. Placental PGs stimulate expression of 17α-hydroxylase/17,20 lyase, which converts pregnenolone to C_{19}-steroids in lieu of progesterone (P_4). The C_{19}-steroids, in turn, are metabolized to estrogens by placental aromatase P450 [35]. The increased estrogens are suggested to augment uterine contractility by antagonizing progesterone receptor (PR) function to maintain uterine quiescence [36]. The decline in placental P_4 production also contributes to the initiation of parturition. While this decline in maternal systemic P_4 levels occurs in sheep, as well as the majority of mammalian species prior to the initiation labor at term [37-40], in humans the decline in circulating P_4 does not occur.

Corticotrophin-Releasing Hormone Secreted by the Placenta Near Term Provides a Fetal Signal for the Initiation of Labor in Humans

In humans, and other primates, the timing of birth is associated with increased expression CRH [41]. Unlike sheep, however, in primates, the placenta provides the source for the increase in CRH production [42-45]. Increased placental CRH stimulates production of ACTH by the fetal-pituitary gland which, in turn, enhances fetal adrenal production of cortisol and dehydroepiandrosterone sulfate (DHEAS) [46]. DHEAS, a substrate for estrogen synthesis, is metabolized and aromatized to estrogens in the placenta [44], which are hypothesized to antagonize P_4 action.

Collectively, these hormonal changes are believed to promote increased intrauterine production of PGs, cervical ripening and uterine contractions leading to labor

at term [23]. Several independent studies support the association between elevated levels of CRH in maternal plasma and the timing of human birth [47-49]. But, although it has been suggested that placental production of CRH may provide a fetal signal for the initiation of labor near term [50-51], CRH has not been found to be elevated in all cases of PTB [34].

The Timing of Birth is Linked to Maturation of the Fetal Lungs

Intriguingly, CRH has been found to stimulate fetal lung maturation. As term approaches, increased cortisol concentrations precede the developmental induction of fetal pulmonary surfactant synthesis. Muglia and colleagues observed that CRH deficiency in mice results in delayed fetal lung maturation and in developmental induction of surfactant protein-A, SP-A, expression [52]. Notably, pro-inflammatory actions of SP-A and their associated phospholipids have been found to stimulate myometrial contractility through increased production of PGs by fetal membranes and the myometrium.Taken together, CRH may potentially mediate the initiation of labor indirectly by enhancing fetal-derived ACTH and adrenal cortisol production and/or by directly stimulating fetal lung maturation and the production of surfactant. In this regard, we and others have postulated that the signal for parturition may arise from the fetus [53-55]. Studies by Condon *et al.* recently suggested that fetal-derived SP-A, a component of pulmonary surfactant, serves to initiate labor at term [1]. (These findings are discussed further in an ensuing section.)

Progesterone Maintains Uterine Quiescence during Pregnancy

It is well established that P_4 functions to maintain uterine quiescence during pregnancy via suppression of myometrial CAP genes, which serve to promote uterine contractility. The finding that maternal P_4 levels in rodents decline precipitously near term contributed to the notion that labor is associated with P_4 withdrawal [56]. Moreover, in a large proportion of mammals the decline of maternal systemic P_4 levels precedes initiation of labor at term. However, in higher primates, such as great apes and humans, P_4 concentrations fail to decline at the end of pregnancy [36, 57], and instead, increase well into labor and decline only after delivery. This has led to the concept that in humans, the biochemical and molecular events leading to labor are associated with a functional withdrawal of PR function. Several studies support this concept. First, administration of anti-progestins to pregnant women were found to cause enhanced sensitivity of the myometrium to oxytocin and prostaglandin $F_2\alpha$ ($PGF_{2\alpha}$), thereby inducing abortion [30]. Next, treatment of pregnant women with anti-progestins results in the induction labor [30, 58], while removal of the corpus luteum in early pregnancy causes fetal loss [59]. Collectively, these data suggest that parturition in humans is induced, in part, by coordinated P_4 withdrawal and estrogen activation of the myometrial tissue.

In the majority of mammalian species, transition of the pregnant uterus at term to an activated state of responsiveness to uteronic factors (*e.g.* oxytocin, prostaglandins) is associated with a decline in maternal P_4 levels [56], however, as mentioned, a decline in circulating P_4 levels is not observed in humans. In light of this, the concept of functional P_4 withdrawal (inactivation) has been proposed. It should be noted that even in rodents,

levels of circulating P_4 at term do not decline below the K_d for binding to PR. Moreover, mice with targeted deletion of the 5α-reductase type 1 gene fail to undergo cervical ripening at term which suggests that cervical metabolism of P_4 is indeed required for P_4 withdrawal and spontaneous labor [60]. Thus, an antagonism of (PR) function may mediate increased uterine contractility in humans, as well as other species.

PR is a ligand-activated transcription factor belonging to the steroid receptor superfamily. Human PRs exist in three isoforms that are transcribed from a single gene; full-length PR-B (116 kDa) and two smaller N-terminally truncated forms, PR-A (94 kDa) and PR-C (60 kDa) [61-62]. In the pregnant myometrium PR levels do not decline prior to the onset of labor. Importantly, a critical role for the functional inactivation of PR in the initiation of labor in women was suggested in experiments in which PR antagonist RU486 enhanced cervical ripening and initiated labor [63]. In support of this, in previous studies in our lab, we observed that parturition in humans and mice was associated with a pronounced decline in uterine levels of a number of coactivators that interact with PR, and with a significant decrease in histone acetylation [64]. In total, these studies suggested that a decline in PR coactivator expression and in histone acetylation in the uterus near term provides a potential mechanism for the impairment of PR function. This, results in downregulation of genes required for the maintenance of uterine quiescence and to increased sensitivity of the uterus to contractile stimuli. To date, however, P4/PR target genes that prevent uterine contractility have not been identified (Figure 1).

Importantly, P_4/PR mediated uterine quiescence appears to be indirect; acting through inhibition of inflammatory pathways and expression of contractile genes within

the uterus and cervix. Therefore, it is postulated that spontaneous labor is initiated by a series of molecular events that inhibit PR function [65].

Term and Preterm Labor are Associated with an Inflammatory Response

Ample evidence now supports the concept that both term and preterm labor are associated with an inflammatory response. Specifically, labor is associated with increased pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) in AF [66], and with infiltration of maternal myometrium, cervix, and fetal membranes by neutrophils and macrophages [1, 4, 67]. This is supported by the finding that systemic administration of IL-1 induces preterm labor in mice [68]. A number of studies have also demonstrated that P_4 has immune suppressive properties [69-71] and can suppress LPS-stimulated production of pro-inflammatory cytokines TNF-α and IL-1β [70]. Intriguingly, during the estrous cycle in rodents, migration of Mϕ and neutrophils into the uterus is stimulated by estrogen and inhibited by P_4 [72-74] . PR-dependence in immune cell migration is suggested by the observation that in PR knockout mice (*PRKO* mice) the antagonistic effect between estrogen and P_4 fails to occur and results in a massive uterine inflammatory response upon estrogen or estrogen plus P_4 treatment [74-75].

While infection associated with chorioamnionitis has been suggested to serve as a stimulus for enhanced leukocyte activation and pro-inflammatory cytokine production leading to preterm labor [76], the signal(s) for the increased inflammatory response associated with labor at term is less clear.

Surfactant Protein Secreted by the Maturing Mouse Fetal Lung Acts as a Hormone that Signals the Initiation of Parturition

Studies by Condon *et al.* suggested the potential role of fetal-derived pulmonary surfactant in signaling the initiation of labor at term. In these studies, SP-A, a C-type lectin that is developmentally regulated in fetal lung, was found to be secreted at high concentrations into amniotic fluid (AF) near term, beginning at 17 days post-coitum (dpc), and reaching maximal levels at term (19 dpc). The gestational increase of SP-A in the AF was associated with increased expression of IL-1β in AF Mϕ and activation of uterine NF-κB in the gravid uterus. Additionally, AF Mϕ cultured with SP-A were found to upregulate expression of IL-1β and NF-κB. Moreover, intra-amniotic injection of SP-A caused preterm delivery of fetuses within 6-24 h. By contrast, injection of SP-A antibody or NF-κB inhibitor into AF delayed labor by more than 24 h.

Using homozygous *Rosa 26 Lac-Z* transgenic males, which express the gene for β-galactosidase in all tissues, fetal AF Mϕ were found to migrate to the pregnant uterus in parallel with the gestational increase in SP-A in AF. In these studies, uteri of WT mothers carrying heterozygous *Rosa 26 Lac-Z* embryos contained β-galactosidase positive cells by 17 dpc. Immunohistochemical analysis of serial sections from these mice also demonstrated that some of the *β*-gal positive cells also expressed F4/80, a Mϕ specific marker. Using dual immunofluorescence, *β*-gal was found to co-localize with F4/80 suggesting that a proportion of the Mϕ that invade the uterus near term are of fetal origin.

Given these findings, it was proposed that augmented production of SP-A by the fetal lung near term activates fetal Mϕ and triggers their migration to the maternal uterus where local production of cytokines, such as IL-1β, results in activation of nuclear factor κB (NF-κB) pathways leading to labor through local alteration of inflammatory gene transcription and/or by blocking PR function, culminating in labor at term [1] (Figure 1).

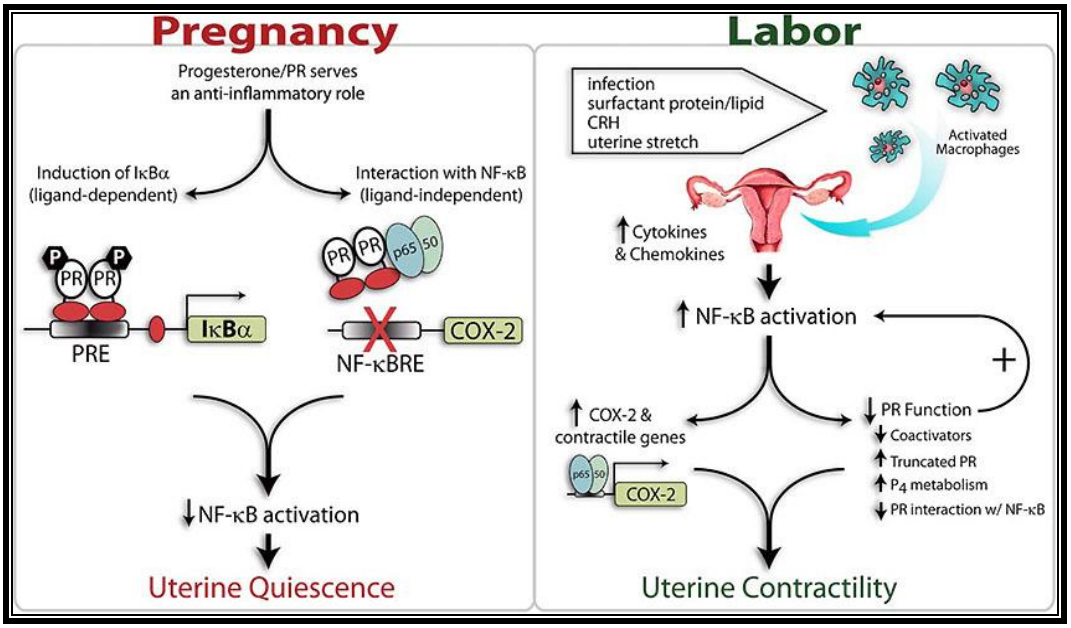


Figure 1. Mechanisms for progesterone/PR regulation of uterine quiescence during pregnancy and for induction of uterine contractility in preterm and term labor. During most of pregnancy, the uterus is maintained in a quiescent state by the PR, which acts in a ligand-dependent manner to up-regulate expression of the NF-κB inhibitor IκBα in myometrial cells. Alternatively, PR can act in a ligand-independent manner (likely via direct protein-protein interaction with NF-κB p65) to block NF-κB activation, DNA

binding, and transactivation of contractile genes within the uterus. Labor can be initiated preterm as a result of bacterial infection, resulting in enhanced migration of macrophages to the maternal uterus with release of cytokines/chemokines and activation of NF-κB. However, at term, enhanced macrophage activation and migration and increased uterine NF-κB activity are likely induced by signals produced by the maturing fetus. These include secretion of surfactant proteins and lipids by the fetal lung into amniotic fluid, augmented production of CRH by the placenta, and enhanced uterine stretch caused by the growing conceptus. Within the uterus, the activated NF-κB directly acts to increase expression of contractile genes and causes an impairment of PR function by affecting: 1) down-regulation of PR coactivators; 2) increased expression of inhibitory PR isoforms; 3) increased metabolism of progesterone to inactivate products, and; 4) direct inhibitory interaction with PR. These concerted events culminate in a further increase in NF-κB activation and expression of contractile genes, leading to labor. (Reprinted with permission. *MiniReview: fetal-maternal hormonal signaling in pregnancy and labor*. Mendelson, C.R. *Mol Endocrinol.* 23(7):947-954 copyright 2009.)

Pulmonary surfactant

Pulmonary surfactant, a glycerophospholipid-rich lipoprotein, contains a small amount of cholesterol and four associated surfactant proteins (SP) whose expression is developmentally regulated in the fetal lung, detectable after 75% of gestation is complete in humans (3rd trimester) [77] and mice (~17 dpc) [1]. Surfactant is synthesized in alveolar type II cells and stored in inclusions known as lamellar bodies. Lamellar bodies are secreted into the alveolar lumens where they are transformed into tubular myelin and mediate the reduction of surface tension at the air-liquid interface of the alveoli, thereby preventing lung collapse at end of expiration [78]. Deficiency in the molecular machinery required for surfactant synthesis associated with premature birth frequently leads to respiratory distress syndrome (RDS) that is currently treated with exogenous commercially available surfactant replacement [79].

The four lung-specific surfactant proteins, which include, SP-A, SP-B, SP-C and SP-D [80], belong to three different superfamilies [81]. SP-A and SP-D are hydrophilic proteins that map to the long arm of chromosome 10 in humans, syntenic with chromosome 14 in the mouse. In humans, SP-A protein is encoded by two genes, *SFTPA1 and SFTPA2*, while SP-D is encoded by *SFTPD* [82]. They are members of the collectin family of proteins [83] and have been found to play a role in innate immunity of the lung. SP-B, encoded by *SFTPB* belongs to a diverse group of lipid-interacting proteins known as saposin-like proteins [84] while SP-C, encoded by *SFTPC*, belongs to

the chondromodulin I (CHMI) family [85]. SP-B and SP-C are highly hydrophobic and play a critical role in the biophysical functions of surfactant [80].

Collectins: Surfactant Protein-A and –D

Collectins are an evolutionarily conserved family of proteins that consists of pulmonary SP-A and SP-D, and the liver-derived serum mannose binding lectin (MBL) [86]. Collectins are referred to as C-type lectins due to their common C-type (calcium dependent) carbohydrate recognition domain (CRD) linked to a collagen arm ending in an N-terminal tail [83, 87]. SP-A and –D share structural similarity in that they are made up of a basic monomeric subunit which oligomerizes into a trimeric oligomer and then further multimerizes to form the native SP-A or SP-D molecule. While, SP-A is an octadecamer, resembling a "flower bouquet-like structure", SP-D is a dodecamer, resembling a cruciform [88]. Details of their differential ligand affinities are incompletely understood. However, their common high affinity for clustered oligosaccharides facilitates their ability to distinguish self from non-self [79]. Interaction of lung collectins with bacteria, viruses and fungi results in either agglutination or opsonization [89]. The best characterized function, opsonization, is mediated by the CRD domain which recognizes a wide variety of pathogen-associated molecular patterns (PAMPs) on microorganisms thereby mediating their phagocytic clearance. However, SP-A and SP-D not only interact with cell surface receptors on microorganisms, they also directly modulate leukocyte functions through interactions with cellular receptors or binding sites and, therefore, act as immune-modulators.

Surfactant Protein A and –D in Pulmonary Immunity

The lung is continuously bombarded by inhaled pathogens and particulates. While the majority is cleared within the upper airways of the lungs, foreign particles that find their way to the alveoli encounter the innate immune system. Since chronic and aggressive inflammatory reactions can result in collateral damage to the surrounding tissues of the lung, compromising the function at the air-liquid interface, pulmonary innate immunity must prevent chronic inflammatory events while providing swift host protection against pathogen invasion and dissemination. The first line of pulmonary host defense consists of alveolar macrophages (AMϕ) and pulmonary surfactant.

The host defense functions of surfactant, primarily mediated by SP-A and SP-D against viruses [90], fungi [91-92] and a broad spectrum of bacteria [93-95], are well documented. Mice carrying a targeted deletion of the *Sftpa* gene (*Sftpa*$^{-/-}$or SP-A$^{-/-}$) are viable, have normal respiratory function but have defects in pulmonary innate immunity. These mice demonstrate high susceptibility to pulmonary infection by P. *aeruginosa* [96], *Haemophilus influenza* [97] , respiratory syncytial virus [98], and Pneumocystis *jirovecii (carnii)* [99], and to group B streptococcus (GBS) following intra-tracheal challenge. GBS infection also results in decreased phagocytic capacity [100], increased levels of lung inflammatory cytokines and splenic dissemination [101]. Lavage fluids from these mice also contain elevated levels of pro-inflammatory cytokines (e.g. TNF-α, IL-6). Intra-tracheal administration of SP-A restores both bacterial clearance and normalized cytokine levels in these mice [97]. Studies of AMϕ isolated from SP-A$^{-/-}$ mice reveal deficiency in bacterial uptake [96-98, 100], decreased production of oxygen radicals and

exaggerated inflammatory responses accompanied by heightened levels of pro-inflammatory cytokines.

In contrast to SP-A$^{-/-}$ mice, those carrying a targeted deletion of the *Sftpd* gene (*Sftpd*$^{-/-}$or SP-D$^{-/-}$) have abnormal lung anatomy and impaired surfactant metabolism [102-103]. They also have increased numbers of apoptotic and activated foamy AMϕ, and manifest emphysema due to increased levels of metalloproteinase-2, -9, & -12 activity, and elevated macrophage-derived oxidant production in alveoli [104]. Defects in host defense are also evident as AMϕ isolated from these mice display decreased phagocytic capacity when challenged with influenza A virus (IVA) and RSV [97, 105] and display elevated levels of pro-inflammatory cytokines post intra-tracheal challenge. By contrast, they are able to clear GBS and *H. influenza* infection normally. Furthermore, restoration of SP-D reverses defects in microbial clearance and inflammation [97, 106]. Collectively, these findings not only serve to underscore the overlapping roles of SP-A and –D in innate immunity, but also highlight their distinct functions in defense of the lung.

Additional roles for SP-A and –D have been elucidated and include, but are not limited to, binding of apoptotic cells to enhance their clearance by AMϕ [107], stimulation of the respiratory burst in AMϕ [108-110], leukocyte recruitment [111-113], protection against hypersensitivity to antigens and allergens [114], and inhibition of cytokine production and lymphocyte proliferation [115-118]. In humans, mutations associated with SP-A and –D have not been linked to disease and increased susceptibility to infection [114]. However, altered levels of these collectins are detected in bronchio-alveolar lavage fluids taken from patients with pathologies linked to chronic

inflammation such as bronchial asthma, acute respiratory distress syndrome (ARDs) and cystic fibrosis [119-121].

SP-A and SP-D Influence Immune Cell Activities

SP-A and –D can enhance bacterial and viral phagocytosis by Mϕ and neutrophils through opsonization or aggregation and can act as activation ligands to upregulate the expression of immune cell surface receptors. It has been reported that direct activation of phagocytic activity in Mϕ is independent of microbial binding. In support of this, both SP-A and SP-D have been shown to mediate increases in cell surface expression of the mannose receptor on macrophages [122-123]. In addition, SP-A has been demonstrated to upregulate cell surface expression of scavenger receptor A (SR-A) in AMϕ [124]. Both MR and SR-A are involved in mediating phagocytosis. Expression of SP-A receptors, on mononuclear cells, is tightly regulated by cytokines and various other agents [125-126]. Thus, SP-A and -D are capable of influencing immune cell activity in the absence of pathogenic stimuli.

SP-A and SP-D molecules interact with antigen presenting cells (APCs) and T-cells, thereby linking adaptive and innate immunity. Briefly, both molecules have been shown to modulate the function of dendritic cells (DC) [127-128] and T cell [115, 129]. SP-A was reported to modulate differentiation of bone marrow-derived dendritic cells (DC) while SP-D enhances bacterial antigen presentation by bone marrow derived DC. SP-A and –D both inhibit T cell proliferation. These findings reveal that SP-A and –D are capable of modulating the immune response of cells of the adaptive immune system. This

may serve to protect the lung further from potentially chronic inflammatory events encountered by daily respiration and inhalation of pathogens that could potentially damage or impair the ability of the lungs to function properly.

SP-A and -D Regulate both Inflammatory and Anti-inflammatory Responses in Alveolar Macrophages

AMϕ exist in a microenvironment containing high levels SP-A and –D proteins. Their quiescence in the absence of infection is exemplified by suppression of both inflammatory cytokine production and antigen presentation [130-132]. When challenged by pathogens, both collectins contribute to innate immune activity by facilitating removal of pathogens and by enhancing protective inflammatory responses. Putative receptors for SP-A and SP-D were identified in several studies. These receptors include, signal-regulatory protein-α (SIRP-α) [133], calreticulin [133-135] Toll-like receptor 2 (TLR2) [136-138], and TLR4 (TLR4/MD2) [137-140]. SP-D is also reported to bind gp-340 [141]. SP-A additionally binds CD93 (C1qR) [142-143] and SP-R210 [144]. Reports regarding the pro- and anti-inflammatory effects of these collectins on alveolar Mϕ [145-149] were initially confusing but have partially been clarified by recent work carried out by Gardai and colleagues [133]. These investigators propose that SP-A and –D mediate their immunomodulatory effects via their binding orientation to receptors SIRP-α and CD91-calreticulin complex on the AMϕ cell surface. They show that inhibition of pro-inflammatory mediators by SP-A is attributed to the binding of the C-type lectin domain to SIRP-α. Under these conditions, tyrosine phosphate SHP-1 is activated, leading to

inhibition of src-family kinases and p38 mitogen-activated protein kinase signaling. In contrast, binding of pathogens, apoptotic cells or cell debris by the lectin domain of SP-A prevents engagement of SIRP-α receptor and instead SP-A binds through its collagenous tail to CD91/calreticulin and stimulates p38-mediated activation of NF-κB, leading to induction of pro-inflammatory mediator expression and activation of AMϕ. Based on these studies, it is postulated that the binding orientation of SP-A and SP-D molecules, with their cognate cell surface receptors, dictate the immunomodulatory responses that are elicited.

Surfactant-dependent air-breathing appears to have evolved at least 400 million years ago in the lungs of primitive air-breathing fish and has been highly conserved suggesting potentially 'older' , and as yet, undefined functions [150] [151]. Certainly, numerous *in vivo* and *in vitro* studies in the last two decades have revealed important roles for SP-A and SP-D in pulmonary immunity, modulation of inflammatory and anti-inflammatory responses, a role in apoptotic cell clearance and, more recently, a role in mediating the signal for labor at term via fetal derived AF Mϕ activation.

Although the significance of the evolutionarily ancient SP-A and SP-D molecules in human physiology is beginning to unfold, their broad spectrum of effector functions remain incompletely defined. Recently, evidence suggests that SP-A production by the fetal lung near term provides a key signal leading to term labor [1]. In light of these findings, it is of great interest to define the phenotypic changes of AF Mϕ associated with SP-A activation to better understand the cellular and molecular mechanisms involved in mediating the signal for parturition at term.

The Mononuclear Phagocyte System Gives Rise to Macrophages

The mononuclear phagocyte system (MPS) is a family of cells originating from hematopoietic progenitors in the bone marrow that progress through a monoblast to promonocyte to monocyte stage [152]. Peripheral blood monocytes enter target tissues where they undergo final maturation to either replenish the resident tissue-specific macrophage (Mϕ) pool at a steady state rate or in response to antigenic stimuli [152-153]. Macrophages are ubiquitous and can be found in virtually every organ: in the bone (osteoclasts), connective tissue (histiocytes), liver (Kupffer cells), lymph nodes, thymus, nervous system (microglial), gastrointestinal tract, spleen, thymus, peritoneum, reproductive tract and lung alveoli [153]. Importantly, each Mϕ population manifests a distinct phenotypic profile (Figure 2).

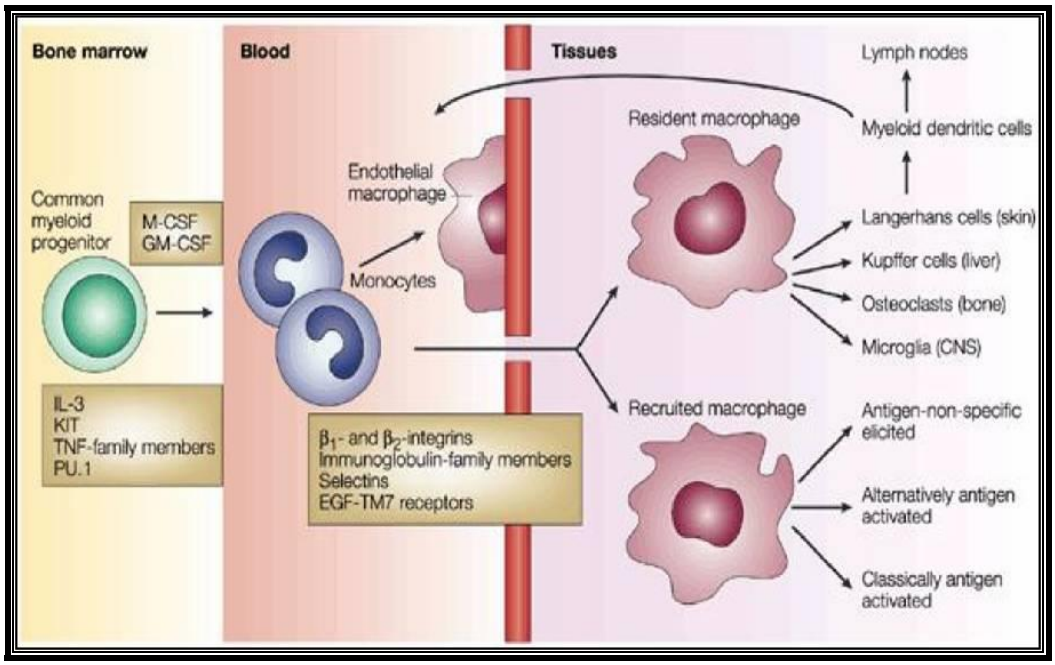


Figure 2. Differentiation, distribution and activation of macrophages *in vivo.* Macrophages arise from common myeloid progenitor cells in the bone marrow that become committed to the monocytic lineage in response to various differentiation signals. Blood monocytes enter tissue compartments from peripheral blood where they undergo final maturation in response to inflammatory stimuli or replace resident macrophages [154]. (Reprinted with permission. Alternative Activation of Macrophages, Gordon, S. *Nat Rev Immunol,* Copy right 2003.)

Macrophage Ontogeny in the Developing Embryo

In the early 1880's Ilya Mechnikov (Metchnikoff) introduced the term phagocyte ("devouring cells") to describe his observations of starfish larvae take up of foreign particles [155]. He later introduced the term "macrophage", meaning "large eaters" in Greek, which is now synonymous with the prodigious uptake of cellular debris and pathogens by this cell type. The presence of Mϕ during embryogenesis has long been recognized, but the existence of distinct embryonic Mϕ populations was reported ~20 years ago by Naito and colleagues. Studies by this group revealed the existence of "primitive" and "definitive" Mϕ populations in the developing embryo. They reported the emergence of an initial "primitive" population, prior to the establishment of blood circulation and erythropoiesis, which bypassed the stage of the circulating monocyte. The later "definitive" population was found to arise from erythromyeloid precursors that pass through the monocyte stage and are reminiscent of adult Mϕ [156-157].

During mammal embryogenesis, hematopoiesis begins within blood islands of the yolk-sac (YS) switches to the fetal liver and subsequently moves to the bone marrow which then becomes the predominant site for hematopoietic activity thereafter and throughout postnatal life. In mice, hematopoiesis in the YS is believed to shift to the liver between 10.5 and 12 days post-coitum (dpc), and then to the bone marrow between 14 to 16 dcp. The first cells generated within the YS belong to the erythroid and myeloid lineage. The "primitive" and "definitive" populations are the earliest detectable Mϕ populations within the YS. In 2005, Bertrand and colleagues carried out studies to

determine the lineage relationship between these embryonic Mϕ populations. Using embryos expressing CX_3CR1^{EGFP} mice, in which EGFP is expressed in monocytes and tissue Mϕ, they identified three successive "waves" of YS Mϕ and described their origin. Their studies demonstrated that the previously reported "primitive" population actually contains two successive "waves" of cells: the first "wave" (7.5-8 dpc) is transient and contains mature maternal-derived Mϕ (GFP^-, $CD45^+$, MAC-1^+, F4/80^+) while the second "wave" (8 dpc) is made up of fetal-derived monopotent precursors which give rise to Mϕ (GFP^+, $CD45^+$, MAC-1^+, F4/80^+). The "third wave" (10 dpc), corresponds to the "definitive" population, and is comprised of erythromyeloid precursors that give rise to Mϕ (GFP^+, $CD45^+$, c-Kit^-, MAC-1^+, F4/80^+) [158]. Both monopotent and erythromyeloid precursors follow the developmental pathway common to adult Mϕ (promonocyte to monocyte stage) [158]. Importantly, the embryonic AF Mϕ characterized herein arise from the "definitive" population.

In mammals, production of Mϕ by the liver and bone marrow is controlled, in part, by colony-stimulating factor (CSF-1). CSF-1 binds to the colony-stimulating factor 1 receptor (*Csf1r*), a high affinity tyrosine kinase receptor, encoded by the *c-fms* proto-oncogene. CSF1r, expressed in Mϕ and trophoblast cell lineages, is vital for differentiation, proliferation, and survival of Mϕ [159-161]. C-*fms* (*Csf1r*) is detectable during embryogenesis in "primitive" and "definitive" Mϕ prior to the expression of the well-characterized Mϕ-restricted transcription factor PU.1 [162]. In the mouse, c-*fms* expression is predominantly restricted to cells of the Mϕ lineage in both the embryo and adult [162-163]. Studies by Hume *et al.* and Dai *et al.* demonstrate that disruption of the

CSF-1(Csf1^{op}/Csf1^{op}) and *Csf1r* (C*sf1r*-/*Csf1r*-) genes result in vast depletion of F4/80-positive Mϕ but does not obliterate them entirely [164-165]. A well recognized function of Mϕ during embryogenesis is clearance of dying cells [166-167]. However, production and secretion of a wide array of factors and their ubiquitous presence within tissues during development lead to speculations of trophic functions. The lack of viability of mice lacking Mϕ altogether further pointed to other potential roles during development. Certainly, studies of c-*fms* and CSF-1 -deficient mice support this concept since they manifest a wide range of defects among various organ systems including bone, pancreas, gonads, mammary glands and the nervous system [164-165, 168-171]. Indeed, recent evidence reported by Sasmono *et al.* lends further support to this notion. Using a previously described transgenic mouse in which the Csf1r promoter drives expression of an EGFP reporter gene (*Csf1r-EGFP*) [172], the role of embryonic Mϕ in the modulation of kidney development was elucidated. Additionally, expression profiling of 15.5 dpc Mϕ populations isolated from kidney, lung and brain tissues demonstrated remarkable similarities. Moreover, the gene expression pattern of these populations were similar to those of Mϕ involved in anti-inflammatory/wound repair (alternatively activated or M2 Mϕ). Moreover, they displayed considerable overlap with tumor-associated Mϕ (TAMs) [173].

It is becoming increasing clear that the roles for Mϕ in the developing embryo, and in the adult, extend beyond early ascribed roles in phagocytosis and as mere sentinels of the immune system. Further studies will undoubtedly serve to reveal the scope of their functional plasticity and trophic functions [174].

Macrophage Heterogeneity

Macrophages are amongst the most diverse cell type of the immune system. Their extensive specialized effector functions and phenotypic heterogeneity are due to differences in their local microenvironment, anatomical location [175], genetic background [176-178] as well as the monocyte precursors from which they arise [179-180]. Mϕ are professional antigen presenting cells (APC) known for their involvement in pathogen recognition and clearance during infection and in removal of senescent and dying cells and immune complexes. Recognition of a wide range of ligands is mediated by plasma-membrane receptors that either result in phagocytosis, endocytosis, or in changes in gene expression or repression [181]. Mϕ secrete various products in response to exogenous and endogenous antigenic stimuli that lead to the induction of specific transient effector functions capable of modulating innate and adaptive immune responses.

Mϕ play critical roles in the initiation and resolution of inflammation, wound healing, in the induction of tolerance, and tumor immunity [182] and exhibit specialized trophic functions during organogenesis [164-165, 168-171]. They are indispensible in tissue homeostasis and are vital for innate and acquired cell-mediated immunity. The Mϕ sub-populations associated with these diverse roles manifest specialized effector functions and exhibit distinct gene expression repertoires. The ability of Mϕ to acquire specific functions in response to temporal changes within various anatomical sites and in response to a wide range of stimuli exemplifies their remarkable versatility and plasticity. Classification of

these populations is currently based on their immunological response to antigenic stimuli and to soluble factors.

Macrophage Activation

Macrophages are activated by their local cytokine milieu or by phagocytic uptake. Depending to the activating stimuli, macrophages are polarized to one of two broadly divided states that follow the activation paradigm of Th1 and Th2 activated T cells. Classically activated (or M1) Mϕ manifest a Th1-like phenotype that promotes inflammation, tissue destruction and cell-mediated adaptive immunity. Though vital for successful elimination of infectious, toxic, and allergenic agents, inflammatory processes must be attenuated once the inciting antigenic challenge is eliminated to avoid collateral damage to the host. Thus, anti-inflammatory programs in Mϕ are temporally and spatially engaged to prevent further destruction and to initiate the resolution of inflammation to allow healing and to re-establish tissue homeostasis. Alternatively activated (or M2) Mϕ manifest a Th2-like phenotype that antagonize M1 mediated cascades and initiate resolution of inflammation and promote healing [154].

Classical Activation

As mentioned, the functional phenotype acquired by Mϕ is a consequence of the signals they receive from their local environment. M1-activation is the best studied state and occurs in a type I cytokine environment. M1 polarization is induced by the concomitant

presence of interferon-γ (IFN-γ) and lipopolysaccharide (LPS) [183]. M1-activated Mϕ responses are geared to mediate resistance against intracellular parasites and tumors and elicit tissue destruction [184-185]. These specialized functions are due, in part, to their ability to secrete a variety of pro-inflammatory cytokines, chemokines, and reactive nitrogen (*i.e.* nitric oxide) [186] and oxygen intermediates (*e.g.* reactive oxygen intermediates (ROI)) [187], which further serve to recruit other inflammatory effector cells to the site of infection/injury thereby perpetuating the inflammatory response [188-189]. Functionally, these cells have heightened endocytic capabilities and display increased levels of MHC class II and co-stimulatory molecules (CD80/CD86) leading to enhanced antigen presentation [185, 190-191]. They are commonly characterized by elevated expression of inflammation-associated cytokines such as IL-1β, IL-6, IL-12, and tumor necrosis factor (TNF)-α (an extensive summary of the cytokine profile was recently reviewed by Van Ginderachter *et al*.) [192] . Secretion of chemokines such as CXCL9/Mig, IP-10/CXCL10, CXCL11/I-TAC, CXCL15/HCC-2, CCL20/MIP-3α, and CXCL13/BCA-1 has also been reported [154, 191, 193]. M1-activated Mϕ also release matrix metalloproteinase (MMP)-1, -2, -9, and -12 thereby contributing to tissue destruction through degradation of collagen, elastin, fibronectin and other extra cellular matrix components [194-196]. Moreover, M1 Mϕ are associated with Th1 lymphocyte responses and are important in cell-mediated immunity to intracellular pathogens [153, 188]. They interact with T and B cells in cell-to-cell interactions or indirectly via release of cytokines, chemokines, enzymes, and arachidonic acid to modulate adaptive cell-mediated immune responses [191].

Direct recognition of microbial Pathogen-Associated Molecular Patterns (PAMPs) (*e.g*. LPS) by TLRs or Nucleotide Oligomerization Domain (NOD) receptors [197-198] leads to a class of M1 polarization currently designated as innate activation. This state is phenotypically similar to M1 in that it is characterized by the secretion of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α as well as by upregulation of co-stimulatory molecules. However, unlike M1 Mϕ, these cells do not manifest increased phagocytic capacity [191] (Figure 3).

Alternative Activation

Alternative (M2) Mϕ activation was originally discovered as a response to IL-4, a type II cytokine [154, 199]. M2-activated Mϕ promote the resolution of inflammation and tissue repair through production of anti-inflammatory cytokines and chemokines. They also manifest high phagocytic, and endocytic capacity and aid in the elimination of tissue debris [192, 200-201]. Generally, they express elevated levels of IL-10, transforming growth factor-β (TGF-β) and molecules that antagonize the actions of pro-inflammatory mediators such as IL-1 receptor (IL-1R) and the decoy receptor IL-1RII. They are poor antigen presenting cells and typically do not express ROI or nitrogen intermediates [202]. Since their initial description, wide variations in M2 activation have been reported. Variants along the M2 continuum of functionally- and phenotypically-related polarization states are now sub-divided into M2a, M2b and M2c sub-categories based on the stimuli that induce them.

M2a Mϕ are induced by IL-4 and IL-13 secreted from Th2 cells, mast cells, and basophils and have anti-inflammatory effects as they down-regulate expression of pro-inflammatory mediators such as IL-1β, IL-6, IL-12, TNF-α and a plethora of other inflammatory-associated mediators [191]. They express high levels of chemokines including CCL13/MCP-4, CCL8/MCP-2 and CCL26/eotaxin-3 that induce the influx of eosinophils, basophils and other Th2 cells. Similar to M1 Mϕ, M2a exhibit elevated expression of MHC class II and co-stimulatory molecules. M2a Mϕ also up-regulate expression of scavenger receptors and C-type membrane lectins (*e.g.* MRC1, SR-A, Dectin-1, DC-SIGN) [191]. Expression of arginase 1 (Arg 1), an enzyme involved in proline and polyamine biosynthesis, is upregulated [203]. Proline promotes extracellular matrix construction while polyamines are involved in cell proliferation [204]. M2a Mϕ additionally express fibronectin 1 (FN-1) and matrix associated proteins which promote fibrogenesis [201].

M2b Mϕ are elicited by LPS or IL-1β that bind TLR4 or the IL-1receptor (IL-1R), respectively, or by ligand recognition of immune complexes by the Fc portion of immunoglobulins G (Fcgamma receptor) [205-206]. While they produce low levels of IL-12, an inflammatory-associated cytokine, M2b produce high levels of IL-10, a type II cytokine and support adaptive immune responses. Interestingly, they also produce high levels of IL-1β, IL-6 and TNF-α. M2b can be distinguished from M2a by expression of sphingosine kinase 1 (SPHK1) [191, 207]. M2c, the last sub-class of M2 Mϕ activation, are induced by IL-10, TGF-β, glucocorticoids, and through other immunological interactions (*e.g.* CD200-CD200R, CD47-CD172α) [153]. They release anti-inflammatory mediators that down-regulate inflammation. M2c are regarded as deactivated Mϕ [191] (Figure 3).

M2 Mϕ are antagonistically regulated by type I cytokines. M2 Mϕ are observed in a variety of helminth infections such as *Schitosoma mansoni* [208], *Heligmosomoides polygyrus* [209], *Trichinella spiralis* [210], *Fasciola hepatica* [211], *Ascaris suum [212]*, *Nippostrongylus brasiliensis* [213] and filarial parasites [214]. M2-activated Mϕ also play a role in the healing phase of acute inflammation, in chronic inflammatory diseases (*i.e.* rheumatoid arthritis, atherosclerosis, and psoriasis [208, 215-217]) and in wound healing [217]. Moreover, these, and other, studies suggest that M2 may participate in the three phases of healing: modulation of inflammatory cascades, angiogenesis, and the elimination of tissue debris and apoptotic cells [200, 218]. In addition to these functions, M2 Mϕ also exert immunosuppressive functions [219-220]. M2 Mϕ have been described in the lung and placenta [221], and in other immune privileged sites where they protect against chronic inflammation to non-pathogenic microorganisms [222]. Pathologies associated with this activation status include asthma and allergies.

The numerous sub-categories of M2-activation are steadily rising. Unique and tissue specific gene expression repertoires are immerging as *in vivo* Mϕ populations continue to be characterized. Currently, identification of M1 and M2 Mϕ populations is largely based on phenotypic properties: their ability to participate in phagocytosis, on expression of specific cytokines, chemokines, proteolytic enzymes and by expression or repression of a variety of Mϕ-specific genes [152, 172, 223-225]. The broad spectrum of M1 and M2 Mϕ activation polarization states demonstrate the plasticity and flexibility of the mononuclear phagocyte system.

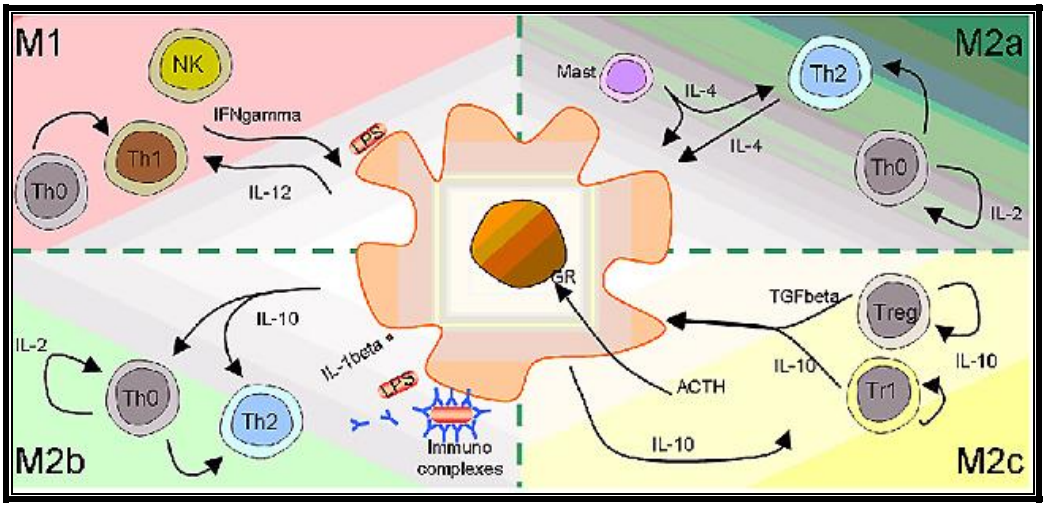


Figure 3. Macrophage polarization paradigm is in agreement with the type I type 2 response tenet. Macrophages are capable of displaying different functional phenotypes, many of which are antagonistic, under the influence of specific mediators. M1: Bacterial LPS in combination with IFNγ elicits the classical activation triggering an IL-12 IFNγ loop. M2a: Il-4 and IL-13 are both capable of eliciting alternative activated macrophages. M2b; Ligation of Fc receptors in the presence of Toll stimuli can induce a polarization antagonistic to M1 which promotes Th2 responses through an IL-10 circuit. M2c; Deactivation is required for the termination of inflammation and is elicited by IL-10, glucocorticoids (GC) or TGF-β. (Reprinted with permission. Macrophage Activation and Polarization. Martinez, F.O., *Frontiers in Biosciences*, 453-461, copyright 2008).

Macrophage Characterization

The F4/80 molecule is expressed on a variety of Mϕ subsets including those found in the lung, liver, the lymph nodes, bone marrow, the thymic cortex, splenic red pulp, and in the brain. Therefore, it is a widely used marker for Mϕ identification in both normal and pathological conditions [226-227]. CD11b, also known as Mac-1, is a cell surface marker used for the identification and analysis of mature Mϕ [228-229]. Many of the molecular markers used to characterize M1 and M2 Mϕ populations have been defined via differential activation of Mϕ *in vitro*. *In vivo* analysis of M1 and M2 Mϕ subsets reveal far more complex phenotypes. Nevertheless, distinctions between these activation states are facilitated by the presence or absence of several molecular hallmarks. M1-activated Mϕ are commonly identified by elevated production of IL-1β, IL-6, IL-12, and/or TNF-α, and by the presence of reactive oxygen species and oxygen radicals (*e.g*. nitric oxide) [191].

The M2 Mϕ repertoire is complex and has been discussed. In general, characterization of M2-activation has been achieved at the biochemical level by the presence of cellular markers such as Arg 1, Ym1, Ym2, FIZZ1, and by the enhanced expression of scavenger receptors such as mannose receptor (MR). As mentioned, type II cytokines (*e.g.* IL-4, IL-13) induce Arg 1. Arg 1 was one of the first markers used to distinguish M2 from M1 Mϕ and was based on their differential metabolism of l-arginine by arginase 1 and inducible nitric oxide synthase (iNOS), respectively [230]. In M1 Mϕ, L-arginine is metabolized by iNOS to release NO. However, because of its high affinity for L-arginine, Arg 1 out-competes iNOS in M2 Mϕ converting it to urea and ornithine. In turn, ornithine

aminotransferase catalyzes the conversion of ornithine into proline and ornithine decarboxylase generates polyamines. Proline and polyamines are important factors in extracellular matrix construction and cell proliferation [204].

Ym1, chitinase 3-like 3, is a mammalian chitinase family member originally described as an eosinophil chemotactic factor produced by $CD8^+$ lymphocytes [231]. Ym1 form large crystals in the lungs of mice with chronic lung pathology [232]. It is proposed to function in the encapsulation chitin-bearing pathogens such as yeast, fungi and nematodes [214]. On the other hand, it is postulated that it may interact with extracellular matrix components which is in line with its potential role in wound healing [233]. In 2002, Welch and colleagues demonstrated that Ym1 and arginase were the most highly up-regulated genes in peritoneal Mϕ in response to IL-4 [234]. Raes *et al.* simultaneously reported Mϕ induction of Ym1 and Ym2 transcripts in response to IL4 and IL-13 [235]. Ym2, chitinase 3-like 4, is highly homologus to Ym1 and is also used in M2 Mϕ identification.

FIZZ1, also known as RELM-α, expression is also correlated with M2-activation. It is a resistin-like secreted protein found to be up-regulated in the lung during allergic pulmonary inflammation [236]. FIZZ1 is a member of a family of secreted cysteine-rich proteins whose functional role remains undetermined. The mannose receptor (MR) is up-regulated in M2 Mϕ. MR is a C-type lectin that recognizes a wide range of antigenic determinants displayed by pathogens including viruses, bacteria, and parasitic worms [153, 237-238].

Mϕ populations manifesting an M1/M2 mixed phenotype have recently been described in tumors. The existence of this unique tumor-associated macrophage (TAM)

phenotype serves to illustrate the diverse activation profiles that are now emerging and highlight the complexity of the physiological microenvironment that influence their induction. Further characterization of the molecular repertoire of differentially activated Mϕ will be useful in diagnosis of diseases where an imbalance between the pro- and anti-inflammatory immune response results in pathology.

Tumor-Associated Macrophages

The tumor microenvironment is central in the activation of TAM. Chemotactic factors at the tumor site, including CSF-1, IL-4, IL-6, IL-10, TGF-β and prostaglandin E2, serve to recruit circulating monocytes and promote monocyte to TAM differentiation. Within tumors, TAM predominant and promote growth, tumor invasion, angiogenesis, and metastasis [191, 239-241]. They display suppressed antigen presenting ability [242], produce very little ROIs and express high levels of scavenger receptors and MR [191]. They also secrete transforming growth factor (TGF)-β and have an IL-10^{high} and Il-12^{low} cytokine repertoire. Additionally, they express several M2-associated markers (*e.g.* Arg 1, Ym1, and FIZZ1) and share a similar functional phenotype with M2 Mϕ. As a direct result of their poor antigen presenting capacity and release of IL-10, TGF-β, and prostaglandins, they act to suppress T cell activation and proliferation. Indeed, several populations isolated from various tumors demonstrate their immunosuppressive properties [243-249]. Interestingly, several reports have described production of pro-inflammatory cytokines and chemokines, most notably, IL-1, IL-6, TNF and CXCL8, in TAM isolated from renal cell carcinoma and

breast carcinoma patients [250-251]. Interestingly, in some forms of established tumors, emerging evidence now demonstrate the presence of TAM populations expressing both pro-inflammatory (M1-like) and immunosuppressive characteristics leading to a ‘mixed’ phenotype classification [252-254]. The mechanisms by which pro-inflammatory cytokine production is mediated in these cells remain unknown but are speculated to be influenced by the hypoxic environment within the tumor [255].

Active Mechanisms of Feto-Maternal Immune Privilege

Immune privilege, described by Medawar and colleagues, is the immunological protection of vital organs or tissues from destruction and damage induced in non-regenerating sites by inflammatory responses [256-258]. Early observations by Medawar noted the prolonged, or indefinite, survival of foreign transplanted tissue within the anterior chamber of the eye, and other immune privileged sites, which is in stark contrast to the rapid rejection observed when foreign tissues are transplanted in non-immune privileged sites [258]. Based on these observations, immune privileged sites were defined as sites in which foreign tissues experience extended survival [259]. Although Medawar initially concluded that this phenomenon was due to “immunological ignorance”, numerous studies now support the concept that immune privilege is a synergy between anatomical, physiological and immune-regulatory processes that modify the capacity of immune system to recognize foreign antigens, to deviate the inflammatory response, and to block the expression of immune-mediated inflammation at the site of privilege [260].

Immune privilege is generally accepted to be an evolutionary adaptation that serves to preserve and protect vital functions such as vision and reproduction.

Over the last three decades the existence of numerous immune privileged sites and tissues has been reported [261-264]. In seminal studies by Kaplan and Streilein and Niederkorn and Streilein [265], the escape of allogenic cells, originally introduced into the anterior chamber of the eye, were found to induce immune deviation by eliciting antibody production (humoral immunity) and down-regulating antigen-specific cell-mediated immunity (a phenomenon is termed anterior chamber-associated immune deviation (ACAID)) [265-266]. Importantly, these studies revealed that antigen specific immune suppression can be generated to provide systemic immunological protection.

The response of immune cells to antigens or immunogens within immune privileged sites is suppressed by the immune system to avoid collateral damage to host organs. These sites include the brain, the eye [258], the pregnant uterus [267], the testicles [268], solid tumors and sites of chronic inflammation [269]. A universally recognized immune privilege site lies within the feto-maternal interface at the placenta. Here, tolerance to the fetus is sustained by anti-inflammatory mediators that promote and sustain immune evasion. Many of the factors that promote immune suppression and deviation have been extensively reviewed elsewhere [7, 260, 270-271], and only a few are discussed here.

In human placentation, fetal-derived extravillous trophoblasts infiltrate decidua to establish a blood supply to the mother. This immunogenic interface is a major target for attack by the maternal immune system. Thus, various fetal mechanisms have evolved

to suppress and evade the maternal immune system to prevent fetal rejection during pregnancy. One of the most important immune evasion strategies used by trophoblasts is the absence of the MHC class Ia molecule which renders the placenta 'invisible' to cytotoxic T lymphocytes (CTL). This does, however, provoke the attention of natural killer (NK) cells which have the capacity to eliminate MHC class I negative cells [272-273]. Consequently, expression of non-conventional MHC Class Ib molecules, HLA-G and HLA-E on trophoblasts act to inhibit maternal NK cell-mediated lysis via interaction with CD94/NKG2 receptor [274-276]. Interestingly, soluble HLA-G, produced by trophoblasts, induces apoptosis of activated $CD8^+$ T cells and suppresses $CD4^+$ T cell proliferation further contributing to immune privilege of the fetus [277].

Other mechanisms that trophoblasts utilize in immune evasion include the expression of cell membrane molecules such as Fas Ligand (FasL:CD95) [278] and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). CD95L and TRAIL contribute to immune suppression during pregnancy by promoting apoptosis of CD95-bearing maternal inflammatory cells [270]. Trophoblasts also produce indoleamine 2,3-dioxygenase (IDO), an enzyme that converts tryptophan to kynurenine intermediates. Since T cells require tryptophan for survival, conversion of this amino acid by IDO depletes local stores resulting the suppression of antigen-specific T-regulatory cells and cell death [279] thereby contributing to the immune privilege of the fetus [7, 271, 280]. Inflammation provoked by complement activation in placenta can lead to fetal loss [281]. The presence of complement regulatory proteins such as Decay accelerating factor (DAF) and membrane cofactor protein (MCP), and their rodent homologue, Crry, are expressed in the placenta. These factors prevent the formation of the complement membrane attack

complex and in this manner, shield the fetus from complement-induced inflammatory injury [260, 282]. The placenta has evolved numerous immunosuppressive and anti-inflammatory molecules to prevent the rejection of the fetal allograft during pregnancy. Collectively, studies have shown that while immune suppression is necessary for fetal gestation, inflammation is equally as important for the process of parturition at term.

Pregnancy is associated with a $CD4^+$ T cell shift from a Th1 to a Th2 phenotype that is believed to be crucial for determining pregnancy outcome since a sustained Th1 state would lead to fetal loss or complications during pregnancy [283]. Several studies have demonstrated that gestation is associated with diminished maternal cellular immunity in favor of humoral immunity [284]. In support of this concept, studies by Mor *et al.* found that pregnancy was comprised of three distinct immunological phases. The phases were characterized by the cytokine profile found in the peripheral blood of the pregnant woman during early, mid and late gestation. In the first, or early phase, significant levels of pro-inflammatory cytokines and chemokines (e.g. IL-8 and monocyte chemotactic protein 1 (MCP-1)) are present but decline near mid pregnancy and increase again as term approaches [285]. These cytokine profiles correspond well with the physiological events associated with each phase of pregnancy. Early gestation is a pro-inflammatory phase in which implantation of the fetus promotes tissue destruction and the mechanisms of immune suppression are invoked. Mid-term gestation is marked by an anti-inflammatory state that allows the fetus time to develop and grow. During late gestation, a resurgence of the pro-inflammatory state contributes to expulsion of the fetus [285]. Together, these studies demonstrate the precise regulation of inflammatory

responses during pregnancy and labor; suppression during gestation and the subsequent escalation of inflammatory processes, due to the loss of immune privilege.

Concluding remarks

The mechanisms leading to parturition remain a mystery. Both term and preterm labor are associated with the inflammatory response. Studies suggest that this inflammatory response is mediated, in part, by leukocyte infiltration of the myometrium[4]. Indeed, fetal-derived AF Mϕ have been demonstrated to migrate into the gravid uterus at term thereby transmitting the fetal signal for the initiation of labor at term through cytokine secretion and NF-κB activation of inflammatory cascades that lead to up-regulated expression of myometrial genes required for uterine contraction culminating in fetal expulsion[1].

Prevention of preterm labor is dependent on obtaining a more thorough understanding of the mechanisms involved in normal labor at term. This will lead to insights into the underlying pathophysiology of preterm labor and shed light on the cross-talk between endocrine, paracrine and immunological systems. More importantly, it will provide improved therapeutic strategies for the prevention and intervention of preterm birth (PTB).

The current studies were undertaken to characterize, for the first time, murine AF Mϕ during late gestation and to assess the changes in their phenotypic properties in association with the developmental induction of SP-A expression by the fetal lung. My

work, therefore, seeks to address three distinct questions. First, what types of phenotypic changes do the amniotic fluid macrophages undergo following SP-A activation that lead/facilitate their migration to the pregnant uterus at term? Second, what effects do *SP-A* and *SP-D* deficiency have on the timing of labor? Finally, how do amniotic fluid macrophages infiltrate the uterus at term?

CHAPTER II:

MATERIALS AND METHODS

Mice

All animal studies were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center. According to the approved protocols, cells and tissues were obtained from mice that were euthanized by inhalation of isoflurane anesthetic (Baxter Healthcare Corp., Gayama. PR) and cervical dislocation.

Timed-pregnant mice

Outbred timed-pregnant CD1/ICR mice were purchased from Harlan Laboratories (Harlan, USA). Mice were time-mated by placing 8 week-old males and females together between 5:00 p.m. and 7:00 a.m. Potential pregnancy age was determined by the presence of vaginal plug the following morning; gestational age was designated as day 0.5 days post-coitum (dpc). Pregnant mice where received either on 12.5 or 14.5 dpc and housed under pathogen-free conditions where they were maintained on a 12-h light, 12-h dark/light cycle and allowed free access to a standard pellet chow.

Timing of labor in mice

To examine the effects of deficiencies in surfactant protein A (*SP-A*$^{-/-}$), surfactant protein D (*SP-D*$^{-/-}$), surfactant protein A and D (*SP-A*$^{-/-}$/*D*$^{-/-}$), Toll-like receptor 2 (*TLR2*$^{-/-}$), and

combined deficiency on Toll-like receptor 2 and TLR 4 (*TLR2$^{-/-}$/4$^{-/-}$*) on the timing of labor, single breeding pairs were housed together overnight and separated in the morning (designated 0.5 dpc). The time of labor was documented upon delivery of the first pup or by the presence of a litter. Timing of parturition in *wild-type* C57BL/6 mice (Mouse Breeding Core Facility, UT Southwestern) was carried out in a similar fashion.

Gene targeted mice

SP-A, SP-D and SP-A/D null mice

Mice homozygous for targeted disruption of the *SP-A* [286], *SP-D* [102]or both *SP-A/D* genes [287] were generously provided by Dr. Samuel Hawgood. It should be noted that since *SP-A* and *SP-D* genes lie 60 Kb apart on mouse chromosome 14, it was necessary for Dr. Hawgood and his colleagues to sequentially target them in embryonic stem cells using graded resistance to G418 to create the double knockout. Deletion of *SP-A* and *SP-D* locus also resulted in deletion of the *Mbl* I gene, encoding the serum Mbl 1 protein, as it lies between the two targeted genes [287]. Deletions were confirmed by polymerase chain reaction (PCR) analysis of tail biopsy DNA using SP-A and SP-D primers listed in Table I. Amplification temperatures are 30 seconds at 94°C, 30 seconds at 63°C, and 31 seconds at 72°C for 30 cycles after an initial denaturing step of 1minute at 94°C. Mice were maintained under pathogen free conditions.

Toll-like receptor 2 and -4 null mice

Mice homozygous for targeted disruption of the *TLR2*^{tm1Kir} and TLR4$^{lps\text{-}del}$ genes were obtained from Jackson Laboratory. Deletion of *TLR2* and *TLR4* were confirmed by PCR analysis of tail biopsy DNA using TLR2 and TLR4 primers listed in Table I. Amplification times/temperatures were 30 sec at 94°C, 30 secs at 63°C, and 90 secs at 68°C for 35 cycles after an initial denaturing step of 3 min at 94°C for TLR2 and 30 secs at 94°C, 30 secs at 53°C, and 50 secs at 72°C for 38 cycles after an initial denaturing step of 3 min at 94°C for TLR4. TLR2 and -4 double-deficient mice were generated by crossbreeding singly deficient mice and verified by PCR. For mating purposes, a single female and male were housed together overnight and separated the next morning. All mice were housed under pathogen-free conditions.

Csf1r-EGFP transgenic mice

Csf1r-EGFP mice express enhanced green fluorescent protein (GFP) under the control of the macrophage specific promoter Colony-stimulating factor-1 receptor (*Csf1r*) [172]. Verification of the transgene was confirmed by PCR analysis of tail biopsy DNA using EGFP primers listed in Table I. The amplification times/temperatures are 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C for 30 cycles after an initial denaturing step of 5 min at 95°C. Mice were maintained under pathogen-free conditions.

Cell isolation and purification

Isolation of murine amniotic fluid cells

Amniotic fluid macrophages were isolated from 15.5, 17.5 and 18.5 dpc mice. To accomplish this, uteri were exposed and amniotic fluid from each amniotic sac was carefully aspirated, avoiding maternal blood contamination, using a 20 gauge needle in a 1.0 ml syringe containing 0.1 ml of phosphate-buffered saline (PBS, pH 7.4) supplemented with 3% FBS. Amniotic fluid contents from all sacs of each pregnant mouse were pooled and incubated with hyaluronidase (SEIKAGAKU Corp., 0.2 U/ml) for 10 min at 37ºC, followed by centrifugation for 5 min at 600 x*g* at 4ºC without a brake to pellet the cells. We found that because of increased amniotic fluid viscosity near term, incubation with hyaluronidase was extremely important for efficient isolation of Mϕ at late gestation. Limulus Amebocyte Lysate (LAL) Assay (Lonza, Switzerland) was used to assess for the presence of gram negative bacterial endotoxin and determine the LPS concentration in the 1:500 dilution used to incubate AF Mϕ. Results demonstrated no detectable LPS at the dilution used to treat AF cells. Red blood cells were removed by treatment with 400 µl of 1X RBC lysis buffer (eBioscience, San Diego,CA.) for 4 min at RT. The total cell number in single-cell suspensions was determined using a hemaocytometer after staining with Trypan Blue (1:1) to determine cell viability.

Purification of murine amniotic fluid macrophages

Following isolation of amniotic fluid, cells were incubated for 10 min at 4°C in Flow Cytometry Staining Buffer (eBioscience) containing rat-anti-mouse monoclonal antibody 2.4G2 (BD Bioscience, San Jose, CA.) to block Fc-mediated nonspecific binding of antibodies to mouse FcγIII/II receptors. Cells were washed with 3 ml of Staining Buffer and stained for 30 min. at 4°C using anti-F4/80-phycoerythrin (anti-F4/80-PE). Following a single wash, cells were pelleted and resuspended in 200 μl of sterile 3% FBS in PBS and passed through a NITEX nylon screen (Tetko Inc., Kansas City, Missouri) to remove cell clumps and debris. The screen was rinsed once using 100 μl of 3% FBS in PBS. Amniotic fluid macrophages were immediately sorted using the BD FACSAria (B.D. Biosciences, San Jose, CA.).

Murine fetal lung single-cell suspension

Fetal lungs harvested from timed-pregnant ICR mice at 15.5, 17.5 and 18.5 dpc ICR were finely minced to a slurry, and each specimen was digested at 37°C for 30 min in a solution containing 1 mg/ml type A collagenase (Roche Applied Science, Indianapolis, IN.) and 30 U/ml DNase (Sigma-Aldrich, St. Louis, MO.) in RPMI 1640 medium (Sigma-Aldrich). Cells were mechanically dispersed by aspirating and ejecting the slurry in a 10 cc syringe (Becton Dickinson and Co., Franklin Lakes, New Jersey) Cells were pelleted by centrifugation at 500 X*g* for 10 min. The supernatant was decanted and each

pellet was then briefly resuspended in 5 ml in cold 1X RBC Lysis Buffer (eBioscience) and incubated for 5 min at RT to remove red blood cells. Lysis was neutralized by addition of 10 ml of serum-free RPMI 1640. Pelleted cells were resuspended in 5 ml of RPMI 1640 and passed through a NITEX nylon screen (Tetko Inc.) which was rinsed with an additional 5 ml of serum-free RPMI 1640. To remove dead cells and debri, ten ml of 40% Percoll (Sigma-Aldrich) was added to the single-cell suspension and centrifuged at 2000 x*g* for 20 min. The supernatant was gently decanted and the cells were resuspended in 3 ml of RPMI 1640.

Isolation of murine alveolar macrophages

Mice were euthanized using isoflurane (Baxter Healthcare Corp.). The thorax was opened and the pulmonary circulation was perfused via injection of the pulmonary artery with pre-warmed (37°C) PBS containing 0.6 mM EDTA (lavage buffer). A small incision was made in the proximal portion of the trachea and a 0.025 inch ID/0.047 inch OD silicone tube (Helix Medical, Carpinteria, CA.) attached to a 20G1/2 needle was inserted. Lungs were lavaged 3 times using 1 ml of the lavage buffer. Lavage fluid was centrifuged at 300 x*g* for 10 min at 4°C to pellet alveolar cells. Contaminating red blood cells were eliminated using cold 1X RBC Lysis Buffer (eBioscience) for 5 min at RT and neutralized with 10 ml of RPMI 1640. Cells were pelleted by centrifugation and counted using a hemaocytometer and Trypan Blue at 1:1 dilution.

Amniotic fluid macrophage cell surface analysis

Flow cytometry and cell sorting

For identification of various leukocyte populations, 10^6 cells from amniotic fluid single cell suspensions, prepared as described above under 'isolation amniotic fluid cells'were incubated on ice for 10 min with rat-anti-mouse monoclonal antibody 2.4G2 (BD Bioscience) to block Fc-mediated binding of antibodies to mouse FcγIII/II receptors. Cells were washed twice with 100 μl of Flow Cytometry Staining Buffer (eBioscience) and stained for 30 min. at 4°C using the following antibodies (eBioscience): anti-CD45-phycoerythrin-Cy5 (anti-CD45-PE-Cy5), anti-F4/80-phycoerythrin (anti-F4/80-PE), allophycocyanin (APC) anti-mouse CD11b (integrin a_M, Mac-1a), fluorescein isothicyanate (FITC) anti-mouse CD14, R-phycoerythrin (R-PE) CD54, allophycocyanin (APC) anti-mouse CD62L (L-selectin, LECAM-1, Ly-22), phycoerythrin (PE) anti-mouse Toll-like receptor 2 (TLR2), Phycoerythrin (PE) anti-mouse Toll-like receptor 4 (TLR4)/MD2, fluorescein isothiocyanate (FITC) anti-mouse Ly-6G (GR-1, GR1), allophycocyanin (APC) rat IgG2a isotype control, phycoerythrin (PE) Rat IgG2a isotype control (12-4321), fluorescein isothiocyanate (FITC) Rat IgG2a isotype control (11-4321), phycoerythrin (PE)-Cy5 rat IgG2a isotype control. Samples were washed with staining buffer, fixed in 2% paraformaldehyde (Sigma Aldrich) in 1X PBS and stored in the dark at 4°C until analysis. Flow cytometric analysis of stained cells was carried out using the FACSCalibur (BD Bioscience). A forward/side scatter live gate was set and

approximately 50-100,000 events were measured for unstained and stained samples. Data analysis was carried out using FlowJo analysis software (Tree Star Inc, Oregan, USA) and CellQuest Pro analysis software (BD Bioscience). Cells stained for sorting were not fixed and sorted immediately by FACSAria (BD Bioscience).

Histological methods

EGFP fetal amniotic fluid macrophage trafficking studies

To determine the route(s) of fetal derived amniotic fluid macrophage migration into the maternal uterus at term, C3H:HeN *wild-type* females (Charles River, Wilmington, MA.) and *Csf1r-eGFP* homozygous males were time-mated resulting in *wild-type* mice carrying EGFP heterozygous pups. Fetal lungs, amniotic fluid, amnion, and uterine tissues were harvested from 15.5, 17.5, and 18.5 dpc C3H:HeN females carrying EGFP heterozygous pups. Harvested tissues were rinsed in ice-cold sterile 1X PBS and fixed in 4 % PFA for 3 h at RT. Tissues were then equilibrated in 10 % sucrose for 2 h, 18 % sucrose for 2 h and left overnight in 30 % sucrose at 4°C. Tissues where cryoembedded in O.C.T. (Sakura, Torrance, CA.) or Tissue Freezing Medium (TFM) (Electron Microscopy Sciences, Hatfield, PA.) using super-cooled isopentane the next morning and stored at -80°C until cut. Five-micron frozen sections were cut in three step size increments every 50 μm using a Leica CM3050 cryostat at -19°C for O.C.T. or -24°C for TFM. Embedding medium was removed from sections by rinsing slides 5 times in 1X PBS. Sections were then air dried and mounted with Mowiol (Polysciences, Warrington,

PA.) before visualization. Images were acquired using the Zeiss LSM 510 META confocal microscope (Carl Zeiss, Thornwood, NY). Spectral imaging and image analysis by the Linear Unmixing algorithm was used to separate the EGFP signal (510 nm) from tissue background autofluoresence. A lambda stack was acquired at 512 x 512 pixels from EGFP alveolar macrophage and GFP negative tissue samples with a Zeiss Plan-Neofluar 40X/1.3NA oil objective (Carl Zeiss, Thornwood, NY). Images were recorded from approximately 500 nm to 575 nm and an emission spectrum was generated by selecting a region of interest for the signal and background. Lambda stacks were generated for all samples analyzed at the same excitation and emission wavelengths. With the known spectral profiles of the EGFP signal and tissue autofluoresence, distinct emission fingerprints were obtained from the images for a true EGFP signal and autofluoresence from sample tissues.

F4/80 staining of murine amniotic fluid cells

Amniotic fluid cells extracted from individual fetuses on 15.5 and 18.5 dpc, as previously described, were pooled from each mother and resuspended in serum-free RPMI 1640. The cells were allowed to adhere to 2 chamber slides (Nalgene Nunc International, Indianapolis, IN.) for 1 h at 37°C followed by two washes to remove non-adherent cells. Rat anti-mouse F4/80 clone CI:A3-1 (AbD Serotec Ltd, Raleigh, NC.) was used in combination with VectaMount (Vector Laboratories, Inc., Burlingame, CA.) according to the manufacturer's instructions.

Surfactant protein-A staining

Immunohistochemistry was performed on the Discovery XT (Ventana Benchmark; Ventana Medical Systems, Inc, Tucson, AZ) using OmniMap horseradish peroxidase (HRP) reagents (Ventana) according to the manufacturer's recommendations. In brief, 4 μm thick sections of formalin fixed, paraffin embedded sections were pretreated with mild cell conditioning media and incubated for 60 minutes at room temperature with a 1:200 dilution of rabbit polyclonal anti-SP-A antibody raised in house [288]. Slides were then treated with anti-mouse HRP multimer and diaminobenzidine (DAB) was used as the chromogen and hematoxylin as the counter stain. Known positive control slides were processed with each batch of slides.

Oil Red O staining

Oil Red O staining was carried out to visualize intracellular neutral lipid. Lung alveolar and amniotic fluid cells were cytospun (Shandon Scientific, Runcorn, UK.) onto slides and fixed in 10% Formalin for 10 min, followed by dH_2O for 15 min. Slides were then incubated with freshly prepared Oil Red O (Electron Microscopy Sciences, Hatfield, PA.), diluted 6 parts ORO to 4 parts dH_2O, for 15 min. Sections were rinsed in distilled H_2O, counter stained with hematoxylin for 30 min, rinsed in tap H_2O for 1 min., air dried, mounted using Vectashield (Vector Laboratories) and coverslipped. Slides were reviewed by light microscopy. Alveolar macrophages were used as positive controls in each run to

assure proper staining. Individual cells containing red cellular inclusions were interpreted as positive.

Diff-Quick staining

Diff-Quick staining, a modified version of the Wright Giemsa stain, was used for the analysis of cellular cytoplasmic (pink) and nuclear (blue) morphology. Amniotic fluid and alveolar cells were cytospun onto slides, air-dried and stained using Diff-Quick (Fisher, Kalamazoo, MI) according to the manufacture's recommendations. Briefly, slides were incubated in Diff Quick fixative for 30 secs, 30 secs in solution II and counterstained with Diff Quick solution I for 30 sec. Slides were rinsed in tap water to remove excess stain, mounted using Vectashield (Vector Laboratories) and coverslipped. Slides were reviewed by light microscopy.

Transmission electron microscopy

Amniotic fluid cells isolated from 15.5, 17.5 and 18.5 dpc mice were centrifuged. Pelleted cells were embedded in 2% Agar noble, fixed in 2% glutaraldehyde and post-fixed in 1% osmium tetroxide. The cell pellet was then dehydrated in graded alcohols, embedded in resin, sectioned and stained with lead citrate and uranyl nitrate. Sections were examined and photographed with a Hitachi 7500 scanning electron microscope.

SuperArray and quantitative real-time PCR analysis

RNA isolation

For gene-expression analysis, total RNA was extracted from FACSAria (BD Bioscience) sorted AF Mϕ populations (>90% purity) using Trizol (Invitrogen, California, USA) and the one step method of Chomczynski and Sacchi [289]. RNA quality was assessed using Experion High Sensitivity Chips (BIO-RAD, Hercules, CA.) for inspection of 18S and 28S ribosomal RNA peaks. Samples were stored -80 °C pending further study.

First Strand cDNA synthesis and qRT-PCR

Complementary DNA (cDNA) was prepared by reverse transcription of 500 ng of mRNA using the RT^2 PCR Array First Strand Kit (SuperArray, Frederick, MD.) following the manufacturer's instructions. PCR array analysis of genes of interest (Table II), as well as a set of 84 gene transcripts (Mouse Inflammatory RT^2 Profiler PCR array, SuperArray) (Table III), was performed using the ABI-Prism 7900HT Real-time PCR system (Applied Biosystems, Foster, CA.). Briefly, a total volume of 25 µl of PCR mixture, containing cDNA and SYBR Green/ROX PCR Master Mix solution (SuperArray), was loaded into each well of the PCR array. cDNA amplification was performed under the following conditions: 10 min at 95° C for one cycle and 15 sec at

95 °C followed by 1min at 60° C for 40 cycles. ABI Sequence Detection System 1.3.1 software (Applied Biosystems) was used to determine the threshold cycle (Ct) value.

SuperArray data normalization and analysis

The comparative Ct method was used to quantify gene expression [290]. Relative gene expressions were calculated by using the $2^{-\Delta\Delta Ct}$ method in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches detection threshold. The normalized ΔCt value of each sample was calculated using endogenous control genes. Fold change values are presented as average fold change = $2^{-\Delta\Delta Ct}$. Importantly, the Ct for CCR8 was used to normalize sample Ct values in the mouse inflammatory gene panel (SuperArray) since the Ct was stable across the gestational time points analyzed. Ct values above 35 were interpreted as below detectable limits. Statistical calculations were based on the web-based program of RT^{2}Profiler^{TM} PCR Array Data Analysis (www.sabiosciences.com/pcr/arrayanalysis).

Illumina MouseWG- 6 v2.0 Expression BeadChip

Probe labeling and Illumina Sentrix BeadChip Array

Biotinylated cRNA was prepared using the Illumina Total Prep Kit (Ambion, Austin, TX) according to the manufacturer's directions starting with total RNA. For microarray analysis, the Illumina Mouse 6 Sentrix Expression BeadChip was used

(Illumina Inc., San Diego, CA.). Hybridization of labeled cRNA to the BeadChip, washing and scanning were performed according to the IlluminaBeadStation500 manual. Briefly, 1.5 ng of amplified, biotin-labeled F4/80^{+} sorted mouse amniotic fluid macrophage cRNA was suspended in a solution of Hyb E1 buffer (Illumina) and heated for 5 min at 65° C, cooled to RT and loaded onto the bead chip . Hybridization was performed in the Illumina hybe chamber followed by incubation in the Illumina hybe oven at 58°C for 17 h, followed by bead array matrix wash for 10 min with 1x High Temperature Buffer (Illumina). The array was blocked for 5 min with 1% (w/v) casein-PBS (Pierce, Rockford, IL.). The array signal was developed by 10 min incubation with streptavidin-Cy3 at a final concentration of 1% (w/v) casein-PBS blocking solution. The Mouse 6 Sentrix Expression BeadChip was washed a final time in Wash E1BC buffer for 5 min and dried via centrifugation for 4 min at 275 x*g*. The array was scanned on the Illumina BeadArray reader, a confocal-type imaging system with 532 (cye3) nm laser illumination. Bead signals were computed with weighted averages of pixel intensities, and local background was subtracted. Sequence-type signal was calculated by averaging corresponding bead signals.

Illumina BeadChip microarray data analysis

Preliminary data analysis and QC was conducted using BeadStudio v3.1.3 0 software (vendor). Non-normalized fluorescent intensity of each probe on the microarray slide was obtained using the DirectHyb gene expression package in BeadStudio software (Illumina, verision 3.1.3). Fluorescent intensity filtering was performed to remove

genes that lacked a minimum relative fluorescence of 64 units in a least one time point. Data from the remaining probes were log transformed and quantile normalized

Statistical analysis

Graphpad Prism 5.0 software (Graphpad Prism San Diego, CA.) was used to determine statistical significance between two groups via unpaired one-tailed Student's *t* test and by one-way ANOVA followed by Tukey's analysis for determining differences among multiple groups The data are expressed as mean ± SEM. * $P < 0.05$, ** $p < 0.005$, ***$p < 0.0005$ were considered statistically significant.

CHAPTER III:

AMNIOTIC FLUID MACROPHAGES DISPLAY AN M1/M2 ACTIVATION STATE

Introduction

The signals leading to the initiation of term and preterm labor remain unclear. A growing body of evidence suggests that parturition is associated with an inflammatory response exemplified by increased levels of pro-inflammatory cytokines in AF, infiltration of myometrium, cervix, and fetal membranes by neutrophils and Mϕ, and with activation of NF-κB and other pro-inflammatory transcription factors in myometrium. In preterm labor, intra-amniotic infection associated with chorioamnionitis may provide the stimulus for increased levels of interleukins (*e.g*. IL-1β) and leukocyte migration. In labor at term, the stimulus and source of the invading leukocytes have not been determined.

In 2004, Condon *et al.* demonstrated compelling evidence that the fetus provides an important signal for initiation of labor near term through the developmental induction of SP-A. In these studies, SP-A expression in mouse fetal lung and secretion into AF was associated with increased expression of IL-1β by AF Mϕ and activation of NF-κB in the maternal uterus culminating in labor at term. Studies using *Rosa 26 Lac-Z* transgenic males bred to wild-type females, and immunohistochemistry identifying β-galactosidase^{+}, F4/80^{+} Mϕ suggested that the Mϕ invading the maternal uterus at term were, in part, of

fetal origin. Although the source of the Mϕ was not determined, it was suggested that they originate from the fetal lung [1]. Based on these findings, it was postulated that augmented production of SP-A by the fetal lung near term activates AF Mϕ, triggering their migration into the maternal uterus, where local production of IL-1β activates NF-κB pathways promoting expression of inflammatory genes and contraction-associated proteins (CAPs) proteins culminating in fetal expulsion at term [1, 65].

The immunomodulatory effects of SP-A are well characterized in adult alveolar Mϕ (AMϕ). Numerous studies have shown that SP-A mediates both pro- and anti-inflammatory responses. Specifically, SP-A inhibited LPS-induced cytokine and nitric oxide production [145, 291], peptidoglycan-induced TNF-α secretion [147], and suppressed reactive oxygen intermediates in response to *Mycobacterium tuberculosis* [148]. Additionally, SP-A stimulated neutrophils chemotaxis indirectly [111], activated complement [292] actin polymerization [293] and phagocytosis in Mϕ [107, 294-295].

On the other hand, SP-A also regulates inflammatory responses. Kremlev and colleagues found that SP-A stimulated production of IL-1β, IL-1α, IL-6, and interferon-gamma (IFN-γ) by human peripheral blood mononuclear cells. In rat splenocytes, peripheral blood mononuclear cells and AMϕ, SP-A enhanced TNF-α expression [296] either alone [297] or in the presence of rough LPS [298]. These seemingly contradictory findings were partially clarified by Gardai and colleagues who propose that SP-A mediates its pro- or anti-inflammatory immunomodulatory effects depending on the binding orientation of SP-A to its cognate receptor, which is, in turn, dependent on whether the receptors are bound to pathogen or to host cells. In addition, it is becoming

increasingly clear that the effects of SP-A are dependent on various factors, including the presence of pathogens, the stimulus encountered, the cell type, and the state of activation of the cell with which it interacts [133, 299].

Based on the seminal studies by Condon and colleagues, it is postulated that interactions of SP-A with AF Mϕ surface receptors at term, or bacterial LPS in preterm, result in the enhanced expression of genes associated with inflammation and Mϕ migration to the uterus. Thus, to gain insight into the phenotypic properties of this unique Mϕ population, studies were undertaken to characterize AF Mϕ activation during late gestation, in association with SP-A expression, and to obtain a profile for genes involved in their migration to the gravid uterus at term.

Results

The density of macrophage increases in amniotic fluid as term approaches

To begin characterizing the AF Mϕ population in late gestation, the total number of cells contained in AF was assessed. The volume of AF aspirated from individual amniotic sacs of single mothers was measured and the total number of cells contained in pooled samples was determined. The volume of AF in the individual sacs of single mothers at 15.5 (n=64), 17.5 (n=59), and 18.5 (n=73) dpc (19.5 = term) declined significantly approaching term. Between 15.5 and 17.5 dpc the average volume declined from ~190 μl to 78 μl ($P< 0.0001$), and remained constant between 17.5 and 18.5 dpc (Figure 4A). This decline was associated with increased viscosity of AF at 18.5 dpc making it necessary to incubate the AF with hyaluronidase (HA) in order to isolate the total cell population. HA catalyzes the hydrolysis of hyaluronic acid thereby lowering the viscosity the AF [300]. The number of cells in pooled AF samples from 15.5 (n=64), 17.5 (n=52), and 18.5 (n=59) dpc single mothers was determined using a hemacytometer and Trypan blue to identify viable cells. The number of cells markedly increased from 1.8×10^6 at 15.5 dpc, to 2.3×10^6 at 17.5 dpc ($P< 0.0001$), and further increased to 3.1×10^6 cells by 18.5 dpc ($P< 0.0001$) (Figure 4B). The total number of cells per μl of AF also was quantified. The number of cells per μl of AF greatly increased from 0.97×10^4 to 3.3×10^4 between 15.5 and 17.5 dpc ($P < 0.0001$), and increased further to 4.4×10^4 cells per μl by 18.5 dpc ($P < 0.0001$) (Figure 4C).

Amniotic fluid macrophages are highly vacuolated and are morphologically similar to fetal lung macrophages

To determine whether the increase in AF cells contained a proportionally increasing Mϕ population, 15.5 and 18.5 dpc AF Mϕ, isolated by adhesion, were immunostained using an antibody directed against the F4/80 cell surface antigen. The proportion of F4/80$^+$ Mϕ was found to increase between 15.5 (Figure 5A) and 18.5 (Figure 5B) dpc in AF near term. Morphological analysis by light microscopy and Diff-Quik staining revealed the presence of numerous vacuoles within the cytoplasm of 15.5 (n=5), and 18.5 (n=5) dpc AF Mϕ giving them a foam cell-like appearance. Briefly, Diff-Quik stain is a modified differential stain used in detailed cytoplasmic evaluation of intra-cytoplasmic mucins and secretory granules. Additionally, the number and size of the vacuoles increased between 15.5 to 18.5 dpc, yielding larger cells by term (Figure 5B). To confirm these findings, F4/80$^+$ AF Mϕ were sorted at 15.5 (n=5), 17.5 (n=5) and 18.5 (n=5) dpc and measured using the Countess® Automated Cell Counter. Consistent with the histological observations, Mϕ increased significantly in size from 11.5 to 14.7 μm ($P = 0.0044$) between 15.5 and 18.5 dpc (Figure 5B).

One phenotypic characteristic of pulmonary alveolar Mϕ (AMϕ) is a foam cell-like appearance [301]. As such, the foam cell-like appearance of the AF Mϕ suggests they may arise from the fetal lung. To further explore this possibility, a morphological comparison between time-matched AF Mϕ and fetal lung Mϕ was carried out. To this end, cells obtained from single-cell suspensions of fetal lung were compared to F4/80$^+$

AF Mϕ isolated at 18.5 dpc. Following Diff-Quik staining, cell morphology was compared by light microscopy. AF Mϕ (Figure 6A) and fetal lung Mϕ (▲) (Figure 6B) contain numerous vacuoles within their cytoplasm and are morphologically similar.

Pulmonary surfactant, exclusively synthesized in alveolar type II cells, is composed primarily of glycerophospholipids and four surfactant proteins, including SP-A [80]. Once secreted into the lung airspace, surfactant is taken up by AMϕ and type II cells [302-303]. *In vitro* studies demonstrated that AMϕ take up and catabolize the lipid and protein components of surfactant [304]. Thus, to determine whether AF Mϕ contained lipids within their cytoplasmic vacuoles, 18.5 dpc AF Mϕ were stained with Oil Red O, a neutral lipid-specific stain, (Figure 7B) and compared to AMϕ isolated from bronchiolar lavage obtained from adult ICR mice (Figure 7A). Results demonstrated the absence of neutral lipids (indicated by the absence of red stain) within the vacuoles of 18.5 dpc AF Mϕ (Figure 8B).

To further examine the proposed uptake of surfactant by AF Mϕ and their trafficking to the maternal uterus, immunocytochemical analysis was performed for detection of SP-A protein on AF Mϕ and within the maternal uterus at term. The results demonstrated the presence of SP-A-positive Mϕ (▲) within the AF (Figure 8A) and the maternal uterus (Figure 8B) at 18.5 dpc (cells in Panels A and B were identified as Mϕ based on their morphological characteristics by pathologists Drs. Linda Margraf and James Richardson). The presence of multi-lamellar bodies within the cytoplasmic vacuoles of AMϕ are well characterized [301]. Thus, to determine whether lamellar bodies were contained within AF Mϕ vacuoles, transmission electron microscopy was

used to survey 15.5 (n=2) (Figure 9A) and 18.5 (n=2) dpc cells (Figure 9B). The images revealed the presence of lamellated bodies (red box) within cytoplasmic vacuoles at 18.5 dpc (Figure 9B), but not at 15.5 dpc, which is prior to the time that surfactant is produced by the fetal lung.

Flow cytometric analysis demonstrates upregulated expression of TLR2, TLR4/MD2 and CD14 in late gestation amniotic fluid macrophages

Fluorescence Activated Cell Sorting (FACS) was used to analyze the expression of membrane receptors and characterize changes in the cellular phenotype of AF Mϕ. The proportion of $CD45^{+}F4/80^{+}$ cells within the total AF population was assessed using antibodies that identified CD45, a pan-leukocyte marker, and F4/80. The results demonstrated a steady increase of $CD45^{+}F4/80^{+}$ cells from 50% at 15.5, to 64% at 17.5 dpc, reaching 70% by day 18.5 dpc (Figure 10A-D). The increase in Mϕ density is in agreement with the immunohistological studies previously carried out (Figure 5B).

Exclusive use of F4/80 or CD11b in the identification of Mϕ subpopulations may identify distinct Mϕ subpopulations. Therefore, the presence of CD11b and F4/80 populations were analyzed to identify the potential existence of distinct Mϕ subpopulations within AF during late gestation. The data revealed the presence of a single double-positive population at 15.5, 17.5 and 18.5 dpc (data not shown). The percent of $CD11b^{+}F4/80^{+}$ cells isolated from late gestation AF increased from 54% to 78% between by 15.5 and 18.5 dpc ($P < 0.0001$) and remained constant at 75% of the

total AF population between 17.5 and 18.5 dpc (Figure 11A). Next, expression of receptors reported to bind SP-A were examined. As previously mentioned in Chapter One, TLR2 [147] and TLR4/MD2 [137, 139] are known to bind SP-A. Additionally, CD14 has also been reported to bind SP-A, although the mechanism of its binding remains unclear [136]. Thus, the expression of these receptors on AF Mϕ was examined at 15.5, 17.5 and 18.5 dpc. The results demonstrated a significant increase in the expression of TLR2 and TLR4/MD2 in $CD11b^{+}$ Mϕ between 15.5 and 17.5 dpc (Figure 11B, C). Expression of TLR2 increased from 45% to 76% between 15.5 and 17.5 dpc ($P < 0.0001$) and remained constant at 78% at 18.5 dpc. TLR4/MD2 expression increased from 17% to 58% between 15.5 and 17.5 dpc ($P < 0.0001$) then declined to 48% between 17.5 and 18.5 dpc ($P = 0.0407$). On the other hand, CD14 expression initially declined from 48% to 43% between 15.5 and 17.5 dpc ($P = 0.0062$) and increased to 63% by 18.5 dpc ($P = 0.0149$) (Figure 11D).

Intercellular adhesion molecule 1 (ICAM-1), also designated CD54, is a 95-kDa member of the Ig superfamily expressed on a variety of cells including Mϕ. Its expression is up-regulated upon stimulation by inflammatory mediators such as cytokines (*e.g*. TNF-α) or LPS. ICAM-1 is a ligand for leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2) and facilitates leukocyte transmigration [305]. Additionally, CD54 expression is indicative of cellular activation [306]. Thus, to gain insight into the migratory capacity and activation status of AF Mϕ, the expression of CD54 was examined. Between 15.5 and 17.5 dpc, CD54 expression increased markedly from 28% to 67% ($P < 0.0001$) and increased further to 72% by 18.5 dpc (Figure 11E).

Amniotic fluid macrophages upregulate classical and alternative activation markers approaching term

To obtain a phenotypic profile of AF Mϕ during late gestation, I used the Illumina Mouse WG-6 Expression BeadChip microarray platform to survey the expression of genes associated with Mϕ activation status, migration and immunoregulatory functions at 15.5, 17.5 and 18.5 dpc. Analysis of F4/80^{+} AF Mϕ gene expression was analyzed using BeadStudio v3.1.3 software using the cutoff criterion of 1.5-fold differential expression and with statistical significance at $P< 0.05$. The results of the profiling showed that genes associated with both Mϕ M1 and M2 phenotypes were upregulated in tandem between 15.5 and 18.5 dpc in F4/80^{+} AF Mϕ. Expression of M1-associated genes included IL-1β and IL-6 (Figure 12A). M2-associated genes included Arg 1, FIZZ1, Ym1, and Ym2 (Figure 12B). Additionally, genes involved in migration, CCR2, CD62L and Pecam1 were upregulated between 15.5 and 18.5 dpc (Figure 12C). Immunoregulatory genes including IL-1RII and PD-L1, also were upregulated (Figure 12D). To confirm the validity of the microarray data, Arg 1 (Figure 13A), Ym1 (Figure 13B), Ym2 (Figure 13C), IL-1β and TNF-α (Figure 13D) expression were analyzed in F4/80^{+} AF Mϕ between 15.5 and 18.5 dpc using quantitative real time PCR. The results confirmed the increased expression of both M1 and M2 Mϕ-associated genes ($P < 0.001$) at term.

SuperArray qRT-PCR based gene profiling demonstrates maximal expression of inflammatory cytokines, chemokines and their receptors at term

To assess the range of the inflammatory genes expressed in AF Mϕ near term, Superarray® qRT-PCR-based inflammatory gene array panels were analyzed. Comparison between 15.5 and 17.5 dpc and 15.5 and 18.5 dpc F4/80^{+} AF Mϕ revealed a remarkable trend in which many of the inflammatory genes upregulated at 17.5 dpc were further upregulated at term (18.5 dpc) (Figure 14). Moreover, the elevated expression of cytokines IL-1β and IL-6 (Figure 15A) was accompanied by increased expression in chemokines including CCL2, CCL5, CCL11, CCL25, CXCL1 and CXCL5 (Figure 15B). Chemokine receptors CCR2, CCR3, CCR9, CXCR3 and CXCR5 expressed at 17.5 dpc were further up-regulated by 18.5 dpc (Figure 16A). Interestingly, expression of a cluster of chemokines, including CCR10, IL-1RI, IL-8Rb, lymphotoxin-alpha (Lta), and Xcr1, was detectable only at 18.5 dpc (Figure 16B).

Discussion

Surfactant protein A is the major pulmonary surfactant protein and plays a key role in pulmonary innate immunity. Surfactant protein-A modulates host interactions with viruses [90], fungi [91], and a wide range of microbial pathogens [93-95] by acting as an opsonin or as an activating stimulus. Interactions with AMϕ have been shown to regulate both pro- and anti-inflammatory actions via its binding orientation [133]. During late gestation, augmented production of SP-A by the fetal lung and secretion into AF has been proposed to activate fetal-derived AF Mϕ, triggering their migration into the gravid uterus where they promote inflammation culminating in labor at term [1]. The expression of IL-1β by AF Mϕ points to an M1-activation status. However, these finding appear to be inconsistent with reports by Wilbanks and Streilein, which demonstrate that AF endows peritoneal Mϕ with anterior chamber-associated immune deviation (ACAID)-inducing properties [228]. Notwithstanding, it is plausible that temporal secretion of SP-A into AF, into an immune suppressive microenvironment, serves to polarize AF Mϕ to an M1 pro-inflammatory state at a time when termination of immune privilege is desirable for mediating fetal expulsion. To gain a better understanding of the dynamic phenotypic changes in AF Mϕ during late gestation, studies were undertaken to characterize their activation state, in association with SP-A production, and to obtain a profile of the genes that accompany their migration to the pregnant uterus at term.

To begin characterizing AF Mϕ during late gestation, the total number of cells contained within the AF compartment was determined at 15.5, 17.5 and 18.5 dpc. The

cell counts revealed a remarkable increase in the total number of cells between 15.5 and 17.5 dpc which further increased by 18.5 dpc (Figure 4C). Immunohistochemistry using F4/80 antibody demonstrated a proportional increase in the density of F4/80^{+} Mϕ in AF between 15.5 and 18.5 dpc (Figure 5A) that was further confirmed by flow cytometric analysis (Figure 11A).

Morphological studies demonstrated the AF Mϕ possessed a striking morphological phenotype characterized by a 'foam cell-like' appearance during late gestation (Figure 5B). Numerous vacuoles within the cytoplasm increased in size and number, causing the cells to be larger at term. Measurement of F4/80^{+} AF Mϕ, indeed, confirmed significant enlargement by 18.5 dpc (Figure 5C). The highly vacuolated appearance observed in AF Mϕ is reminiscent of lung AMϕ morphology [301, 307-308]. Comparison of time-matched F4/80^{+} AF Mϕ and fetal lung Mϕ demonstrated their conspicuous similarity at 18.5 dpc (Figure 6). Importantly, the increase in size and density of AF Mϕ, in parallel with increasing SP-A concentrations within AF, are consistent with reported effects of surfactant on AMϕ. In studies by Kramer *et al.,* serial intra-tracheal instillation of exogenous surfactant was shown to increase the density and size of AMϕ recovered from the lungs of mice 6 h following the last instillation of surfactant. Amazingly, the number of AMϕ recovered by bronchioalveolar lavage increased by 82% compared to untreated animals. Moreover, with administration of exogenous surfactant, the AMϕ became 'larger' with increased numbers of vacuoles characteristic of lipid-laden or foam Mϕ [309]. In the present study, the presence of lamellated bodies within the cytoplasmic vacuoles of AF Mϕ demonstrates the uptake of

surfactant. It is important to note that lamellar bodies may be taken up within the fetal lung, where AF Mϕ are suggested to originate. Alternatively, secreted surfactant may be phagocytized within the AF compartment. The presence of SP-A^+ Mϕ within the AF and maternal uterus at 18.5 dpc further serves to confirm SP-A-AF Mϕ interactions (Figure 8A,B). Taken together, these studies strongly suggest that SP-A induces changes in AF Mϕ phenotypic properties and lend support to the contention that AF Mϕ originate in the fetal lung.

In the studies carried out by Kramer and colleagues, changes in the morphological phenotype of AMϕ following intra-tracheal administration of surfactant were not associated with changes in their activation state. This observation was based on the unchanged expression levels of CD14, CD16, CD54, SR-AI, and SR-AII. Briefly, CD14, in addition to being an accessory protein involved in LPS signaling, also plays a role in apoptosis and modulation of inflammation [310]. CD16 is a low affinity Fc receptor FCγRIII component [311] and CD54 (ICAM-1) is involved in leukocyte transmigration and cellular activation [312]. Scavenger receptor A type I (SR-AI) and SR-AII are scavenger receptors known to be involved in cell adhesion [313]. The finding that the AMϕ did not change their activation status is not surprising in light of the studies by Gardai *et al.* which suggest that in an unbound state (absence of pathogen), the CRD domain of SP-A inhibits Mϕ activation by binding to SIRP-α, which in turn inhibits p38-mediated activation of NF-κB [133]. In support of this notion are numerous studies that have established that, in the absence of infection, AMϕ manifest an M2 phenotype as a direct result of the high SP-A concentrations within the lungs [130-133, 314]. However,

SP-A has also been demonstrated to induce transcription of pro-inflammatory mediators in the absence of pathogen. This is dependent on the cytokine milieu within the microenvironment, as well as other factors previously discussed in Chapter One, including binding orientation.

TLR2 and/or TLR4/MD2 may mediate the temporal activation of amniotic fluid macrophages near term

TLRs are a family of evolutionary ancient mammalian pattern recognition receptors (PRRs) expressed across all vertebrate species that are designed to recognize specific molecular patterns unique to bacterial, viral and fungal pathogens. Binding of PPRs to pathogen-associated molecular patterns (PAMPs) signal infection ("danger signal") and activate molecular cascades that control transcription of pro-inflammatory genes [315]. TLRs also bind to endogenous non-pathogenic ligands (e.g. exogenous and endogenous heat-shock protein 60 and 70 [316-317]) underscoring their involvement in normal physiological processes.

To date, 11 mouse and 10 human TLRs have been identified. Distinction among TLRs is based on recognition of cognate ligand(s). TLR2 and TLR4 recognize bacterial cell wall components lipoteichoic acid (LTA) and lipopolysaccharide (LPS), respectively. Upon binding ligands, TLR2 and TLR4 initiate a signaling cascade directing expression of various pro-inflammatory genes such as IL-1, IL-6 and TNF-α, as well as induction of other cytokines, chemokines, such as KC (IL-8 in humans), MCP-1, and type 1 interferon (IFNs) genes [318-319]. Signaling is dependent on recruitment of intracellular molecules

such as myeloid differentiation factor (MyD88), a common adaptor involved in several TLR signal transduction pathways. This results in activation and nuclear localization of the transcription factor NF-κB [320]. Unlike TLR2, TLR4 can also signal through MyD88-independent pathways [321-322]. In TLR4 receptor signaling, the presence of LPS associated with CD14 or MD-2 initiates a pro-inflammatory signaling cascade [137]. LPS-induced TLR4 signaling results in activation of mitogen activated protein (MAP) kinase and NF-κB, leading to secretion of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α [140].

Harju and colleagues recently demonstrated that during ontogeny in mice, TLR2 and TLR4 mRNA levels increase 7-fold within the fetal lung between 15 dpc and term [323]. Whether this increase is due to upregulated expression in lung tissue or AMϕ was not determined. As mentioned in Chapter One, TLR2 [147] and TLR4 [137, 139] also serve as receptors for SP-A. CD14 has also been reported to bind SP-A, although the mechanism of its binding is not yet known [136]. Using flow cytometric analysis, a remarkable increase in the expression of TLR2 (Figure 11A) and TLR4/MD2 (Figure 11B) was observed on AF Mϕ between 15.5 and 18.5 dpc. In addition, levels of CD14 (Figure 11D) also increased at term. The enhanced expression of TLR2 and TLR4/MD2 correlates well with the presence of SP-A protein within AF. Interestingly, studies by Henning *et al.* demonstrated that SP-A differentially regulates transcriptional and post-transcriptional TLR2 and TLR4 expression in human Mϕ [324]. In terms of CD14, the potential reasons for its decline between 15.5 and 17.5 dpc, and subsequent up-regulation by 18.5 dpc, are not immediately obvious, but may be due to a latent post-transcriptional

response to SP-A binding. Further studies will be required to define the mechanisms underlying these observations. The concomitant increased expression of TLR2 and TLR4/MD2 in AF Mϕ and SP-A secretion into the AF during late gestation suggests a temporal mechanism for activation of inflammatory response genes. The enhanced expression of CD54 between 15.5 and 18.5 dpc on AF Mϕ (Figure 11E) supports this notion as its augmented expression not only points to their migratory capacity but is also indicative of a switch to an inflammatory state. Taken together, these data may explain how temporal activation of AF Mϕ is achieved as term approaches.

Amniotic fluid macrophages upregulate expression of M1 and M2-associated markers

Macrophage heterogeneity is the hallmark of the mononuclear system. The extent of Mϕ plasticity is reflected by the complex repertories characterized *in vivo*. The Th1/Th2 paradigm for Mϕ activation has previously been discussed in Chapter One. Briefly, classical, or M1, activation of Mϕ occurs in response to type I cytokines, endogenous 'danger' signals (*e.g*. heat shock proteins) or upon recognition of microbes through recognition of PAMPs by PRPs. This leads to anti-proliferative and cytotoxic activities, resulting partly from their ability to secrete reactive oxygen species and pro-inflammatory cytokines (*e.g.*, IL-1, IL-6, and TNF). Upon neutralization of the inciting stimulus, Mϕ anti-inflammatory effector functions are temporally and spatially engaged to initiate the resolution of inflammation to prevent collateral damage to the host. In this regard, alternatively activated, or M2, Mϕ promote healing and tissue repair through

production of anti-inflammatory cytokines, chemokines (*e.g.* IL-10, TGF-β) and molecules that antagonize pro-inflammatory mediators (*e.g*. IL-1RII (IL-1 receptor decoy)). The molecular markers used to differentiate between the two activation states include expression of IL-1, IL-6, and TNF-α, for M1 Mϕ, and Arg 1, FIZZ1, Ym1 and Ym2, for M2 Mϕ.

In the present studies, microarray gene profiling of late gestation F4/80^{+} AF Mϕ demonstrated upregulated expression of M1 and M2-associated genes approaching term. Specifically, expression of M1-associated cytokines, IL-1β and IL-6, were dramatically upregulated between 15.5 and 18.5 dpc (Figure 12A). Additionally, expression of M2-associated markers, including Arg 1, FIZZ1, YM1, and YM2, were more modestly upregulated in tandem. Although Condon *et al*. [1] previously demonstrated IL-1β expression by AF Mϕ at term, a parallel expression in M2 markers was unexpected. Quantitative real time PCR of Arg 1, Ym1, Ym2, IL-1β and TNF-α, served to confirm their enhanced gene expression levels between 15.5 and 18.5 dpc (Figure 13). These data suggest that AF Mϕ possess an M1/M2 'mixed' phenotype near term.

Interestingly, IL-1RII and PD-L1 were also upregulated between 15.5 and 18.5 dpc (Figure 12D). IL-1RII is a type I interleukin-1 decoy receptor with a short cytoplasmic tail that when bound by its cognate ligands, IL- 1α and IL-1β, does not transduce a signal thereby antagonizing the actions of pro-inflammatory mediators. Expression IL-1RII is characteristically elevated in M2 Mϕ [325]. Programmed-death ligand-1 (PD-L1) is a ligand for the co-stimulatory molecule PD-1. PD-1 plays an inhibitory role in regulating peripheral T cell activation. It is expressed on various cells

including Mϕ [326]. In mice, its expression can be induced on resident Mϕ by LPS, IFN-γ or polyinosinic-polycytidylic acid. Further up-regulation of PD-L1 can be induced by exposure to LPS or IFN-γ. Studies of the mechanisms involved in PD-L1 expression revealed that the induction by LPS and IFN-γ is regulated by TLR4 and STAT1 signaling following activation [327]. Consequently, PD-L1 expression is linked to M1 activation. The enhanced expression of IL-1RII and PD-L1 in late gestation lends further support to the M1/M2 'mixed' phenotypic profile of AF Mϕ in late gestation and implies their immuno-regulatory capacity.

The AF microenvironment contains both pro- and anti-inflammatory cytokines and chemokines. In studies conducted by Orsi and colleagues [328], the cytokine networks in AF and in the maternal circulation of mice during late gestation were shown to behave in a distinct manner. In the maternal serum, pro-inflammatory cytokines, such as IFN-γ, IL-1β, IL-6, and TNF-α, predominated at term. However, this Th1 shift was not reflected in the AF. Instead, AF contained a mixture of pro- and anti-inflammatory associated cytokines and chemokines (*e.g.* IL-4, IL-6, IL-10, IL-12(p40), MCP-1, and KC) [328]. Although these findings reflect some of the phenotypic characteristics observed in late gestation AF Mϕ, they do not reveal how temporal M1 activation is promoted. Moreover, as mentioned, Wilbanks and Streilein demonstrated that AF endows Mϕ with ACAID-inducing properties [329], which encompass deviation of inflammatory responses. Therefore, the AF cytokine milieu cannot fully account for the M1/M2 'mixed' activation status observed in Mϕ during late gestation and strongly argues for the role of SP-A as an inciting stimulus.

Hyaluronan in amniotic fluid

The glycosaminoglycan hyaluronan (HA)**,** a component of the extracellular matrix (ECM), is a negatively charged high-molecular-weight (HMW) straight chain polymer consisting of repeating units of the disaccharide GlcNAc(1→4)GlcUAβ(1-3) and can reach up 10^6 saccharides (4×10^2 - 2×10^4 kDa) in length [330-331]. In mammals, HA it is primarily synthesized on the cytoplasmic surface of the plasma membrane of fibroblasts by three HA synthases (HAS) and is exported as it is synthesized [332-334]. HMW-HA is abundant in the basement membrane of the lung, where it makes up 10% of the proteoglycan content [335], the vitreous humor of the eye [336], the synovial fluid of joints, the intercellular space of the epidermis, in skeletal tissues, the umbilical cord, in the heart valve and in amniotic fluid [337-338]. It is thought to play a crucial role in water homeostasis, plasma protein distribution, joint lubrication, and in the maintenance of matrix integrity [339]. In vivo, HMW-HA is present in healthy intact tissue and is anti-inflammatory, immunosuppressive, anti-angiogenic, and does not activate immune cells [340-341]. This is thought to be due, in part, to its ability to coat the cell thereby preventing cell-to-cell interactions and ligand accessibility with its cognate cell surface receptors [342]. It has also been shown to be involved in various normal physiological processes such as ovulation, embryogenesis and wound healing [330, 343-344]. Additionally, it is proposed to attenuate inflammation at sites of tissue injury, to inhibit LPS-induced pro-inflammatory cytokine production by monocytes and Mϕ [345-346] and

has been demonstrated to play an immunosuppressive role in the amniotic fluid during development [330, 338].

During cellular stress, tissue injury or infection, HMW-HA becomes depolymerized and fragmented resulting in the production of low-molecular-weight (LMW)-HA fragments. In contrast to HMW-HA, production of discrete intra- and extracellular LMW-HA fragments, <1000 saccharides, by oxygen radicals, hyaluronidase, β-glucronidase, and hexosaminidase, leads to the generation of LMW-HA fragments, which can serve as host-derived danger signals. Some of these LMW-HA fragments have been demonstrated to be angiogenic, pro-inflammatory, and immunostimulatory [347-348]. However, very small LMW fragments, < 4 saccharides, have the capacity to ameliorate the effects of the larger LMW-HA and are anti-apoptotic and can induce the expression of heat-shock proteins. How these small LMW-HA fragments are generated is unknown. What is clear is that breakdown of HMW-HA to LMW-HA fragments results in a broad range of fragment sizes that possess a vast array of opposing physiological functions including, but not limited to, the induction of sterile inflammation, immune reactions, angiogenesis, neoplastic cell migration and metastatic spreading of malignant tumors [330, 349]. Studies to determine how these fragments induce such a wide-range of physiological responses are ongoing.

In *in vitro* studies by JinXiang *et al*., LMW-HA (<24 oligosaccharides) was shown to activate Kupffer cells (liver macrophages) via the TLR4 signaling pathway in a dose-dependent manner that was, in part, dependent on p38 MAPK activation [350]. Others have shown that LMW-HA fragments (4.7×10^5 Da, and 2.8×10^5 Da) have the

ability to induce inflammation-associated chemokine gene expression (i.e. MIP-1α, MIP-1β, MCP-1, RANTES, IL-8) in the murine alveolar macrophage cell line MH-S, the monocytic cell line THP and in human alveolar Mϕ obtained by bronchioalveolar lavage from patients with idiopathic pulmonary fibrosis [351]. Yet, in other studies, LMW-HA, <500 kDa, and many in the 100-250 kDa range, were shown to induce inflammatory responses on inflammatory but not in in vivo resident macrophage populations [351-355]. Thus, it appears that LMW-HA fragments have a diverse range of opposing biological functions that are not only influenced by the fragment size but also by the cell type and the receptor with which they interact [348].

In human, Dahl and colleagues demonstrated that the concentration of HA in the AF collected during amniocentesis and at term from normal deliveries declines from approximately 20μg/ml between the 16-20th week to 1 μg/ml at term in humans [338, 356]. Using gel chromatography, they later revealed that HMW-HA predominates at 16 weeks of gestation, while samples isolated at 40 weeks contained a both HMW- and LMW-HA (>10^6 to <10^5 daltons) fragments [356]. The precise range of the LMW-HA in the AF was not determined as fragments less than 10^3 were not characterized. Nevertheless, the presence of HMW-HA and the fact that human AF possess wound healing properties [343, 357-358], and can attenuate the inflammatory responses in Mϕ [329], demonstrates its anti-inflammatory properties. Additionally, the required use of hyaluronidase to solubilize late gestational mouse AF for characterization of Mϕ in the present study, points to the predominance of HMW-HA in the AF during late gestation. Together, these data suggest that HA in the AF functions to protect the fetus from overt

inflammatory responses during gestation. However, further investigation will be required to confirm this supposition and to explore the possibility that HA contributes to the observed changes in AF Mϕ activation status.

In the studies presented herein, a survey of the expression of inflammation-related genes revealed a remarkable expression pattern in which many of the genes upregulated at 17.5 dpc were further up-regulated by 18.5 dpc (Figure 14). It. is possible that these changes in gene expression are directly due to AF Mϕ activation by SP-A. Indeed, the general pattern of enhanced inflammatory gene expression follows the temporal pattern of SP-A production by the fetal lung. The robust expression of various inflammatory cytokines, chemokines, and chemokine receptors observed in AF Mϕ by qRT-PCR demonstrate an escalating response to an inciting stimulus. For example, the profiling results demonstrated the enhanced expression of many well-characterized inflammatory cytokines (*e.g.* IL-1β, IL-6) (Figure 15A), chemokines (*e.g.* CCL2, CCL5, CCL11, CCL25, CXCL1, CXCL5) (Figure 15B), and chemokine receptors (CCR2, CCR3, CCR9, CXCR3, CXCR5) (Figure 16A). Interestingly, a cluster of chemokine receptors, including CCR10, IL-1R1, IL-8Rb, lymphotoxin a (Lta) and Xcr1 (Figure 16B) were upregulated at 18.5 dpc, when SP-A concentrations reach maximal levels. Together, these data serve to demonstrate the vigorous expression of AF Mϕ inflammatory genes within an immune privileged site. Moreover, AF Mϕ may play an active role in promoting inflammation by producing a wide range of chemokines and promoting the chemotaxis of immune cells to the AF compartment. Additionally, their

chemokine receptor expression profile suggests their capacity to be recruited to other sites of inflammation (*e.g.* myometrium).

PECAM-1, L-selectin, and CCR2 may mediate amniotic fluid macrophage migration to the gravid uterus at term

Adhesion molecules regulate leukocyte trafficking. Platelet-endothelial-cell adhesion molecule 1 (PECAM-1), also known as CD31, is a glycoprotein belonging to the Ig superfamily of cell adhesion molecules. PECAM-1 is expressed on a variety of cells including Mϕ and plays a critical role in transmigration. As implied by its name, it is involved in leukocyte-endothelium interaction and transendothelial migration during inflammation [359-360]. CD62L (L-selectin) is expressed on leukocytes and is important for their tethering and subsequent rolling on endothelial cells. L-selectin is known to facilitate leukocyte migration into secondary lymphoid organs and to sites of inflammation [361]. In AF Mϕ, PECAM-1 and L-selectin expression is upregulated between 15.5 and 18.5 dpc (Figure 12C) pointing to their capacity for transmigration at term.

As previously discussed in Chapter One, several studies have demonstrated leukocyte infiltration of uterine tissues near term [67, 362]. Macrophages account for 22% of the invading population in the gravid uterus of mice [363] and are postulated to contribute to the local cytokine production leading to inflammation and the initiation of labor [1, 4]. In human myometrium, monocyte chemoattractant protein-1 (MCP-1), also known as CCL-2, is highly expressed during labor [364]. MCP-1 is a chemoattractant

that locally mediates leukocyte migration into tissues where it is expressed [365-366]. Moreover, it is proposed to contribute to the initiation of normal labor via recruitment of Mϕ [367]. MCP-1 interacts with C-C chemokine receptor 2 (CCR2). Interestingly, AF Mϕ demonstrate enhanced expression of CCR2 between 15.5 and 18.5 dpc (Figure 12C). It is therefore possible that augmented expression of CCR2 allows AF Mϕ to respond the increasing MCP-1 gradient within the gravid uterus and in this manner, initiates or contributes to the termination of immune privilege.

Amniotic fluid macrophages display an M1/M2 'mixed' activation state at term

The data presented herein, demonstrate that AF Mϕ undergo both morphological and phenotypic changes in association with SP-A expression in AF. The expression of M1 and M2-associated markers suggests that AF Mϕ posses a M1/M2 'mixed' phenotype near term. Moreover, their phenotypic profile implies that they are a distinct subclass of Mϕ capable of modulating both pro- and anti-inflammatory functions simultaneously in response to dynamic changes within the AF milieu. Furthermore, enhanced expression of adhesion molecules, PECAM-1, L-selectin and ICAM-1, points to their capacity for transmigration in response to increasing levels of MCP-1 in the laboring uterus.

The discovery that SP-A polarizes AF Mϕ to an M1 state, in the absence of infection or antigenic stimuli, and also within an immune privileged site, is intriguing for several reasons. First, during pregnancy, numerous fetal-derived mechanisms are invoked to sustain immune privilege and immune suppression. These mechanisms are designed to

thwart inflammation, thereby protecting the fetus from the maternal immune system throughout gestation, ensuring its viability. It is, therefore, not surprising that a maternal shift away from a $CD4^+$ T cell pro-inflammatory Th1 phenotype to an immunoregulatory Th2 phenotype is crucial in determining pregnancy outcome [283]. What is unexpected is the presence of an M1 Mϕ population within an immune privileged site that would be predicted to polarize local Mϕ to an M2 phenotype. Second, the ability of SP-A to activate AF Mϕ, triggering their egress from the fetal compartment into foreign maternal tissues, suggests that this Mϕ population is phenotypically unique. Finally, infiltration of the pregnant uterus at term by this fetal-derived subpopulation suggests that AF Mϕ may play a role in abrogating immune privilege. As a consequence of entering foreign maternal tissues, AF Mϕ may both provoke and invoke an inflammatory response and, in this manner, promote or contribute to the initiation of the inflammatory cascade leading to labor at term. This notion is consistent studies of Condon *et al.* in that infiltration of the maternal uterus by fetal Mϕ serves to initiate labor at term.

To further explore the unique properties manifested by AF Mϕ near term, it will be important to determine whether this phenotypic profile is reflective of a dynamic change in a single population, from M2 to M1, or whether two discrete subpopulations coexist within the AF compartment. It is also possible that they indeed represent a novel Mϕ subpopulation, in which case further characterization is warranted.

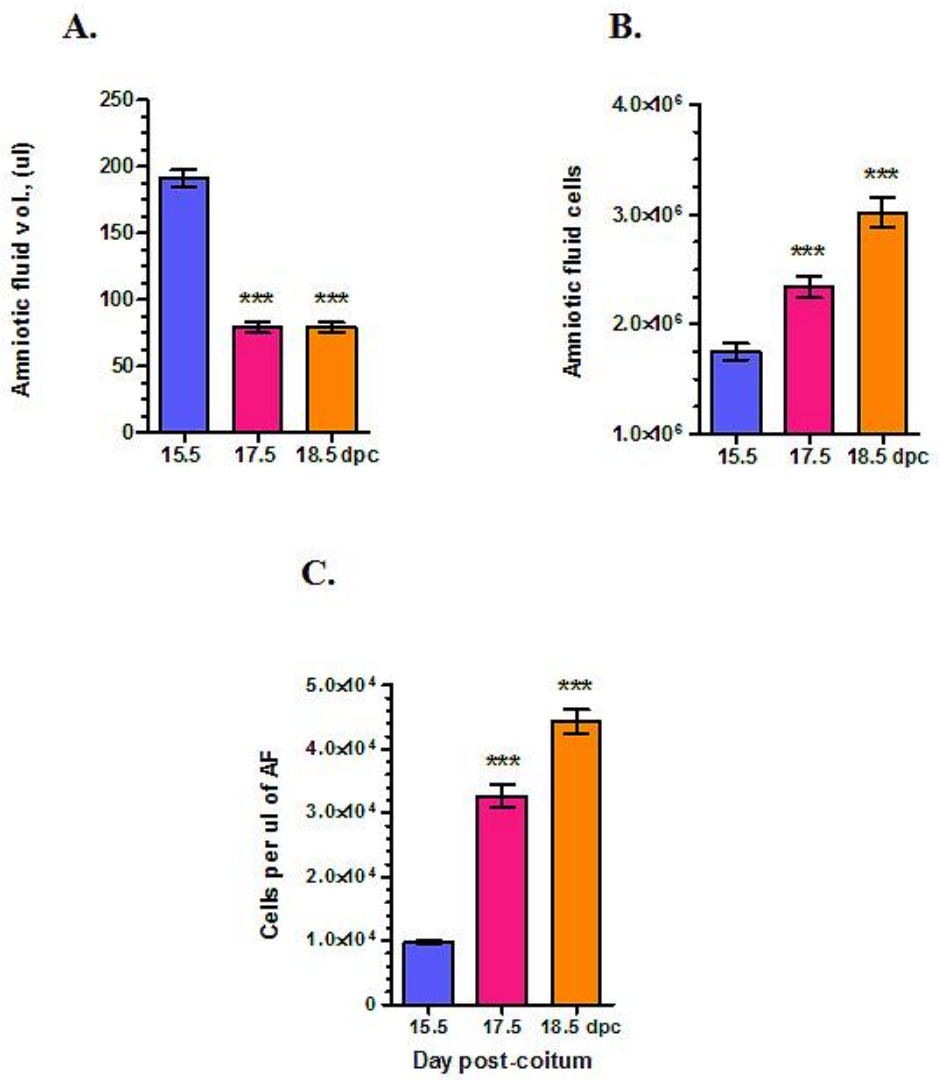
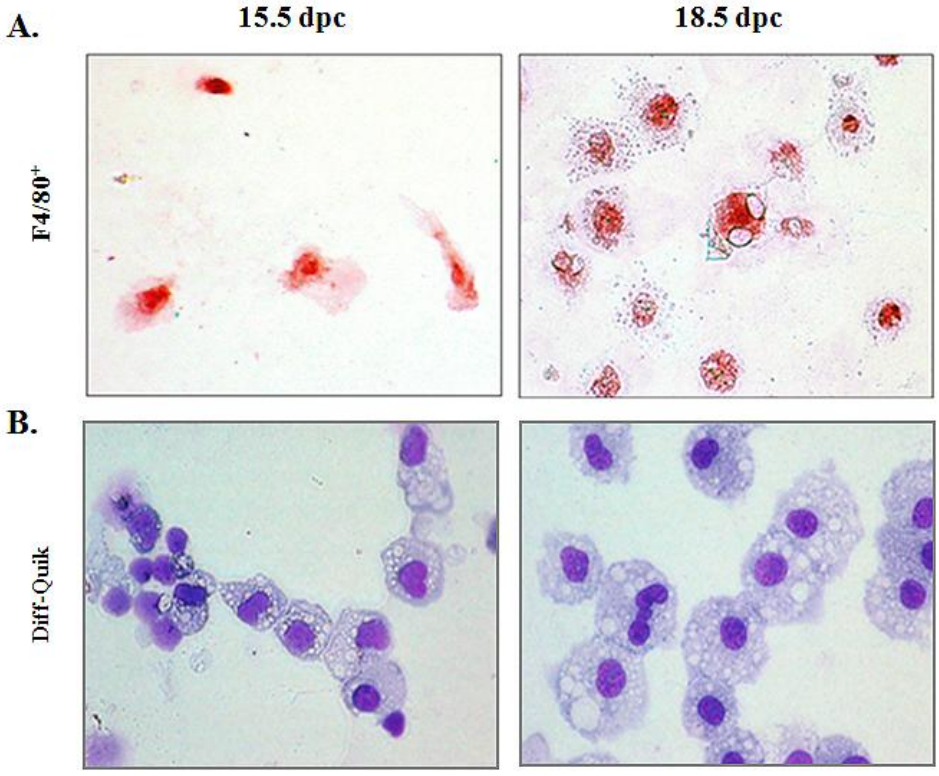


Figure 4. The total number of cells in AF increases as term approaches. The volume of pooled AF aspirated from all individual amniotic sacs of each pregnant mouse at 15.5 dpc (n=64), 17.5 dpc (n=59), and 18.5 dpc (n=73) was measured (A). The total number of cells in each pooled AF sample was determined using a hemacytometer and Trypan Blue exclusion (B). The number of cells per μl of AF was determined at 15.5 dpc (n=65), 17.5

dpc (n=33), and 18.5 dpc (n=74) (C). Significance between two groups was determined using the unpaired one-tailed Student's *t* test. Differences between multiple groups were assessed by one-way ANOVA followed by Tukey's analysis. The data are expressed as mean ± SEM. ***P < 0.05 is considered statistically significant.


A.
15.5 dpc
18.5 dpc
F4/80+
B.
Diff-Quik

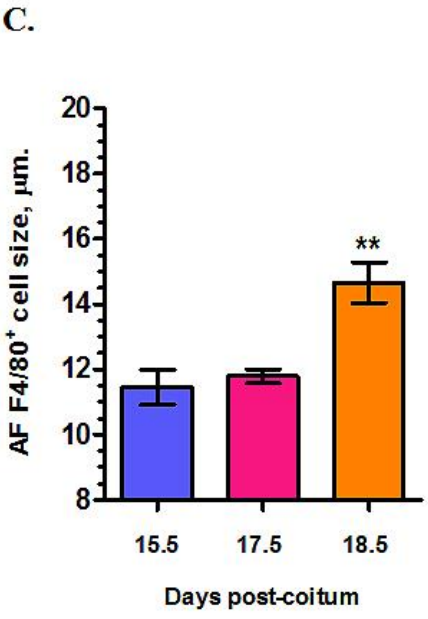

C.
AF F4/80+ cell size, µm.
20
18
16
14
12
10
8
**
15.5
17.5
18.5
Days post-coitum

Figure 5. The density and size of F4/80^{+} macrophages in amniotic fluid increase in late gestation. AF cells isolated at 15.5 and 18.5 dpc were either cytospun onto slides or allowed to adhere to two-chamber slides for 1 h. Adherent cells were immunostained using antibody directed against the F4/80 at 15.5 (n=3) and 18.5 dpc (n=3) (A). For morphological analysis, AF cells were isolated and F4/80^{+} cells were sorted using a FACS Aria cell sorter. Cell morphology was examined at 15.5 dpc (n=5) and 18.5 dpc (n=5) followed by Diff-Quik staining (B). The sizes of F4/80^{+} AF Mϕ at 15.5 (n=5), 17.5 (n=5) and 18.5 (n=5) are compared using the Countess® Automated Cell Counter (C). All images are at 20X magnification. Significance between two groups was determined by the unpaired one-tailed Student's *t* test.

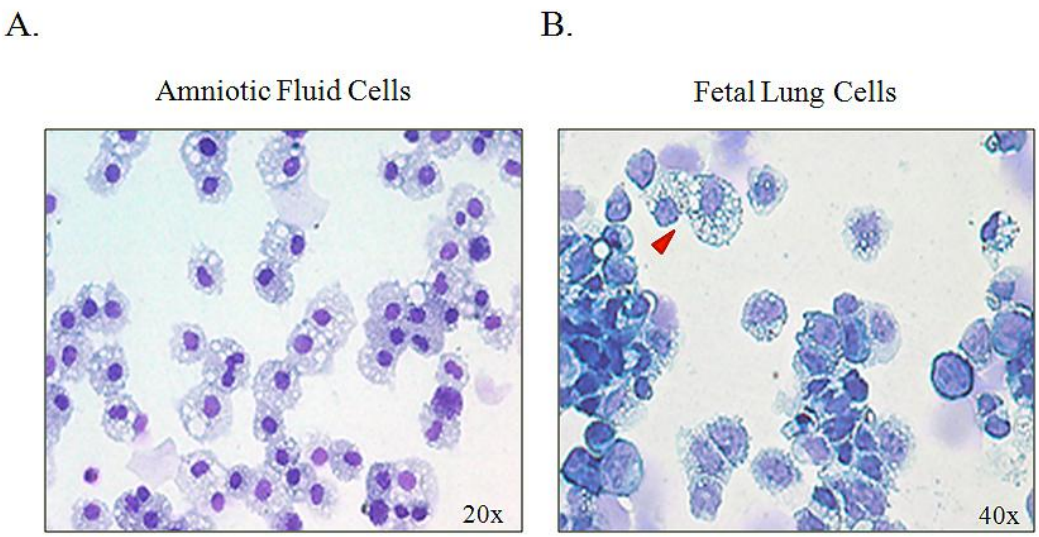


Figure 6. Amniotic fluid Mϕ are morphologically similar to fetal alveolar Mϕ. AF macrophages isolated from 18.5 dpc mice (A) were compared to fetal lung Mϕ (red arrowhead) (B) in single cell suspensions obtained by collagenase digestion. Cells were cytospun onto slides, stained using Diff-Quik and examined by light microscopy.

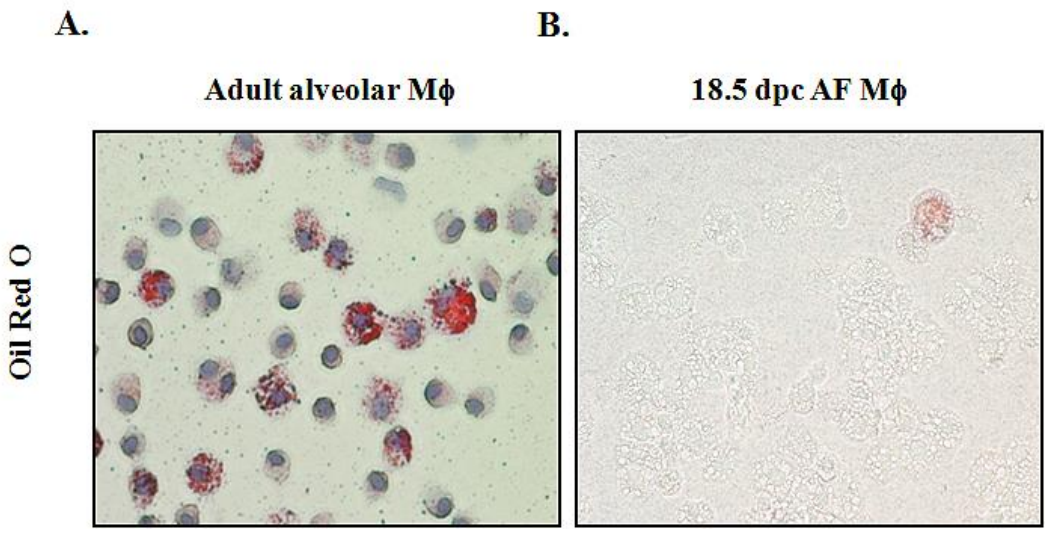


Figure 7. Amniotic fluid macrophages do not contain lipid inclusions in their vacuoles at 18.5 dpc. Adult alveolar macrophages and AF macrophages were isolated at 18.5 dpc by adhesion and stained with Oil Red O. The presence of lipids within the vacuoles of adult alveolar macrophages (A) is indicated by the red color. The majority of AF macrophages did not stain positively for the presence of lipids. Both images are at 20X magnification.

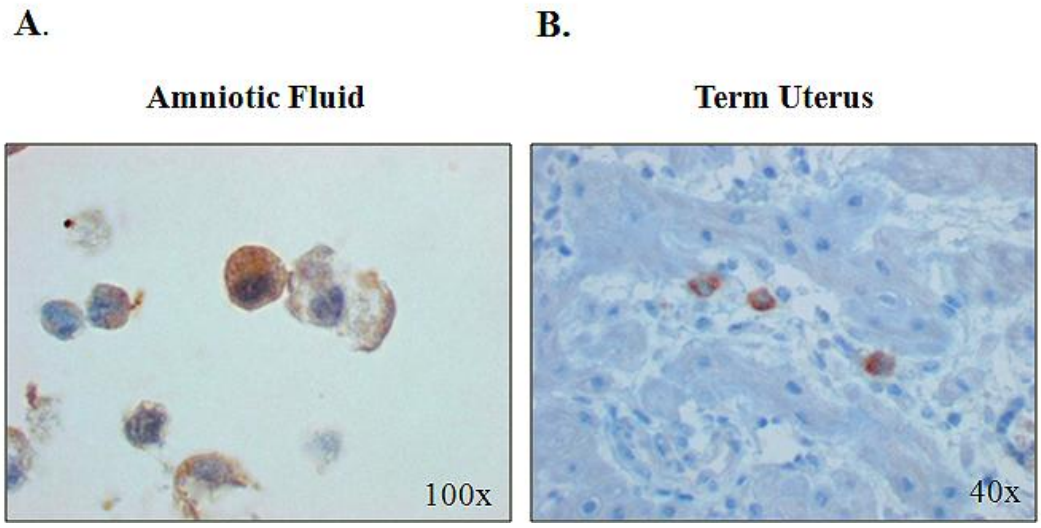


Figure 8. SP-A-positive cells are detectable in amniotic fluid and uteri of 18.5 dpc mice. Amnioitc fluid cells (A) and uterine sections (B) isolated at 18.5 dpc were embedded in paraffin and processed for SP-A staining. Known positive control sections of maternal lung and term fetal lung were processed with each batch of slides (data not shown). SP-A-positive cells are red.

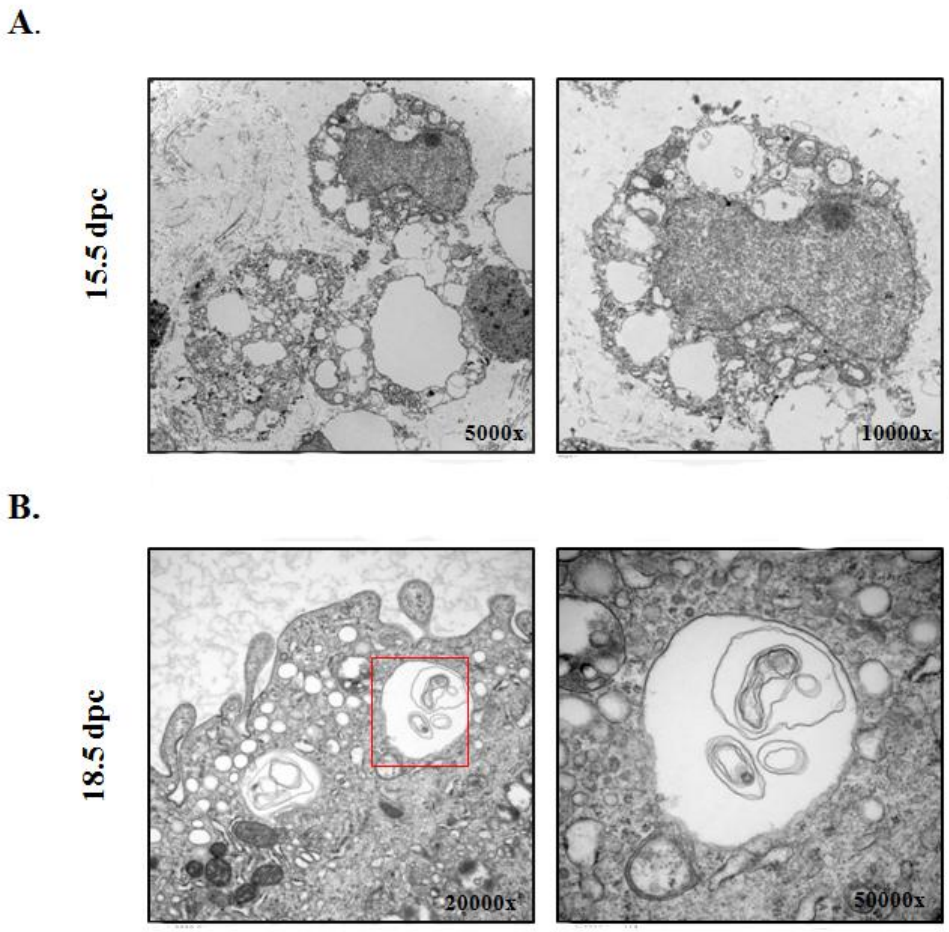


Figure 9. Transmission electron microscopy demonstrates the presence of lamellated bodies within vacuoles of amniotic fluid macrophage at term. Amniotic fluid cells isolated at 15.5 (A) and 18.5 (B) dpc were pelleted, embedded in agarose, fixed in glutaraldehyde overnight and processed for electron microscopy. Lamellated bodies were not present within the cytoplasmic vacuoles of 15.5 (A) dpc AF Mϕ, but present in 18.5 (B) dpc AF macrophages [red square].

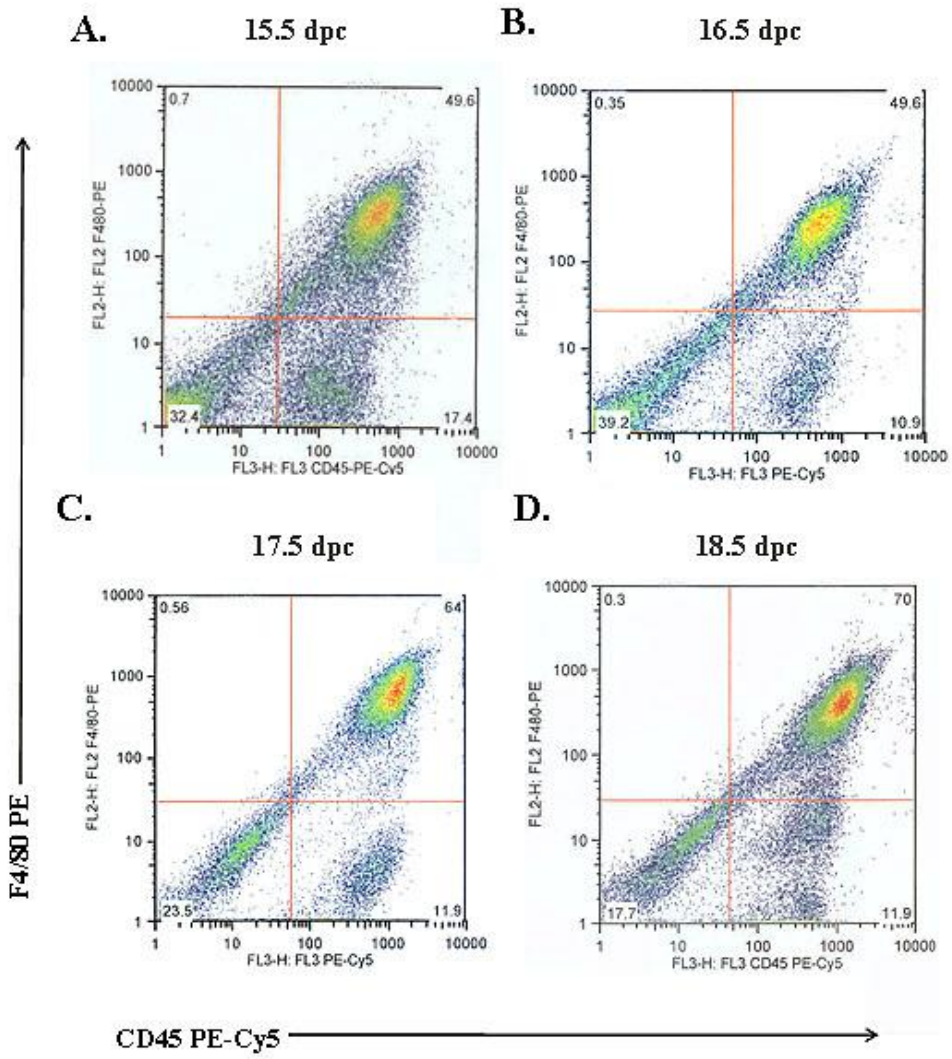


Figure 10. The proportion of $CD45^{+}F4/80^{+}$ cells within amniotic fluid steadily increases as term approaches. Amniotic fluid cells isolated at 15.5 (A), 16.5 (B), 17.5 (C) and 18.5 (D) dpc mice were stained with CD45 and F4/80 antibodies. The right quadrant indicates the percent of CD45, F4/80 double-positive cells at each gestational time point. At 15.5 and 16.5 dpc 50% of the total AF population are Mϕ. This proportion increases by 17.5

dpc to 64% and to 75% by 18.5 dpc. Each panel is representative of 5 individual experiments.

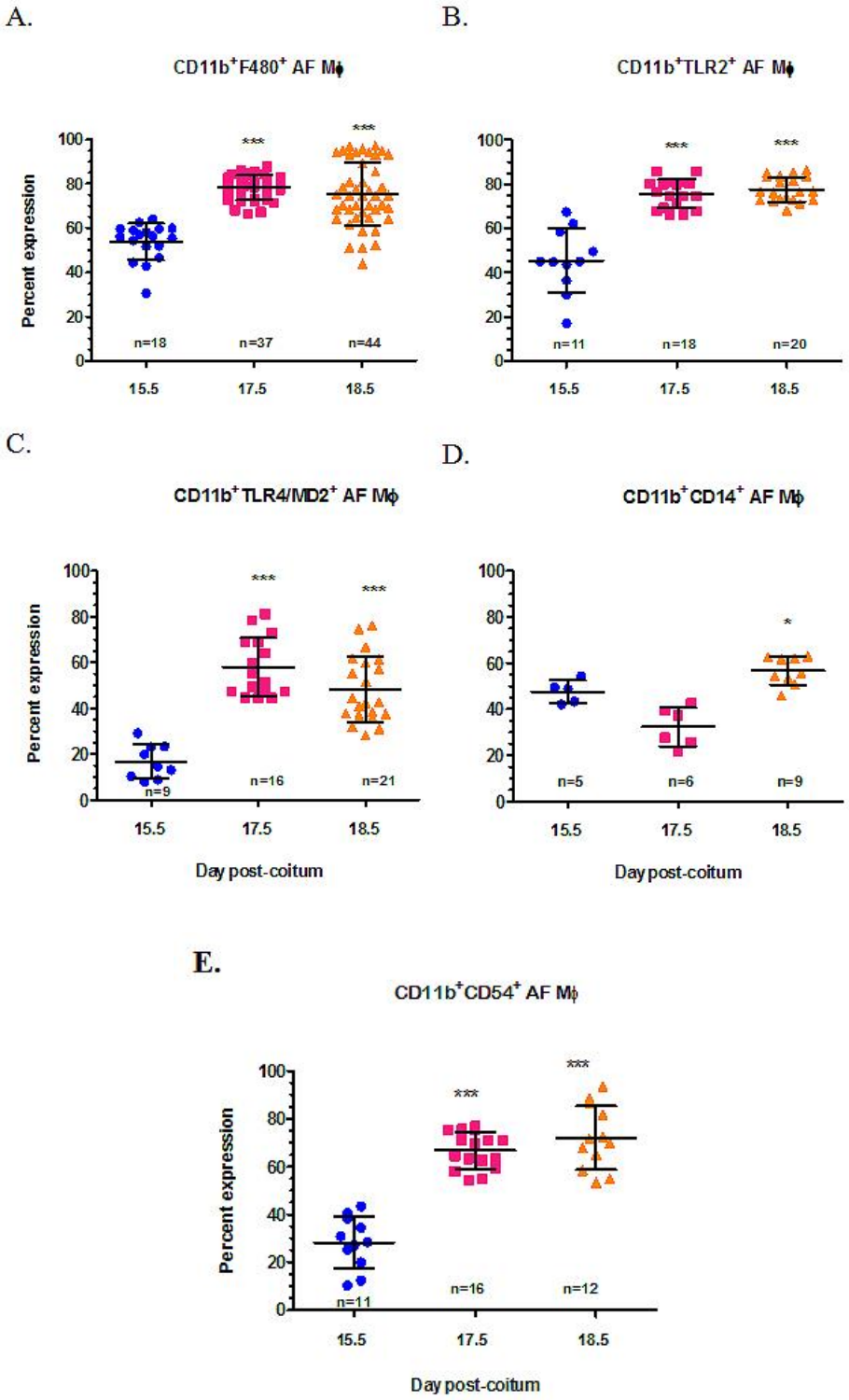

A.
CD11b+F480+ AF Mϕ
Percent expression
100
80
60
40
20
0
***
***
n=18
n=37
n=44
15.5
17.5
18.5
B.
CD11b+TLR2+ AF Mϕ
***
***
n=11
n=18
n=20
15.5
17.5
18.5
C.
CD11b+TLR4/MD2+ AF Mϕ
Percent expression
***
***
n=9
n=16
n=21
15.5
17.5
18.5
Day post-coitum
D.
CD11b+CD14+ AF Mϕ
*
n=5
n=6
n=9
15.5
17.5
18.5
Day post-coitum
E.
CD11b+CD54+ AF Mϕ
Percent expression
***
***
n=11
n=16
n=12
15.5
17.5
18.5
Day post-coitum

Figure 11. AF macrophages manifest upregulated expression of TLR2, TLR4/MD2, CD14 and CD54 in late gestation. AF cells isolated from 15.5, 17.5 and 18.5 dpc mice were analyzed by flow cytometry for expression of CD11b (A), TLR 2 (B), TLR 4/MD2 (C), CD14 (D), and CD54 (E). Live cells were gated based on forward and side scatter characteristics. Statistical significance between two groups was analyzed via unpaired one-tailed Student's *t* test and by one-way ANOVA followed by Tukey's analysis for determining differences among multiple groups with $P<0.05$ being significant. The data are expressed as mean ± SEM. *$P < 0.05$, ** $P < 0.005$, ***$P < 0.0005$.

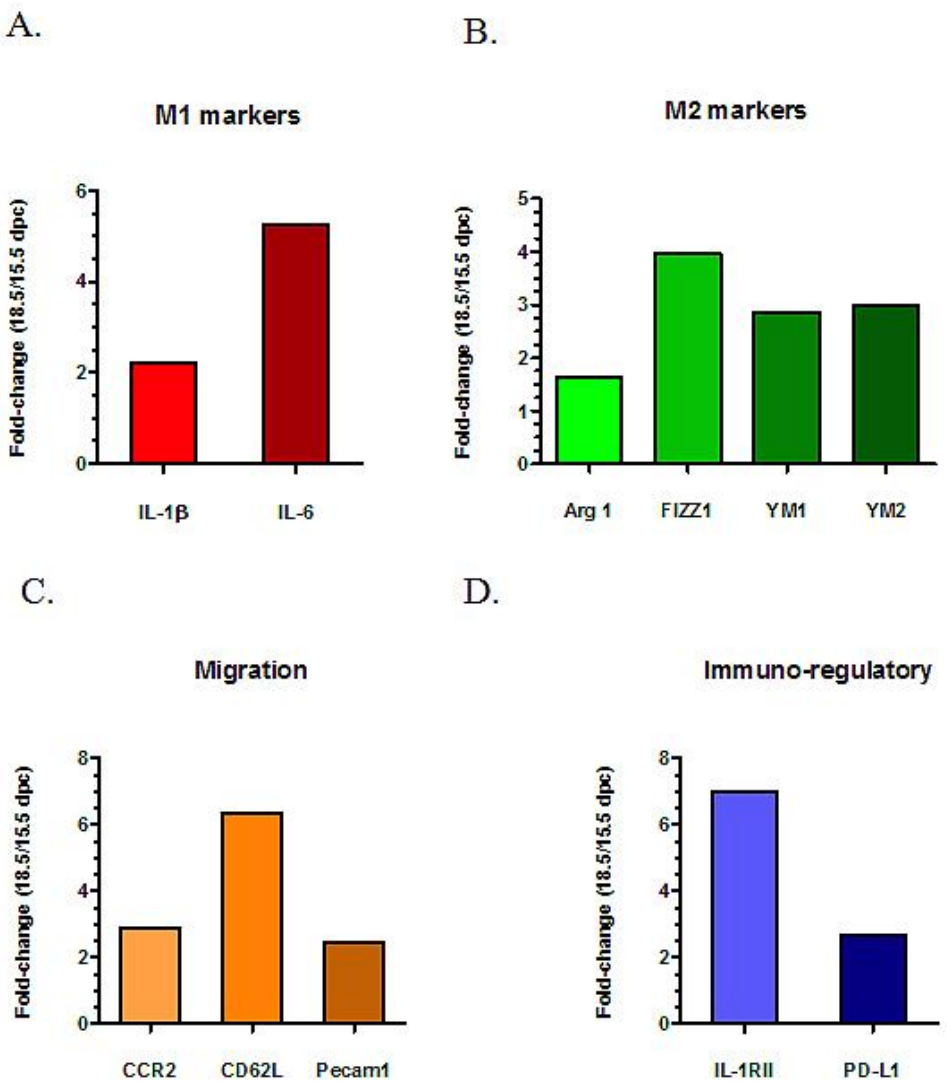


Figure 12. Microarray analysis of late gestation F4/80^{+} AF macrophages reveals upregulated expression of classical and alternative activation markers approaching term. F4/80^{+} AF macrophages isolated at 15.5, 17.5, and 18.5 dpc were sorted using a FACS Aria cell sorter. Total RNA was extracted, amplified and analyzed using Illumina mouse

microarray. Duplicate samples at each gestational time point were interrogated and averaged for each time point. Samples were clustered by functional similarity. Expression of M1 markers (A), M2 markers (B), migration (C), and immunoregulatory-associated genes (D) demonstrated increased expression of clustered genes between 15.5 and 18.5 dpc.

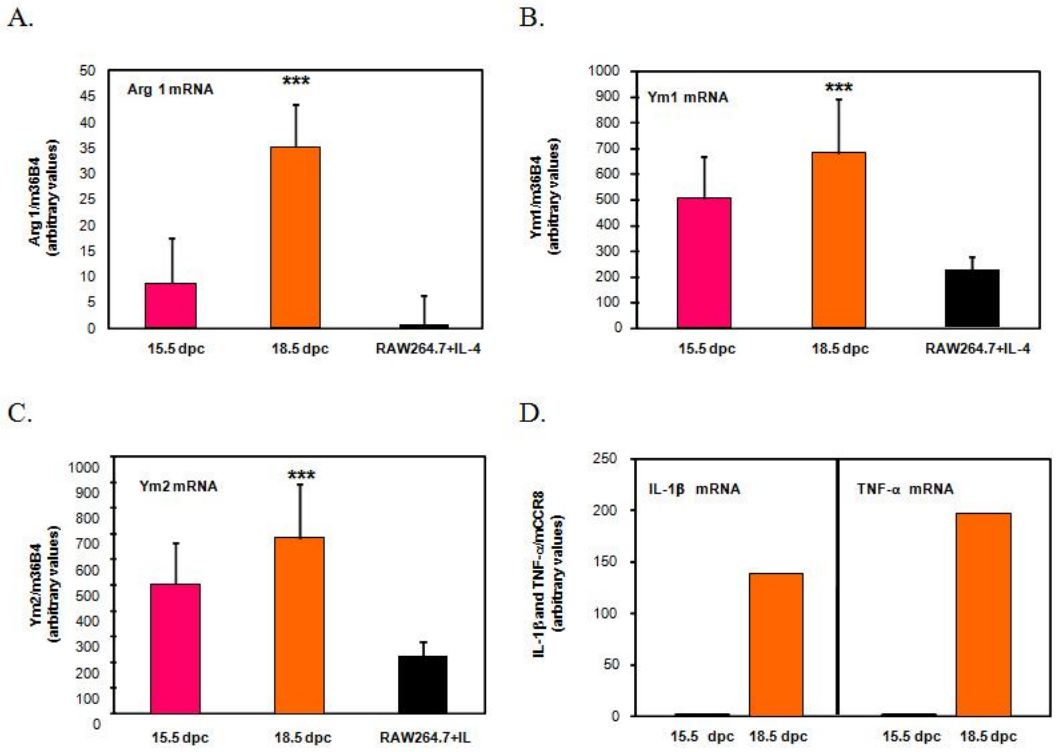


Figure 13. qRT-PCR confirms expression of classical and alternative activation markers in F4/80^{+} AF macrophages. AF Mϕ isolated from 15.5 and 18.5 dpc mice by FACs Aria cell sorting were analyzed for expression of Arg 1(A), Ym1 (B), Ym2 (C), IL-1β and TNF-α (D). Gene expression levels were compared to 15.5 dpc by the comparative Ct method. RAW264.7 cells, polarized to an M2 state by incubation with IL-4, were used as controls (black bars). Data were normalized to m36B4 (a-c) and CCR8 (d). Significance between two groups was determined by the unpaired one-tailed Student's *t* test with $P<0.05$ considered as significant. The data are expressed as mean ± SEM. ***$p < 0.0005$.

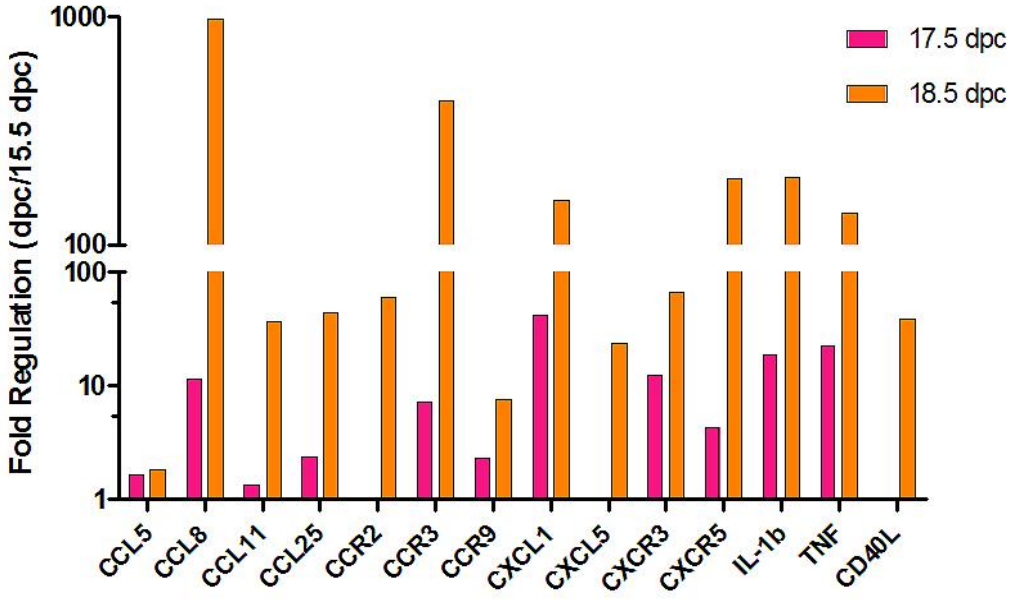


Figure 14. Inflammation-related gene expression increases between 15.5 and 17.5 dpc reaching maximal levels by term. SuperArray qRT-PCR based inflammatory gene analysis of F4/80^{+} AF Mϕ was used to profile cytokines, chemokines, and chemokine receptor expression between 15.5, 17.5 and 18.5 dpc. Bars indicate fold-change relative to 15.5 dpc AF Mϕ.

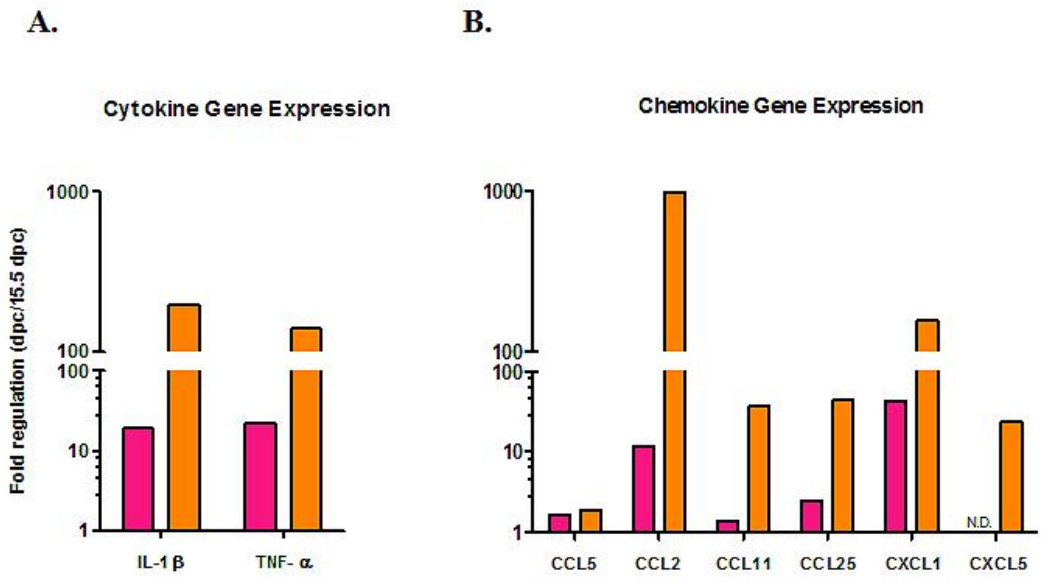


Figure 15. Inflammatory cytokines and chemokines reach maximal expression levels at the end of gestation. SuperArray qRT-PCR-based inflammatory gene analysis of AF Mϕ cytokines (A) and chemokines (B) expressed at 17.5 and 18.5 dpc. Bars indicate fold-change relative to 15.5 dpc AF Mϕ.

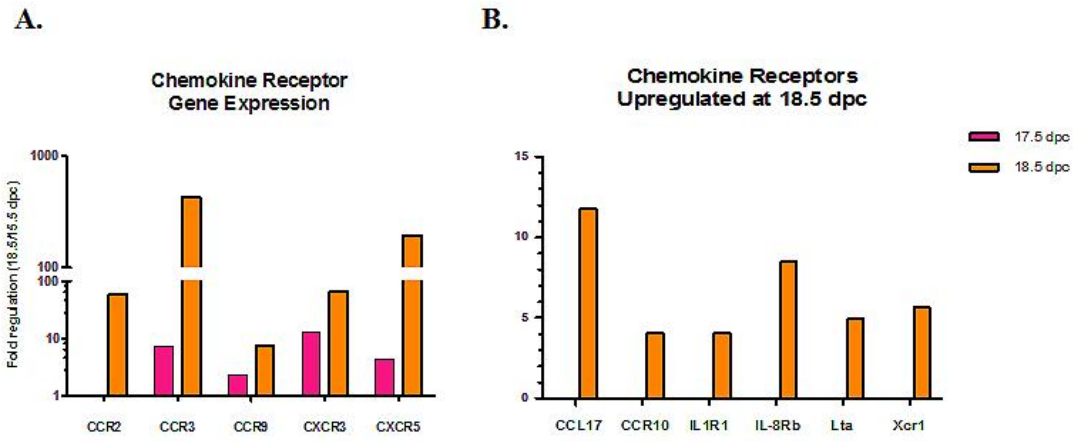


Figure 16. Inflammation-associated chemokine receptors demonstrate maximal expression at term. SuperArray qRT-PCR based inflammatory gene analysis of AF Mϕ chemokine receptors upregulated between 17.5 and 18.5 dpc (A) and those upregulated at 18.5 dpc. Bars indicate fold-change relative to 17.5 dpc AF Mϕ (B).

CHAPTER IV:

SURFACTANT PROTEIN-A AND –D DEFICIENCY DOES NOT SIGNIFICANTLY AFFECT THE TIMING OF LABOR AT TERM

Introduction

Work in our lab demonstrated that SP-A secreted by the fetal lung into AF, plays a key role in initiation of parturition at term [1]. In these studies, developmental induction of SP-A expression in mouse fetal lung and secretion into amniotic fluid after 17 dpc was associated with increased expression of IL-1β by AF Mϕ and with activation of NF-κB in the maternal uterus culminating in labor at term. Studies using *Rosa 26 Lac-Z* mice revealed that fetal AF Mϕ migrate to the uterus in parallel with increased AF SP-A suggested that a proportion of the Mϕ that invade the uterus near term are of fetal origin. Purified SP-A was found to stimulate IL-1β and NF-κB expression in cultured AF Mϕ. Moreover, intra-amniotic injection of SP-A induced preterm delivery within 6-24 h while injection of an SP-A antibody or NF-κB inhibitor (SN50) into amniotic fluid resulted in delayed labor by >24 h. Thus, it is postulated that augmented production of SP-A by the fetal lung near term causes activation and migration of fetal-derived AF Mϕ into the maternal uterus, where increased production of cytokines activate NF-κB pathways that induce expression of inflammatory genes and contraction-associated proteins (CAPs) proteins leading to fetal expulsion at term [1]. Therefore, it is proposed that interactions between AF Mϕ surface receptors with SP-A, at term, initiate changes in AF Mϕ phenotypic properties, resulting in enhanced expression of genes involved in mediating

migration and promotion of inflammatory cascades within the pregnant uterus. Based on these findings, I hypothesized that mice deficient in SP-A would manifest a delay in the time to parturition. To test this supposition, parturition was timed in mice carrying a targeted single deletion in SP-A (SP-$A^{-/-}$) or –D (SP-$D^{-/-}$). Because of possible functional redundancy of these surfactant proteins, parturition timing also was assessed in mice with combined deletion of SP-A and –D (SP-$A/D^{-/-}$).

SP-A and-D have been reported to bind to Toll-like receptors (TLRs) 2 and TLR4 [136-140]. Expression of TLR2 and TLR4 on cells involved in the innate and adaptive immune responses (*i.e.* Mϕ and dendritic cells) are well documented and have been previously discussed in Chapter 3. A growing body of evidence suggests that interactions between the immune and reproductive systems involve TLRs. Indeed, functional TLRs are expressed in reproductive tissues such as the cervix [368], placenta [369-371], amnion [372], decidua [373], myometrium [374], and at the maternal-fetal interface [375-376]. In the setting of infection, TLR ligands, such as LPS and LTA, have been clearly demonstrated to cause preterm labor. Interestingly, the biological processes facilitating parturition in pathological preterm and normal labor involve common inflammatory cascades, in which elevated levels of IL-1β, IL-6, IL-8 and TNF-α in the cervix, fetal membranes and myometrium are characteristic [377]. Increases in pro-inflammatory factors lead to leukocyte recruitment (*e.g.* Mϕ and neutrophils) [1, 4, 67], prostaglandin synthesis, and matrix metalloproteinase production within reproductive tissues, thereby, facilitating labor [378]. Notably, preterm birth can be induced in animal models by injection of IL-1β or TNF-α [68]. In the setting of infection, the inciting signal

for PTL is clear. However, with labor at term, the stimulus for triggering these cascades remains unknown. It is important to acknowledge that the role of immune cell infiltration and inflammatory gene expression during normal cervical ripening is controversial as emerging data now reveal that induction of cervical ripening through activation of immune pathways is sufficient but not necessary [379-382].

Due to the inflammatory hallmarks of labor, an involvement of TLR2 and 4 in normal term labor has been proposed. As mentioned, SP-A and –D are known ligands for TLR2 and TLR4 and may play a critical role in the initiation of labor at term. As discussed in Chapter One, TLR2 and 4 can modulate either pro- or anti-inflammatory responses when bound by SP-A [137-139, 383]. Therefore, due to their potential involvement in initiating the signal for labor via SP-A binding, I hypothesized that deficiency in either TLR2 and/or TLR4 may lead to a delayed labor phenotype. To test this idea, timed parturition studies were carried out using *TLR2* singly deficient (*TLR2*$^{-/-}$) and *TLR2* plus *TLR4* doubly-deficient (*TLR2/4*$^{-/-}$) mice.

Results

SP-A and SP-D deficiency in mice does not affect the timing of labor

To test the hypothesis that fetal lung production and secretion of SP-A into the AF during late gestation is necessary for signaling labor at term, homozygous virgin mice carrying targeted deletions of *SP-A* (n=27), *SP-D* (n=11) and *SP-A/D* (n=17) genes were bred to genetically like males and the time of labor was assessed. Timing of parturition in mice homozygous for deletions in *SP-A* (~19.4 dpc), *SP-D* (~19.4 dpc) and for *SP-A/D* (~19.5 dpc) genes did not manifest a delay in labor at term (19.5 dpc) (Figure 17a). Obvious physical defects in neonates were not detectable and litter sizes were normal (Figure 17c). Due to the possibility that increased uterine stretch might play a primary and dominant role in the timing of labor during first pregnancies [384], the time to parturition in second pregnancies was also documented (Figure 17b). Deficiency in *SP-A* (~19.7 dpc, P=0.39) and *SP-A/D*$^{-}$ (~19.9 dpc, P =0.06) did not delay labor at term in second pregnancies compared to WT (~19.6 dpc) and litter sizes were normal (Figure 17d).

TLR2 deficient mice manifest a delay in labor at term

To determine whether *TLR2* and *TLR4* are involved in mediating the initiation of labor at term, the time to parturition was assessed in *TLR2*$^{-/-}$ (n=20) and *TLR2/4*$^{-/-}$ (n=20) deficient mice. Interestingly, compared to WT C57BL/6 counterparts (~19.5 dpc),

TLR2$^{-/-}$ mice (~19.93 dpc, P = 0.003) manifested a delay in labor while *TLR2/4*$^{-/-}$ mice (~19.7 dpc) did not display a statistically significant delay in the time to parturition (Figure 18a). In addition, the average neonatal weight of pups delivered by *TLR2*$^{-/-}$ (n = 49, P< 0.000) are significantly greater compared to their WT counterparts (n=31) (Figure 18b).

Discussion

Other pulmonary surfactant-associated factors may initiate labor at term

Condon *et al*. previously obtained evidence that SP-A, when secreted by the fetal lung near term, plays a critical role in the initiation of parturition [1]. In those studies the developmental increase in SP-A expression in mouse fetal lung and secretion into AF after 17 dpc was associated with increased expression of IL-1β in AF Mϕ and with activation of NF-κB in the maternal uterus. Based on this, it was postulated that augmented production of SP-A by the fetal lung near term causes activation and migration of fetal derived AF Mϕ to the maternal uterus, where increased production of cytokines activate NF-κB-regulated inflammatory pathways leading to labor. In light of the potential role of SP-A in the initiation of labor, it was of great interest to functionally test the requirement of SP-A and SP-D in the labor cascade. I hypothesized that deficiency in SP-A would lead to a delayed parturition phenotype.

To test this idea, homozygous mice with targeted deletions in *SP-A*, *SP-D* and *SP-A/D* genes were bred to syngeneic males and the time to labor was assessed. Results revealed no delay in the time to parturition in any of surfactant deficient mice. To eliminate the possibility that uterine stretch might serve as the primary and overriding signal in first pregnancies [367, 385-386] due to increased elasticity of the uterus, labor was timed in WT, *SP-A*$^{-/-}$and *SP-A/D*$^{-/-}$ second pregnancies reasoning that mitigation of the stretch factor would allow other signals (*e.g.* SP-A) to reveal their requirement in initiating labor at term. Results demonstrate that the time to parturition in second pregnancies was unaffected by SP-A or SP-A/D deficiency compared to WT counterparts. Therefore, deficiency of *SP-A* and *SP-D*, in mice, is not sufficient to delay parturition at term and a compensatory effect does not exist between SP-A and SP-D proteins in mediating labor timing of labor. It should be noted that since *SP-A* and *SP-D* genes lie only 60 kb apart on mouse chromosome 14, it was necessary for Dr. Hawgood and his colleagues to sequentially target them in embryonic stem cells using graded resistance to G418 in order to create the SP-A and SP-D double knockout. Deletion of *SP-A* and *SP-D* locus also resulted in deletion of the *Mbl* I gene, which encodes the serum collectin protein Mbl 1, as it lies between the two targeted genes [287]. Thus, the results obtained from SP-A/D$^{-/-}$ mice also demonstrate that *Mbl 1* deficiency has no deleterious effects on parturition timing.

These unexpected results suggest the possible role of some other component of pulmonary surfactant in parturition timing. However, unpublished observations from our lab in which female mice that were haplodeficient for the steroid receptor coactivator (SRC)-1 and SRC-2 bred to genetically-like males manifested a delayed labor phenotype

in association with decreased expression and secretion of SP-A by the fetal lungs. The SRC-1/SRC-2 deficiency was associated with a decreased inflammatory response within the maternal uterus, a delay in luteolysis and maintance of elevated circulating progesterone levels [387]. Notably, offspring from these crosses were reported to die at birth from atelectasis due to apparent surfactant deficiency [388]. Since SP-A is not required for survival, the delay in parturition, suggests the possible role of some other component of pulmonary surfactant in signaling labor. Further studies are underway to identify the surfactant component(s) responsible for these marked defects.

Other investigators have also postulated that some component of pulmonary surfactant can initiate labor at term. Johnson and colleagues proposed that platelet-activating factor (PAF), a highly bioactive phosphoplipid component of fetal lung surfactant secreted into amniotic fluid near term, may activate myometrial contractility [389]. In support of this idea, Elovitz *et al*. demonstrated that PAF is capable of inducing preterm delivery in mice [390]. Lopez-Bernal *et al*. observed that pulmonary surfactant isolated from human amniotic fluid stimulated prostaglandin E production in discs of human amnion and proposed that surfactant phospholipids secreted by the fetal lung into amniotic fluid provides a source of arachidonic acid necessary for prostaglandin synthesis [53]. In other studies, an unidentified substance(s) in amniotic fluid was found to stimulate prostaglandin E2 synthesis in human amnion cells [55]. Thus, further studies investigating the role of surfactant associated complexes are warranted as they may reveal the identity of the fetal derived factor responsible for signaling normal labor at term.

The delayed parturition phenotype of TLR2 deficient mice provide a potential mechanistic link between innate immunity and labor

Since SP-A has been reported to bind to TLR2 and TLR4, I hypothesized that SP-A binding to one or both these receptors initiates the cascade of the inflammatory signaling pathways in AF Mϕ that promote increased myometrial contractility and culminate in parturition. It was predicted that deficiency of either TLR2$^{-/-}$ and/or TLR2/4$^{-/-}$ would result in a delayed parturition phenotype. Thus, to define a role, if any, of *TLR2* and *TLR4* in mediating labor at term, the time to parturition in *TLR2*$^{-/-}$ and *TLR2/4*$^{-/-}$ was assessed. In comparison to WT C57BL/6, mice which deliver at ~19.5 dpc, TLR2$^{-/-}$ mice manifested a significant delay in the timing of labor. This delay was supported by the finding that the neonatal weight of pups born to these mothers was significantly greater compared to their wild-type counterparts (Figure 18B). While labor was somewhat delayed in *TLR2/4*$^{-/-}$ mice, suggesting some degree of dysregulation, the delay was not statistically significant (Figure 18A). To the best of my knowledge, I provide herein, the first functional phenotype in TLR2 deficient mice of delayed parturition.

Future studies will focus on the functional effects of TLR2 deficiency in AF Mϕ polarization status during late gestation and on uterine contractile gene expression in term reproductive tissues, such as myometrium. It is possible that in the absence of TLR2 expression on AF Mϕ, activation does not occur thereby compromising the polarization and induction of the inflammatory cascades that result in their migration into the maternal myometrium and culminate in labor at term. Alternatively, TLR2 deficiency in reproductive tissues may block the initiation and/or amplification of the inflammatory

signaling within the uterus and cervix at term. In support of this notion are findings by Gonzalez *et al*. of increased TLR2 and TLR4 mRNA and protein expression within the pregnant myometrium and cervix of CD-1 mice during late gestation [391]. In other studies, Youseff and colleagues reported an increase in TLR2 and TLR4 mRNA levels at term in human myometrium. Moreover, they observed a concomitant increase in Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1), a protein known to amplify TLR signaling. Notably, TLR2 protein expression was found to be significantly higher in laboring samples compared to non-laboring. These studies further demonstrated that treatment with progestin inhibited TLR2-induced inflammatory mediators *in vitro* [374]. Taken together, these data strongly suggest a potential functional mechanism for the link between the innate immune system and the labor process.

Taken together, the fact that SP-A and SP-D deficient mice deliver normally at term, combined with observations that SRC-1 and SRC-2 deficient mice manifest delayed parturition in conjunction with surfactant insufficiency of pups at birth, suggests that some other component of pulmonary surfactant may be responsible for signaling labor Additionally, the discovery that TLR2 deficiency in mice results in a delayed parturition phenotype provides a potential mechanistic link between innate immunity and the inflammatory processes leading to labor. As such, further investigation into the role of TLR2 in mediating inflammatory and/or labor associated genes (*i.e.* upregulation of CAPs) at term will be of substantial benefit as it will lead to a better understanding of the roles of TLRs and their signaling cascades during spontaneous term and idiopathic preterm labor. Such studies could potentially lead to novel tocolytic targets for prevention of preterm labor.

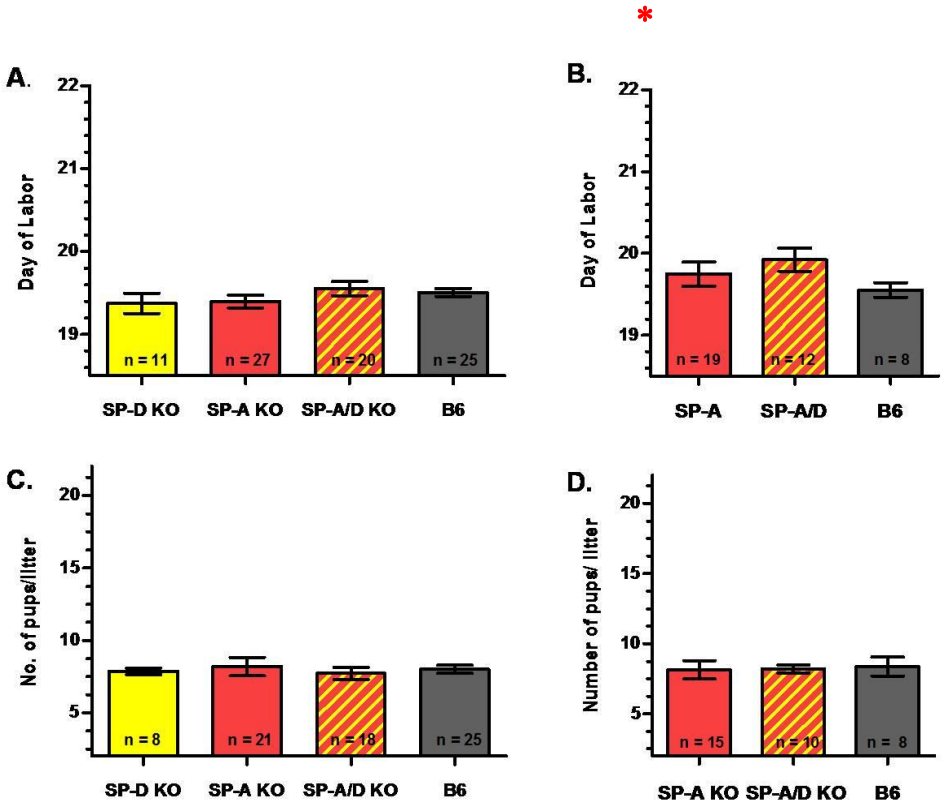


Figure 17. The time to parturition in first (**A**) and second (**B**) pregnancies in SP-A$^{-/-}$, SP-D$^{-/-}$ and SP-A/D$^{-/-}$ mice do not significantly differ compared to wild-type C57BL/6 (B6). The number of pups per litter in first (**C**) and second (**D**) pregnancies were similar to wild-type B6 litter size (B6). Values are expressed as means ± SEM.

*Since the submission of this thesis in April, continued breeding experiments now reveal a statistically significant delay in the time to parturition in SP-A/D$^{-/-}$ second pregnancies (20.15 dpc ($P < 0.03$)) compared to wild-type C57BL/6 (19.5 dpc).

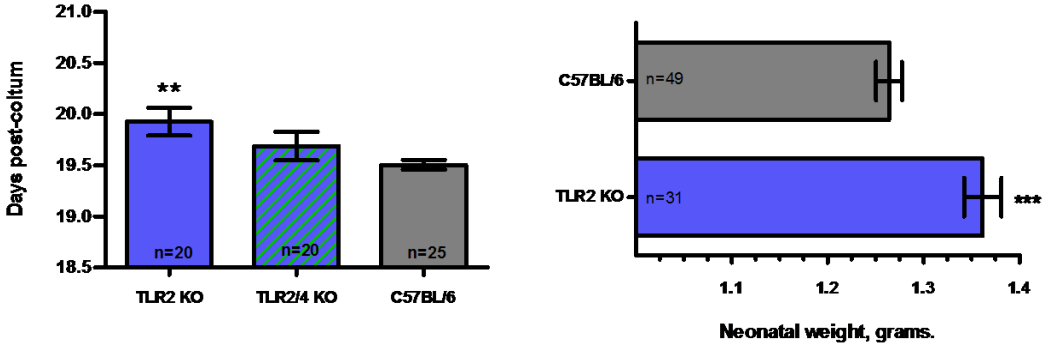


Figure 18. *TLR 2*$^{-/-}$ mice (n = 20) manifested a delay in parturition (**A**), and delivered pups with greater neonatal weight (n =31) (**B**). Values are expressed as means ± S.E.M. ** P = 0.003, *** P < 0.0004.

CHAPTER V:

FETAL DERIVED AMNIOTIC FLUID MACROPHAGES EXPRESSING *Csf1r*-EGFP ARE NOT DETECTABLE WITHIN THE PREGNANT UTERUS AT TERM

Introduction

Infiltration of the myometrium by leukocytes during spontaneous labor at term occurs in humans and mice . It is postulated that this event is critical for the onset of labor. Invading leukocytes are believed to provide a source of inflammatory mediators, such as IL-1, TNF-α and prostaglandins; all key molecules known to play a role in mediating the pro-inflammatory cascades in the myometrium leading to uterine contractile gene expression and culminating in labor [67, 76]. It has been generally accepted that the infiltrating immune cell population is of maternal origin. However, using a transgenic *Rosa 26 Lac-Z* (*B6; 129S-Gt*(*rosa*)*26sor*) (*Lac-Z*) model, a proportion of the Mϕ population that infiltrates the myometrium at term was demonstrated to be of fetal origin [1]. In those studies, AF Mϕ, uteri, and embryos isolated from WT ICR females carrying ≈ 50% *Lac-Z* heterozygous embryos were assayed for β-gal activity at 17 dpc (19 dpc = term). AF Mϕ isolated from *Lac-Z*-positive embryos displayed β-gal activity and the uteri of WT ICR mothers carrying *Lac-Z*-positive embryos were positive for β-gal activity, in direct contrast to uteri of WT mothers carrying WT embryos, which were negative. Dual immunofluorescence staining for F4/80 and *β*-gal in sections of uteri isolated from WT

mothers carrying *Lac-Z* positive embryos, were reported to contain cells positive for β-gal activity co-localized with F4/80^{+} cells, suggesting that a proportion of the Mϕ that infiltrate the pregnant uterus at term are of fetal origin. Importantly, infiltration of fetal-derived AF Mϕ into the gravid uterus was observed to be associated with the secretion of SP-A as previously discussed in Chapter One. To date, the mechanisms whereby fetal-derived Mϕ penetrate the term uterus remain unknown.

Feto-maternal cell trafficking is a well documented phenomenon. During pregnancy fetal cells have been demonstrated to enter maternal blood and tissues and can persist for decades [393-395]. Importantly, fetal microchimerism is theorized to play an important role in tolerizing the mother to the fetus via fetal and placental antigen presentation [396-399]. Engraftment of allogenic fetal cells in maternal tissues (microchimerism) is also believed to contribute to the development of immune pathologies such as systemic sclerosis [400], Hasimoto thyroiditis, and Graves' disease [401]. Studies of microchimerism commonly utilize mouse models due to their hemochorial placentation, similar to humans, and because of the availability of transgenic mice carrying fluorescent transgene reporters which facilitate the tracking of fetal cell movement during gestation. Detailed studies conducted by Vernochet *et al.* and Bianchi *et al.* using mice expressing enhancer green fluorescent protein (EGFP) transgene reporters have previously demonstrated the range and sensitivity of such models in locating, quantifying, and characterizing fetal cell migration into maternal tissues *in vivo* [402-404].

The studies presented herein were undertaken to determine the trafficking route(s) whereby fetal-derived AF Mϕ penetrate term myometrium. To this end, mice carrying the

Csf1r-EGFP transgene reporter were used. Expression of this reporter is driven by the promoter for the *c-fms* proto-oncogene, which encodes the cell surface tyrosine kinase colony-stimulating factor receptor (CSF-1r), which is selectively expressed in macrophage and trophoblast cell lineages. Colony-stimulating factor (CSF-1) is a Mϕ lineage-specific growth factor that binds to CSF-1r thereby activating differentiation, proliferation and survival of Mϕ from liver- and bone- marrow-derived hematopoietic progenitors [172]. *C-fms* (*Csf1r*) mRNA is present in the earliest yolk sac phagocytes and is expressed in the embryo as well as in adult mice in a macrophage-specific manner. Notably, $EGFP^{+}$ cells detected within the tissues of transgenic embryos co-localized to sites known to contain cells expressing c-*fms* mRNA, as well as other macrophage-specific gene markers [162-163], thereby confirming macrophage-restricted expression of this transgene *in vivo*. Thus, the *Csf1r*-EGFP model provides an optimal method for analyzing AF Mϕ trafficking during late gestation and could potentially reveal temporal changes in their route(s) of egress to the maternal uterus.

Results

EGFP-positive macrophages are present within late gestation fetal amniotic fluid and fetal-derived tissues

To determine the route of fetal macrophage migration into the pregnant uterus at term, transgenic mice expressing EGFP in a macrophage-specific manner were utilized. Expression of the macrophage fluorescent signal arises from a unique, paternally inherited transgene that can be used to identify fetal cells within maternal tissues. In these studies *Csf1r-EGFP* homozygous males were mated to syngeneic C3H:HeN (inbred) wild-type (WT) females to examine the trafficking route(s) that this population uses to enter the term uterus.

To confirm the presence of $EGFP^{+}$ cells in the developing embryos during late gestation, AF surrounding heterozygous ($EGFP^{-/+}$) embryos of individual mothers at 18.5 dpc were isolated and fetal-derived Mϕ were sorted based on EGFP-fluorescence using fluorescence activated cell sorting (FACs). After setting a live gate (P1) around the viable cell population (A), the presence of $EGFP^{+}$ Mϕ was confirmed within AF (P3) at 18.5 dpc (B), (Figure 19**)**.

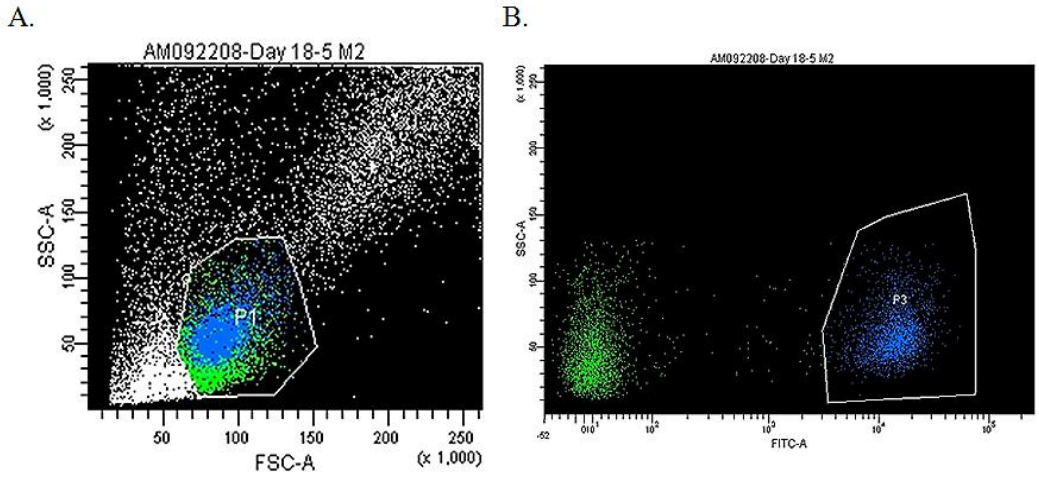


Figure 19. Examination of EGFP-positive macrophages in 18.5 dpc amniotic fluid by FACS analysis. Amniotic fluid (AF) cells isolated from wild-type (WT) syngeneic C3H:HeN mothers caring C*sfr1*-EGFP heterozygous fetuses were sorted by FACS Aria. Based on three forward versus side scatter properties, a live gate (P1) was placed around the viable cell population in 18.5 dpc (A) samples. The presence of fetal-derived macrophages within the live cell population (blue) at 18.5 (B) dpc confirmed the presence of EGFP-fluorescent cells (P3). Each image is representative of results obtained in two independent experiments.

In the report by Condon *et al.*[1], it was postulated that the AF Mϕ that infiltrate the maternal uterus at term emanate from the fetal lung [1]. The presence of $EGPF^{+}$ cells within the fetal lungs of 15.5, 17.5 and 18.5 dpc fetuses was investigated. Lungs of heterozygous ($EGFP^{-/+}$) embryos were isolated from syngeneic WT mothers at 15.5, 17.5 and 18.5 dpc and processed for imaging. Numerous $EGFP^{+}$ Mϕ were present throughout the fetal lungs at 17.5 (Figure 20A) and 18.5 dpc (Figure 20B). To explore the possibility that AF Mϕ extravasate directly from AF through the amnion and into the maternal uterus, 15.5, 17.5 and 18.5 dpc amnion was isolated from WT mothers carrying heterozygous $EGFP^{-/+}$ pups. $EGFP^{+}$ cells were clearly visible in clusters or studded along the length of the tissue facing the fetus at 17.5 (Figure 20C) and 18.5 (Figure 20D) dpc. Their distribution along the amnion appeared random as they were not present with any notable frequency or in any obvious pattern. Within the umbilical cord, fetal-derived Mϕ were detectable but sporadically distributed and EGFP signal was not present in all inspected sections at either 17.5 (Figure 20E) or 18.5 dpc (Figure 20F).

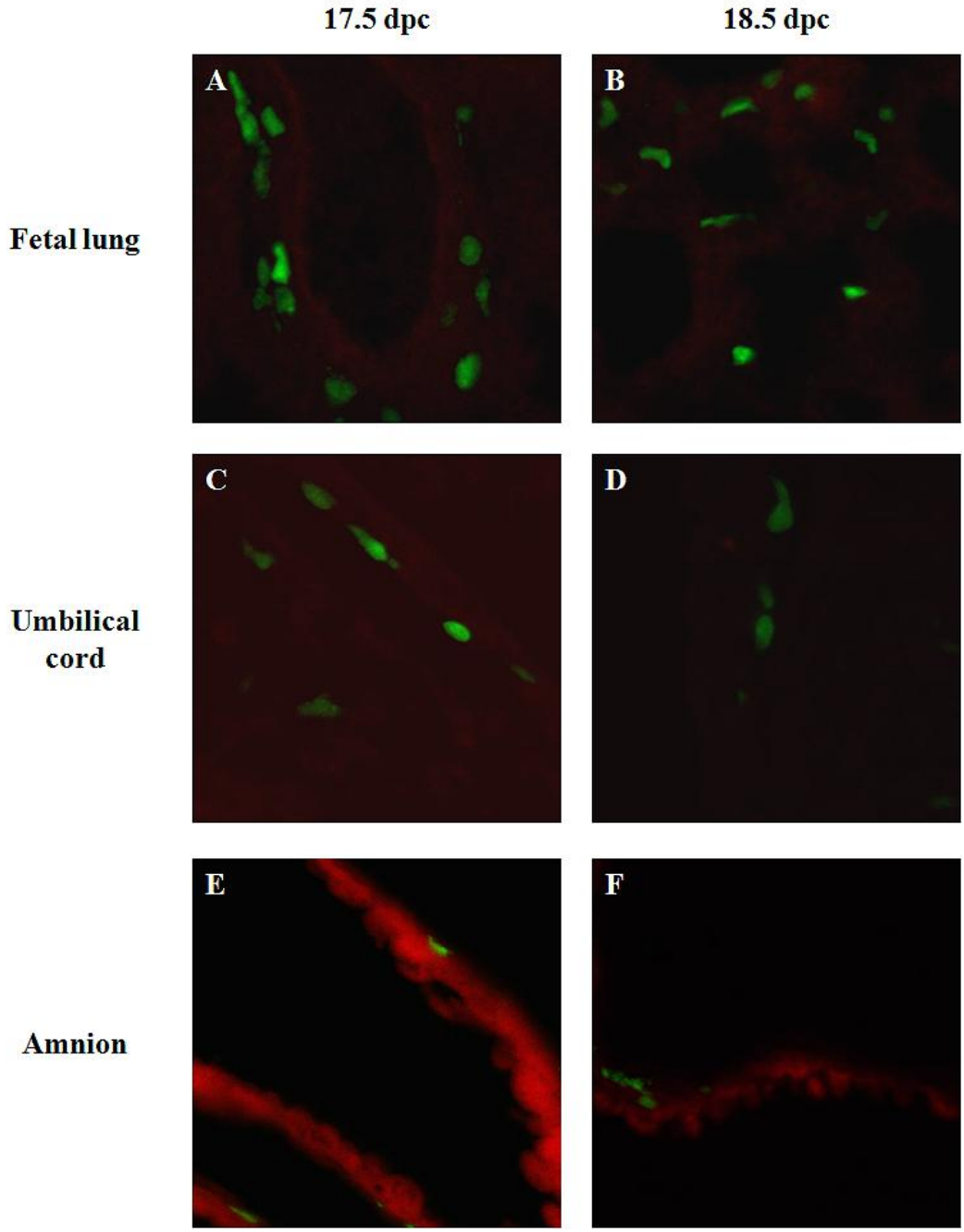

17.5 dpc
18.5 dpc
Fetal lung
Umbilical cord
Amnion
A
B
C
D
E
F

Figure 20. EGFP$^+$ macrophages are present in fetal tissues during late gestation. Tissues from syngeneic WT C3H:HeN mothers carrying *Csf1r*-EGFP heterozygous fetuses at 17.5 and 18.5 dpc were isolated and the presence of EGFP$^+$ cells was verified. All tissues were fixed in 4% paraformaldehyde, equilibrated in a series of sucrose gradients and embedded in optimal tissue freezing medium. Frozen sections were cut in stepwise increments and prepared for imaging. The presence of EGFP$^+$ cells was determined via fluorescence microscopy. Lungs isolated from *Csf1r*-EGFP heterozygous fetuses contained EGFP$^+$ cells at 17.5 (A) and 18.5 (B) dpc. Examination of amnion revealed the presence EGFP$^+$ cells, indicated by the green color, along the fetal side of the tissue at 17.5 (C) and 18.5 dpc (D). EGFP$^+$-macrophages were also detectable in the umbilical cords of 17.5 (E) and 18.5 dpc (F) pups. Each image is representative of 3 separate analyses at the indicated time points. Tissues from four to six pups per mother were examined for the presence of EGFP signal. Panels A-E and F were imaged at 63X and 40X magnification, respectively.

EGFP-positive macrophages are not present within the maternal uterus at term

To determine the potential point of fetal-derived Mϕ entry into the gravid uterus, four to six uterine segments corresponding to the positions of individual fetuses within each WT mother carrying $EGFP^{-/+}$ pups were isolated and processed for imaging. In contrast to the readily detectable EGFP-fluorescent cells within the uteri of *Csf1r*-EGFP homozygous females crossed to like males (Figure 21A), and those of *Csf1r*-EGFP homozygous females crossed with syngeneic WT males (Figure 21B), $EGFP^{+}$ cells were undetectable in all maternal uterine segments isolated from WT females carrying *Csf1r*-EGFP heterozygous fetuses at 17.5 (Figure 21C) and 18.5 dpc (Figure 21D).

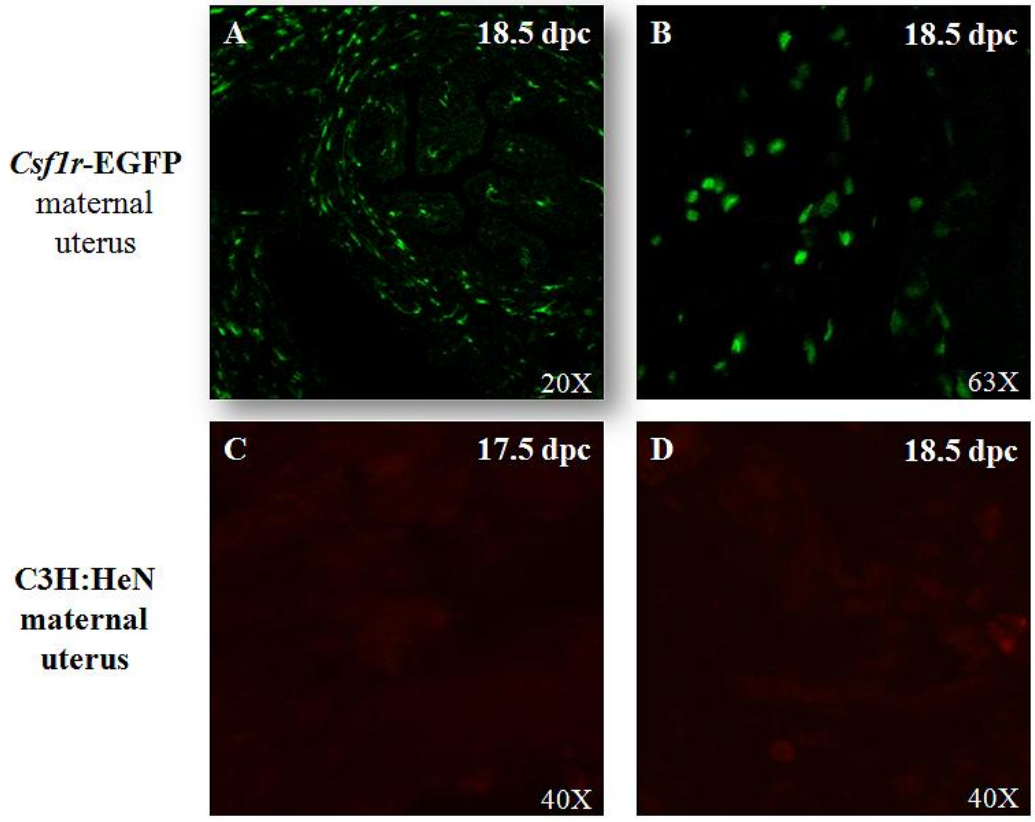


Figure 21. EGFP-positive cells are undetectable in the uteri of wild-type C3H:HeN mothers carrying C*sf1r*-EGFP heterozygous fetuses. $EGFP^{+}$ cells were readily detectable within uterine segments isolated from 18.5 dpc *Csf1r*-EGFP homozygous females crossed to like males (A) and in segments isolated from *Csf1r*-EGFP homozygous females crossed to WT syngeneic males (B), but not in uterine samples isolated from WT females carrying *CSF1r*-EGFP heterozygous fetuses at 17.5 (C) or 18.5 dpc (D).

Discussion

Infiltration of the maternal uterus by fetal AF Mϕ was previously demonstrated using *Rosa 26 Lac-Z* transgenic mice [1]. The studies presented herein were undertaken to determine the trafficking pattern of AF Mϕ to the maternal uterine tissue at term. To this end, syngeneic WT C3H:HeN mice carrying C*sf1r*-EGFP heterozygous pups were used to trace fetal Mϕ movement into the maternal uterus during late gestation. $EGFP^+$ Mϕ were present within AF, fetal lungs, amnion and umbilical cords isolated from the pregnant mice at 17.5 and 18.5 dpc, but were undetectable in corresponding uterine segments. Several reasons may account for their absence: 1). Fetal Mϕ do not infiltrate the uterus at term, 2) the $EGFP^+$ signal from the infiltrating population is below the limit of detection, and 3) the fetal-derived Mϕ population that migrate to the gravid uterus at term arise from an CSF-1-independent population.

Fetal macrophages are not detectable in the gravid uterus at term

Development of the *Csf1r*-EGFP transgenic strain by Sasmono *et al*. has facilitated isolation of embryonic $EGFP^+$ Mϕ populations from various tissues and organs during early embryogenesis and in adult mice [172]. In 2007, Rae *et al*. used this model to image Mϕ production and infiltration during organogenesis. The macrophage-restricted expression of EGFP also facilitated Mϕ isolation and led to the phenotypic characterization and comparison of various tissue-specific Mϕ populations during early to mid-gestation [172].

Such extensive studies served to support the appropriateness of this model for tracking fetal-derived Mϕ into non-transgenic maternal tissues. Based on the data presented herein, the absence of detectable $EGFP^+$ cells within the gravid uterus suggests that fetal-derived Mϕ do not infiltrate the term uterus. This raises the possibility that in earlier studies using *Rosa 26 Lac-Z* mice, the co-localization of $F4/80^+$ and *β*-gal-positive cells in uterine tissues isolated from WT mothers carrying *Lac-Z* positive embryos, may have been due to the presence of contaminating fetal tissues, such as amnion. Alternatively, confocal fluorescence imaging of $F4/80^+$ and *β*-gal signals from uterine sections could have suggested the apparent co-localization of these two potentially independent signals but could not have definitively proven this using this method. While confocal microscopy of uterine tissues can be used to determine whether a signal is present, signal emissions from different cellular populations in various tissue planes cannot be distinguished. To demonstrate unequivocally that the two signals were indeed co-localized, Z-stack image acquisition could have been used to examine the dual fluorescence within the uterine sections. Z-stacks are images collected at different focal plane depths that are processed through specialized software to generate a 3-dimentional image that can conclusively determine the source of fluorescent signals when present.

It is noteworthy, that in feto-maternal trafficking studies using EGFP transgenic strains, Bianchi and colleagues previously found flow cytometric (FCM) detection of EGFP-positive cells within maternal WT tissues and organs to be more sensitive than *in vivo* imaging or real-time PCR strategies [404]. A comparative study by Fujiki *et al*. also demonstrated that FCM detection of EGFP-fluorescent cells provides a highly sensitive

method for quantitative and qualitative evaluation of small numbers of fetal cells within a wide range of non-transgenic maternal organs [405].

Alternatively, the absence of $EGFP^+$ Mϕ within the uteri of WT mothers may be explained by the existence of CSF-1-independent mononuclear phagocyte populations. In osteopetrotic (*op/op)* mice harboring a mutation in the gene encoding CSF-1, a key macrophage growth factor, there was an absence or reduction of various Mϕ populations. Interestingly, Mϕ populations within the thymus, lymphoid organs and skin were unaffected [171, 406]. It has been theorized that other local factors may be able to substitute for CSF-1 [172] but this has not been conclusively demonstrated. It is possible that the AF Mϕ population previously shown to infiltrate the uterus is CSF-1-independent. Interestingly, in microarray analysis of AF Mϕ at 15.5, 17.5 and 18.5 dpc, expression of the receptor for CSF-1, CSF1r, was down-regulated of with advancing gestation, further supporting the possibility that the fetal AF Mϕ population that invades the maternal uterus may be CSF-1-independent. This finding should be taken into consideration in future trafficking studies when using *Csf1r*-EGFP transgenic mice. Taken together, the data generated in the present studies, suggest that EGFP-fluorescent fetal AF Mϕ do not infiltrate the term uterus but does not rule out the possibility that other fetal-derived sub-population(s) can enter the term uterus.

Feto-maternal trafficking studies demonstrate that fetal-derived cells infiltrate the decidua at term

It is important to note, that although $EGFP^{+}$ Mϕ were not found within the uterine tissues of WT mothers carrying $EGFP^{+/-}$ fetuses at term, the ubiquitous presence of β-gal activity within the gravid uteri of WT mothers carrying *Rosa 26 Lac-Z* heterozygous pups supports the contention that some fraction of a fetal-derived cell population infiltrates the maternal uterus at term. Furthermore, numerous studies of microchimerism have already demonstrated feto-maternal bidirectional trafficking during late gestation. Recently, Vernochet *et al*. investigated feto-maternal trafficking via the placenta using EGFP transgenic (Tg) mouse models. In those studies, various combinations of crossings were carried out in order to investigate the bi-directional cell trafficking between mother and fetuses via the placenta using a strategy that also made it possible to study the effects of differences in histocompatibility. These included the crossing of EGFP-Tg C57Bl/6 ($H2^{b}$) (inbred) [407], and EGFP-Tg ICR (outbred) [408] strains with non-Tg B6 ($H2^{b}$) and FVB ($H2^{q}$) mice. Importantly, EGFP was expressed in all tissues of the transgenic animals. Three combinations of crossings were analyzed: syngeneic (B6 background × B6 background); allogeneic (B6 background × FvB background), and ICR background × ICR background. In syngeneic matings (non-Tg B6 females × homozygous EGFP Tg B6 males), migration of EGFP-Tg fetal cells into non-Tg maternal decidua, harvested between 6 and 19 dpc, revealed fetal cell migration into the maternal decidual layer of the uterus. This was also observed in allogeneic (non-Tg FvB females × homozygous EGFP Tg B6 males) crosses. The numbers of fetal cells were found to be comparable in these crosses. Between 10 and 12 dpc, groups of fetal cells formed ‘rings’ in the decidua, and after 13 dpc fetal cells invaded the first third of the decidua and increased significantly between 10 and 16 dpc, remaining constant thereafter (the number of fetal cells between 17-19 dpc was not

significantly different). In outbred crosses, fetal cells were found to invade the first third of the decidua after 13 dpc, but unlike syngeneic and allogeneic crosses, their numbers increased significantly until the end of gestation, although the total number of cells were far fewer than that observed in the syngeneic and allogeneic crosses. Under all conditions described, only a few $EGFP^{+}$ fetal cells were observed within the myometrium. Based on their findings, the investigators concluded that passage of fetal cells into the decidua begins at 10 dpc and increases during the second half of gestation, that the fetal-maternal interfaces are similar in syngeneic and allogeneic placentas, and that genetic differences between mother and fetus influence the frequency of the fetal cell trafficking. Additionally, they suggested that some of the bi-directional cell passage between the fetus and mother occurs via the placenta [403]. These studies lend support to our previously reported finding that a fetal-derived population enters the maternal uterine compartment at term.

Given the results of the *Csf1r*-EGFP trafficking studies presented herein, several potentially meaningful observations and questions come to light. First, based on the *Rosa 26 Lac-Z* studies and those carried out by Vernochet *et al*., it is evident that some fetal-derived sub-population does indeed infiltrate the maternal uterine tissues at term. Second, it appears that feto-maternal trafficking activity is predominately localized to the decidual layer of the maternal uterus which could account for the absence of *Csf1r*-EGFP heterozygous fetal cells within the myometrial tissue of the WT mothers examined in the present studies. It also raises the question as to whether it is necessary for the fetal-derived population to penetrate the myometrial layer in order to trigger the inflammatory response cascades associated with labor. Release of inflammatory mediators within the decidual layer may be sufficient to propagate inflammatory signals leading to myometrial

contractility at term. Third, because a degree of trafficking between the fetus and mother has been shown to occur at the placental interface, extravasation of the fetal population may be concentrated within this site. As such, targeting the placental-decidual tissues in future imaging studies may serve to reveal points of entry during late gestation. Finally, future trafficking studies should employ the use of transgenic models in which EGFP expression is not restricted to a specific cell-lineage. This would facilitate isolation and identification of all fetal-derived populations found to invade the maternal tissues of WT mothers at term. Additionally, FCM may provide an alternative method for detection of small numbers of small numbers of cells that fall under the detection limit of current imaging strategies.

Fetal macrophage trafficking in human myometrium at term

It is important to acknowledge that in human studies conducted by Kim *et al*., fetal Mϕ were not found to be present in lower uterine segment myometrial samples obtained from women in labor at term. In these studies, human placental bed biopsies taken from patients in labor and carrying male fetuses were analyzed for the presence of fetal-derived Mϕ within the myometrium. Identification of fetal-derived Mϕ was ascertained by immune-stain for CD68 or CD14. Macrophages within the sampled specimens were isolated by laser capture microdissection and their origin was determined by PCR detection of the male-specific amelogenin allele. Chromogenic in situ hybridization (CISH) using a Y chromosome-specific probe was also used in histological sections to test the presence of male Mϕ [409]. In similar studies, Leong and colleagues also failed to detect fetal-derived

Mϕ within lower uterine segment myometrium obtained from laboring women carrying male fetuses. Using CISH and a Y chromosome-specific probe, the presence of fetal-derived Mϕ was not detectable [410]. These studies concluded that the mechanisms for signaling labor at term in humans are distinct from those reported in mice.

Notably, in those studies, the anatomical sites examined were restricted to the lower myometrial segment and did not include the fundus where the major inflammatory changes that promote uterine contractility take place [64-65]. In mice, distinction between fundal versus lower uterine segments do not exist. Therefore, direct correlations between human and murine uterine anatomical sites should be approached with caution and generalizations should be avoided. The possibility that human fetal-derived cells migrate to specific areas within the fundus, or to specific cell layers within the uterus (*i.e.* decidua), cannot be excluded by the studies described above. Overall, these studies serve to highlight some of the challenges associated with human and mouse fetal-maternal trafficking studies and underscore the need for more comprehensive methods for analysis of human uterine tissues.

Mouse models are an invaluable tool for gaining insight into biologically relevant pathways

Use of mouse models for the study of physiological processes in humans is controversial. Many cite inherent differences between these species as the primary reason for their exclusion. However, mice are invaluable to the investigative process irrespective

of their conserved or divergent pathways. In analysis of conserved pathways, the benefits of their use are clear. When prohibited use of human tissues, cells or biological processes, due to ethical issues or scarcity, limit or preclude investigation of critical biological pathways, the mouse becomes an invaluable asset. In the area of human reproductive investigation, availability and access to healthy reproductive tissues at various stages of pregnancy and labor can be a limiting factor. However, use of the mouse model for the study of parturition is an optimal alternative as accumulating evidence suggests that a decline in PR function near term provides a common mechanism among species for enhancing the inflammatory response in the uterus and cervix near term. Additionally, the use of mouse models provide alternative approaches in addressing various aspects of the mechanisms involved in preterm and term labor and have several advantages: they are relatively inexpensive, have a short gestational period that can be precisely timed, and can be genetically manipulated to discern essential mediators in various pathways. To date, the use of mouse models has led to seminal discoveries in the relationship between inflammation and labor. Undoubtedly, continued use of mouse models will yield further understanding of the physiology of pregnancy and labor across species. The significance of discoveries clarifying the integrated immunological interactions between the mother and fetus at term cannot be overstated.

CHAPTER VI

DISCUSSION

The mechanisms leading to labor at term remain incompletely defined. Both term and preterm labor are associated with an inflammatory response that is, in part, suggested to be mediated by leukocyte infiltration of the myometrium at term. The leukocytes invading the myometrium are proposed to provide a critical source of pro-inflammatory mediators that promote inflammatory cascades in the myometrium leading to uterine contractile gene expression and culminate in labor. The studies described herein sought to characterize the murine AF Mϕ population shown to infiltrate the gravid uterus at term and to assess the changes in their phenotypic properties in association with the developmental induction of SP-A by the fetal lung. The data demonstrate upregulated expression of classical and alternative activation markers in AF Mϕ in tandem as term approaches and suggests that this unique population has the capacity to modulate both pro- and anti-inflammatory functions simultaneously; highlighting their unique phenotypic properties.

During fetal development, several fetal-maternal mechanisms exist to sustain immune privilege and immune suppression thereby preventing fetal rejection by downregulating inflammation. It has previously been demonstrated that during early to mid-gestation, fetal-derived Mϕ possess an M2-anti-inflammatory phenotype that further contributes to immune suppression and to the maintenance of immune privilege. As term approaches, the increased secretion into amniotic fluid of SP-A, and/or in surfactant-associated factors, along with the enhanced expression of TLR2 and TLR4 receptors on

AF Mϕ, may serve to polarize this cellular population. This, in turn, induces the expression of pro-inflammatory cytokines, chemokines and their receptors, resulting in an inflammatory response that may lead to a break in tolerance which may contribute to the initiation of labor.

The results of the parturition studies in surfactant protein deficient mice point to the possible role of other surfactant-associated components in signaling labor at term. Alternatively, these data may instead reflect the complexity of the overlapping mechanisms that have evolved to ensure species propagation. The delayed parturition phenotype observed in TLR2 deficient mice suggests a potential mechanistic link between innate immunity and labor at term.

Insights into the molecular pathways involved in the timing of normal parturition at term will shed light on the intricate roles and cellular molecular cross-talk between the endocrine and immunological system and may provide key therapeutic targets for prevention of preterm labor.

Future studies

Given the results suggesting that AF Mϕ possess a mixed M1/M2 phenotype, it will be important to determine whether this phenotypic profile is due to a dynamic change in a single population, from M2 to M1, or whether two discrete subpopulations coexist within the AF compartment. Alternatively, the AF Mϕ population may indeed represent a novel Mϕ class, in which case further characterization is warranted.

Phenotypic characterization and comparison AF Mϕ isolated from SP-A deficient mice could further serve to determine whether the changes in their morphological and phenotypic properties are a result of SP-A induction. Moreover, analysis of AF Mϕ from TLR2 deficient mice may potentially reveal the role of this receptor in SP-mediated activation of inflammatory pathways at term.

In light of the fact that surfactant protein-A and -D deficiency do not result in a significant parturition defect, it is of substantial interest to explore the possibility that other surfactant-associated factors, such as platelet-activating factor, a highly bioactive phospholipid component of lung surfactant secreted into amniotic fluid near term [389], may lead to the activation of AF Mϕ culminating in myometrial contractility at term. The availability of platelet-activating factor receptor-deficient mice [411] could potentially provide immediate insight into this possibility. The revelation that TLR2 deficient mice manifest a delayed parturition phenotype suggests a role for this receptor in mediating the inflammatory signal(s) for labor at term. Therefore, the effects of TLR2 deficiency on the expression of contraction-associated genes (*e.g.* connexin 43, oxytocin) in the myometrium is of substantial interest, as it may reveal the nature of the parturition defect.

Although fetal-derived EGFP-positive Mϕ were not detectable in the myometrium of pregnant mice at term, the results do not exclude the possibility that other fetal-derived cells enter the maternal decidua prior to labor. Thus, to identify this unique population, ROSA26-EGFP mice [405], which express EGFP ubiquitously, could be used in conjunction with flow cytometric analysis to verify and quantify their presence. The characterization of this unique population may lead to insights into how a fetal-derived

population contributes to the maintenance of immune privilege and then participates in its termination.

The signals for the initiation of labor at term and at preterm are complex and have, for the most part, remained elusive due to the intricate redundancy of the mechanisms that ensure successful species propagation. The survival of the semi-allogenic fetus requires the precise balancing of immune privilege and maternal immunity. The temporal-spatial induction of SP-A, and/or other surfactant-associated factors, near term provide a mechanism by which maternal immunity can be preserved while simultaneously activating a selective pro-inflammatory cascade transmitted by the AF Mϕ that triggers labor at term.

The implications associated with the studies of Condon and colleagues extend beyond the role of AF Mϕ in initiating labor at term. The existence of a potentially novel Mϕ population that can be coerced to mount an inflammatory response in an otherwise immunosuppressive microenvironment is also extremely relevant in cancer where M2 Mϕ are known to play a pathogenic role. In tumors, M1 polarization of local TAM populations may potentially lead to the suppression of angiogenesis, tumor growth and metastasis. A better understanding of how immune privilege may be broken in tumors to promote their rejection is of considerable interest.

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APPENDIX A

PRIMER TABLES

Table I. Murine genotyping primers

Gene	Primer
SP-A	5′ -GGG TGG GAT TAG ATA AAT GCC TG-3′
	5′ -GCA TTA GAC GAC AGA ACT CCA GCC-3′
	5′ -TAC TGA GAG ATG TGT GCT TGG TGA G-3′
SP-D	5′ -TGG TTT CTG AGA TGG AGT CGT G-3′
	5′ -CAG TGG ATG GAG TGT GCA GAG A-3′
	5′ -GTG GAT GTG GAA TGT GTG CGA G-3′
EGFP	5′ -CTG GTC GAG CTG GAC GGC GAC G-3′
	5′ -CAC GAA CTC CAG CAG GAC CAT G-3′
TLR-2	5′ -CTT CCT GAA TTT GTC CAG TAC A-3′
	5′ -GGG CCA GCT CAT TCC TCC CAC-3′
	5′ -ACG AGC AAG ATC AAC AGG AGA-3′
TLR-4	5′ -GCA AGT TTC TAT ATG CAT TCT C-3′
	5′ -CCT CCA TTT CCA ATA GGT AG-3′
	5′ -GCA AGT TTC TAT ATG CAT TCT C-3′
	5′ -CTG ACT CTG TGA CAT CCA TAA C-3′

Table II. SuperArray Primers

cat. Number	Gene symbol	UniGene #
PPM31770A	Arg 1	Mm.154144
PPM03005E	FIZZ1	Mm.33772
PPM35206A	MGL1	Mm.252405
PPM33801A	MGL2	Mm.222465
PPM25130A	YM1	Mm.387173
PPM346	YM2	Mm.244998
PPM03109E	IL-beta	Mm.222830
PPM03015A	IL-6	Mm.2856
PPM02928B	NOS2	Mm.277153
PPM03145E	CX3CR1	Mm.12876
PPM03176A	CCR2	Mm.6272
PPM02975A	CCR8	Mm.442098
PPM57735E	18S	NA
PPM03561A	36b4	Mm.371545
PPM03560E	TBP	Mm.244820

Table III. Genes analyzed using the mouse inflammatory cytokine and receptor RT^2 Profiler PCR Array

ABCF1	BCL6	BLR1	C3	CASP1	CD1	CCL11	CCL12	CCL17	CCL19	CCL2	CCL20
CCL22	CCL24	CCL25	CCL3	CCL4	CCL5	CCL6	CCL7	CCL8	CCL9	CCR1	CCR2
CCR3	CCR4	CCR5	CCR6	CCR7	CCR8	CCR9	CRP	CX3CL1	CXCL1	CXCL10	CXCL11
CXCL12	CXCL13	CXCL15	CXCL4	CXCL5	CXCL9	CXCR3	CCR10	IFNG	IL10	IL10RA	IL10RB
IL11	IL13	IL13RA1	IL15	1L16	IL17B	IL18	IL1A	IL1B	IL1F6	IL1F8	IL1R1
IL1R2	IL20	IL2RB	1L2RG	1L3	IL4	IL5RA	IL6RA	IL6ST	IL8RB	ITGAM	ITGB2
LTA	LTB	MIF	SCYE1	SPP1	TGFB1	TNF	TNFRSF1A	TNFRSF1B	CD40LG	TOLLIP	XCR1
GUSB	HPRT1	HSP90AB1	GAPGH	ACTB	MGDC	RTC	RTC	RTC	PPC	PPC	PPC

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