Taking your breath away

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**Taking Your Breath Away:
The Influence of Antenatal Inflammation
on Fetal Lung Development**

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Chapter 1

General Introduction

Preterm birth

Preterm birth, defined by The World Health Organization as birth before 37 weeks of gestation, can have an enormous influence on the health of the infant at birth and later on in life (1, 2). Besides being a serious health problem, preterm birth also has a significant social impact on families of preterm infants (1). Preterm birth rates vary across countries. In 2013, 7.6% of all births were preterm in The Netherlands (3), whereas preterm birth rates varied from roughly 5% in certain European nations to 18% in several African countries in 2010 (4). The number of preterm born infants worldwide is estimated to be 15 million yearly. Complications after preterm birth are the number one cause of death among children under the age of five. In 2013, preterm birth complications accounted for approximately 1 million deaths (2). Although survival rates among preterm infants improved due to advances in medical technology and neonatal care, there is still a high risk of morbidity such as neurodevelopmental problems and childhood wheezing disorders (1, 5). Intensity of required neonatal care, disability severity and mortality risk are inversely related to the gestational age of the preterm baby (4). Based on the gestational age at birth, infants are categorized as extremely preterm (<28 weeks), very preterm (28 weeks to <32 weeks) or moderate/late preterm (32 weeks to <37 weeks) (2).

While most preterm births occur spontaneously, some preterm births are a result of caesarean section or early induction of labor (2). Preterm birth can be divided into three sub-categories: 1) delivery due to maternal or fetal indications via either induced labor or caesarean section (~30%); 2) preterm premature rupture of membranes (PPROM) followed by vaginal or caesarean delivery (~25%); or 3) spontaneous preterm labor with intact membranes (~45%). The latter two categories are regarded as spontaneous preterm births (6).

Preterm labor is considered to be a syndrome which can be initiated by several mechanisms, including stress, overdistension of the uterus, infection or inflammation, utero-placental hemorrhage or ischemia, and other immunologically mediated processes (6, 7). In most cases the exact mechanism is unknown, although a lot of risk factors are indicated to contribute to the transition from uterine quiescence towards PPRM or preterm labor (6). Risk factors for preterm birth are a history of giving preterm birth, a short cervical length, smoking, and multiple pregnancies like twins and triplets (2, 6). However, one of the most important risk factors is intra-uterine infection, accounting for up to 50% of preterm births <28 weeks (8, 9). Since intra-uterine inflammation often contributes to preterm birth, this thesis will concentrate on antenatal exposure to inflammation and subsequent immune responses in the

preterm infant. In specific, this thesis focuses on pulmonary inflammation and lung development after intra-uterine inflammation, as the lungs are directly exposed to contaminated amniotic fluid *in utero* due to the breathing movements the fetus practices (10, 11).

Chorioamnionitis

Chorioamnionitis is defined as an intra-uterine infection or inflammation of the chorion and the amnion (fetal membranes) and the placenta, and is caused by invasion of microbes or due to other pathological processes (8, 12, 13). Because intra-uterine infection is often chronic and clinically silent until the membranes rupture or preterm labor starts, it is hard to identify in women (8). After birth, chorioamnionitis is diagnosed by histological examination of the placenta (gold standard), biochemical criteria or microbiological findings (14-17). The incidence of histological chorioamnionitis is inversely related to the gestational age of the preterm infant at birth, making the presence of histological chorioamnionitis more common in extremely preterm infants (18). Clinical chorioamnionitis is defined by one or more clinical symptoms, such as maternal fever, uterine tenderness, maternal or fetal tachycardia and foul amniotic fluid (15, 16).

Intra-uterine infection can occur when microorganisms invade the uterus via the ascending route from the vagina and cervix, the retrograde route from the abdominal cavity through the fallopian tubes, haematogenous placental spread or via contamination of the needle during amniocentesis or chorionic-villus sampling (6, 8, 19) (*Figure 1*).

Figure 1. Different routes of intra-uterine infection. Microorganisms can invade the uterus via the ascending route from the vagina and cervix, the retrograde route from the abdominal cavity through the fallopian tubes, haematogenous placental spread or via contamination of the needle during amniocentesis or chorionic-villus sampling (6).

The ascending route from the lower genital tract is the most common route (6, 20). In this case, the infection starts with a change of the microbial flora in the vagina or cervix (stage I) (*Figure 2*). The microorganisms enter the intra-uterine cavity and are localized between the chorion and the membranes (stage II) before they continue through the amnion to reach the amniotic cavity and start an intra-amniotic infection (stage III). Once the microorganisms are present in the amniotic cavity, they can invade the fetus (stage IV) via the gastrointestinal tract, skin, ears or respiratory tract (19, 20). If they reach the fetal circulation, intra-uterine infection can be followed by a fetal inflammatory response syndrome (FIRS), which is characterized by systemic inflammation and elevated fetal plasma interleukin (IL)-6 concentration (10, 20-23). FIRS can affect multiple organs such as the lungs, brain and the gut (*Figure 2*) and therefore chorioamnionitis is currently considered to be a multi-organ disease of the

fetus (10, 20, 21). Although the lungs are not yet functioning as breathing organs during intra-uterine growth, their normal development is crucial for gas exchange and life after birth (24), explaining why this thesis focuses on the lungs.

Figure 2. Different stages during the ascending route of intra-uterine infection and fetal target organs during a fetal inflammatory response syndrome. Stage I is marked by a change of the microbial flora in the vagina or cervix. The microorganisms enter the intra-uterine cavity and are localized between the chorion and the membranes (stage II). Through the amnion the microorganisms reach the amniotic cavity and start an intra-amniotic infection (stage III). Once the microorganisms are present in the amniotic cavity, they can invade the fetus (stage IV). If the intra-uterine infection is followed by a fetal inflammatory response syndrome (FIRS), multiple organs can be affected. Adapted from (20, 21).

Lung development

The respiratory system consists of tubes which conduct air to a massive gas-exchange system composed of alveoli (25). For the development of these structures, lung development is divided into five stages (24, 25). A schematic overview of these stages and their corresponding developmental events is shown in *Figure 3*.

Figure 3. Stages of human lung development. Adapted from (26). A schematic overview of the embryonic, pseudoglandular, canalicular, saccular and alveolar stage and the corresponding developmental events is shown as a function of gestational (in utero) and postnatal age.

Embryonic stage (3 - 8 weeks of gestation)

Lung morphogenesis starts with the embryonic stage around 3 weeks of gestation with formation of the lung bud (24, 25). This lung bud extends from the foregut into the surrounding mesenchyme and divides into two bronchial buds, which will later on develop into the left and right main bronchi (24, 25, 27, 28). By the fifth week, these two primary bronchial buds branch into three stems on the right and two stems on the left, corresponding to the right and left lung lobes (24, 25). Mesenchymal cells around the airways form blood vessels, cartilage, smooth muscle and other types of pulmonary connective tissue (25). At the end of this stage, the first bronchopulmonary segments and primitive pulmonary arteries and veins are formed (24, 25).

Pseudoglandular stage (8 - 16 weeks of gestation)

The pseudoglandular stage starts at 8 weeks and continues to week 16. Its name is derived from the fact that the lungs, in cross-section, resemble gland-like structures surrounded by dense mesenchyme (25). During this stage, airways continue to branch and form the conducting parts of the lungs up to the level of the terminal bronchioles (24, 25). Although after 16 weeks all divisions are more or less complete, gas exchange would not be possible yet. The reason for this is that only the conducting parts and not the respiratory components involved in gas exchange are present so far (25, 27, 28). Therefore, fetuses who are born during this stage will not survive (28).

Pulmonary arteries have the same branching pattern as the airways since they develop in parallel (25, 27). Cartilage and glands start to form around major airways, while smooth muscle forms around airways and large vessels (24, 25). Differentiation of the epithelium and mesenchyme initiates in proximal airway regions. The differentiation process then progresses to more distally located cells (25). Epithelial cells start to produce lung fluid, which may then either flow into the amniotic fluid due to fetal breathing movements, or is swallowed by the fetus (24, 29, 30).

Canalicular stage (16 – 26/28 weeks of gestation)

During the canalicular stage, each terminal bronchiole divides into two or more respiratory bronchioles, after which each further divides to eventually produce three to six alveolar ducts (25, 28). Some terminal sacs (which will later on develop into the alveoli) already form at the end of the airways (28). All the air spaces which are derived from one terminal bronchiole (the respiratory bronchioles, the alveolar ducts and the terminal sacs) are together called an acinus (24, 31). Capillaries invade the mesenchyme around these acini (24). The capillaries are closely located to the walls of the respiratory bronchioles, giving rise to the air-blood barrier which is necessary for gas exchange after birth (24, 25, 27). The canalicular stage is therefore characterized by extensive angiogenesis. Due to this vascularization, dense capillary networks are formed and potential airspaces become 'canalized' (25).

The epithelium, which in the end will line the alveoli, is composed of two types: the type I alveolar cells (also called type I pneumocytes) and the secretory type II alveolar cells (27). During the canalicular stage, the type II pneumocytes start producing surfactant, which is a mixture of proteins, phospholipids and cholesterol (24, 28). Surfactant covers the epithelial surface to reduce surface tension and prevent collapse of the lungs when breathing is initiated after birth (24, 27).

Throughout lung development, type II pneumocytes are one of the first cell types to be formed (27). Type I cells differentiate out of type II cells and are necessary for gas exchange after birth (24, 25, 27). During this differentiation process, a part of the cubic type II cells loses their secretory function, starts to flatten and eventually terminally differentiates into type I cells (24, 25, 27). Differentiation into type I pneumocytes and formation of the capillary network enables the fetus to survive outside the uterus after 24-26 weeks (24, 28). However, intensive care is necessary and infants may suffer from respiratory distress due to surfactant deficiency and a still immature respiratory system (27, 28). Since cranial parts of the lungs mature more rapidly than their caudal counterparts, there is some overlap between this and the next stage (25).

Saccular stage (26/28 – 32/36 weeks of gestation)

During this stage, more terminal sacs or saccules are formed. Their epithelial layer becomes very thin and around 26 weeks this layer is mainly composed of type I cells to facilitate gas exchange. Scattered between the type I cells are the round type II pneumocytes for surfactant production (28). Secondary crests with a double capillary network divide the terminal sacs at the end of the airways into smaller units (24, 25). The beginning of septation by these vascularized septa, a clear decrease in the thickness of interstitial tissue, an increase in the capillary bed and thinning of the epithelium all contribute to an increased lung surface area and efficient gas exchange (25). At the end of this stage, interstitial fibroblasts start producing extracellular matrix components, such as collagen and elastin fibers (24, 25). The number and size of type II cells increase and surfactant lipids accumulate within these cells (25).

Alveolar stage (32/36 weeks of gestation - postnatal)

During the last weeks of pregnancy, formation of alveoli (called 'alveolarization') exponentially increases the respiratory surface area of the lungs (24, 27). During this process, secondary septa divide sacculi into smaller subunits, the alveoli (24). Initially, these septa are quite thick but they will remodel into mature and thin septa which are capable of gas exchange (28). The double capillary networks within alveolar septa fuse into a single capillary system and alveoli become larger as they mature (25, 28). During alveolarization distal focal deposits of elastin define the positions where alveoli will be formed (32).

The exact time point at which the alveolar stage starts and ends is under debate. While some fetuses already show alveoli at 32 weeks of gestation, general consensus is that the alveolar stage starts at 36 weeks (25). At birth, on average 50 million of the eventually >300 million alveoli are present with a gas-exchange surface area of ~3-4 m² (25, 33). Alveolarization continues postnatally to about 1-2 years of age during which the surface area increases to 75-100 m² (24, 25, 33). Although alveolarization is largely completed after these first years of life, new alveoli are still likely to be formed during childhood at a much slower pace (28, 33). Septal maturation extends up until 2-3 years of age, in which double capillary networks continue to restructure into single capillary beds (25, 33). After 2 to 5 years of age, the lungs continue to grow in proportion to the rest of the body, but this growth is mainly due to increased alveolar volume and not number (25).

Involvement of the Wntless-Int (Wnt) pathway

Lung development is a complicated time- and spatially-regulated process in which multiple factors are involved, such as growth factors, transcription factors and extracellular matrix components (34). The Wntless-Int (Wnt) family consists of growth factors which are essential for pulmonary development (34). Wnt signaling can be divided into three different intra-cellular pathways: the non-canonical Wnt/Ca²⁺ pathway, the planar cell polarity pathway and the most important one, the Wnt/ β -catenin pathway (34, 35). When the canonical Wnt/ β -catenin pathway is inactive due to absence of Wnt signaling, glycogen synthase kinase 3 β (GSK-3 β) is active and phosphorylates the protein β -catenin (36). The binding of Wnt to its receptors inhibits GSK-3 β activity (34, 36). β -catenin phosphorylation and subsequent degradation is then prevented, after which β -catenin can accumulate in the cytoplasm (34-36). β -catenin translocates into the nucleus where it binds to transcription factors to initiate transcription of downstream target genes (34-36).

Pulmonary expression of both β -catenin and the different Wnts is tightly regulated in epithelial and mesenchymal cells (34). Epithelial-mesenchymal interactions are essential for lung cell proliferation and differentiation (34, 35). In the human lungs, expression of β -catenin and the canonical Wnt ligands Wnt2 and Wnt7b is already detected at 7 weeks of human gestation (37). This suggests that involvement of Wnt/ β -catenin signaling starts early during lung development. Transgenic and knockout mice models have indicated the essential role of Wnt/ β -catenin signaling during lung development (34, 38-41). It was shown that deletion of β -catenin throughout embryonal development led to a whole variety of pulmonary defects: a

shortened trachea, decreased branching and reduced peripheral mesenchyme (40). In addition, after embryonal deletion of β -catenin the formation of terminal alveolar saccules was reduced and lungs were primarily composed of conducting airways, suggesting a role for β -catenin in alveolar development (38, 39). Hyperactivation of β -catenin is not beneficial as well, since it resulted in enlarged air spaces and abnormal differentiation of cells resembling alveolar type II cells inside conducting airways (38). When *Wnt7b* was knocked out, lung hypoplasia with a reduced amount of distal mesenchyme was observed and the mice died directly after birth due to respiratory failure. Furthermore, the pulmonary expression of smooth muscle α -actin was abnormal within these mice, suggesting that *Wnt7b* is required for growth and development of the pulmonary mesenchyme and vasculature (41).

Adverse lung development & bronchopulmonary dysplasia

Since the fetus already practices breathing movements *in utero*, amniotic fluid contaminated with microorganisms or cytokines can reach the lungs during an intra-uterine infection. Exposure via this direct route or translocation of cytokines from the circulation into the fetal lungs can induce pulmonary inflammation (10, 11, 42-46). Several postnatal factors such as resuscitation, oxygen toxicity or mechanical ventilation can amplify and extend this inflammatory response, disrupting alveolar and vascular lung development (44). Therefore, chorioamnionitis is associated with an increased risk for chronic lung disease if the preterm infant is postnatally exposed to sepsis or extended mechanical ventilation (47).

A chronic lung disease that mainly affects preterm infants is bronchopulmonary dysplasia (BPD). It is diagnosed postnatally when an infant is oxygen dependent for at least 28 days after birth (48, 49). Severity of BPD is defined as mild, moderate or severe, based on the required respiratory support (48, 49). BPD results from disruption of alveolar and vascular development and is therefore characterized by impaired vascular development, as well as by alveolar simplification and enlargement due to impaired alveolarization (50, 51). Gentler ventilation techniques, exogenous surfactant therapy and the use of maternal corticosteroids attributed to the fact that infants with lower birth weights can currently be successfully resuscitated. Since it is now possible for these infants to survive, lung injury can also arise at earlier lung developmental stages (49, 52). Preterm infants who are at the greatest risk for BPD are born at an extremely preterm age between 24 and 28 weeks, at which the lungs are in the late canalicular or early saccular stage (2, 53). For these infants, final

pulmonary maturation steps occur *ex utero*, when their lungs are exposed to the external environment (54).

There is increasing evidence that BPD is, at least partially, a result of an imbalance between pro- and anti-inflammatory mechanisms (55). This imbalance that favors pro-inflammatory cytokines over anti-inflammatory ones, is considered to be essential for lung injury (55). Therefore, deficient inhibition of high inflammatory cytokine responses after birth in chorioamnionitis-exposed infants may increase the risk of developing BPD (56). Due to its multifactorial pathogenesis (e.g. oxygen exposure, mechanical ventilation, genetic susceptibility, fetal growth restriction, dietary deficits), it is challenging to prevent and treat BPD (57). Since inflammation appears to play a major role in the pathogenesis of BPD, treatment options like corticosteroids focus on prevention of pulmonary inflammation and subsequent lung injury (56, 57). We hypothesize that early intervention, when the fetus is still *in utero*, may be beneficial in inhibiting inflammation in the setting of chorioamnionitis (58, 59) and by doing so possibly prevent the development of BPD.

Corticosteroids

Antenatal corticosteroids are regularly administered to mothers at risk for preterm birth to reduce neonatal death and respiratory distress syndrome (RDS) (60). RDS is a pulmonary insufficiency disorder, which mainly occurs in preterm infants due to lack of surfactant and due to structural immaturity of the lungs at birth (61). Glucocorticoids do not only accelerate fetal lung maturation and surfactant production, but also prevent inflammation (60, 62). Therefore, a single course of maternal corticosteroids is recommended as a standard treatment when an infant is anticipated to be born prematurely (60). Maximal benefits from corticosteroids are obtained when they are administered within a time frame of 1-7 days before birth (63). Since preterm fetuses are frequently exposed to both chorioamnionitis and corticosteroids, the combination of exposure to inflammation and corticosteroids is quite common in clinical practice (64, 65).

Even though currently the use of corticosteroids is essential in the clinic, their use may not be optimal. Glucocorticoids beneficially accelerate lung maturation and surfactant production, but they can be detrimental for alveolarization (60, 62). Therefore, new and better therapies are required with the same favorable effects as corticosteroids, but without any detrimental side-effects on neonatal pulmonary outcome.

Outline of this thesis

Development of healthy and functional lungs is crucial for life after birth. Since preterm infants are born before the *in utero* stages of lung development are complete, they have a higher chance to develop respiratory complications after birth or later on in life (5, 61). This is especially the case when the infant is exposed to intra-uterine inflammation. Although chorioamnionitis is associated with a reduced incidence and severity of RDS, it can increase the risk for BPD because intra-uterine inflammation interacts with postnatal stimuli such as mechanical ventilation and sepsis (47, 66). To prevent postnatal complications such as BPD, and to develop more effective treatment strategies, a better understanding of how lung development is affected by intra-uterine inflammation is essential. Therefore, the general aim of this thesis is to gain more insight how fetal pulmonary alveolar and vascular development are disrupted by exposure to chorioamnionitis. Furthermore, it was assessed how the use of maternal corticosteroids interferes with some of these processes. To study the pulmonary effects of intra-uterine inflammation, an ovine model was used, since sheep lung development closely resembles human lung development (67, 68). While alveolarization is initiated in sheep and humans before birth, it starts postnatally in rodents (67). Furthermore, sheep have a relatively long gestational age (term ~147 days) and are large animal models, making fetal instrumentation possible.

Preterm fetuses are frequently exposed to both chorioamnionitis and corticosteroids antenatally. Since chorioamnionitis is often clinically silent, the order of exposure may be unknown and may vary (64, 65). In **chapter 2**, the interaction and the effect of the order of exposure to intra-uterine inflammation and corticosteroids was assessed. More precisely, we focused on fetal lung development by studying the essential canonical Wnt/ β -catenin pathway. To induce intra-uterine inflammation, lipopolysaccharide (LPS) from *Escherichia coli* was given intra-amniotically. The corticosteroid betamethasone was given intra-muscularly to the ewe, either before or after LPS exposure. Expression levels of different Wnt isoforms and of proteins involved in the downstream Wnt/ β -catenin signaling cascade were studied as outcome parameters.

Previously, it was shown in our chorioamnionitis model that intra-uterine inflammation resulted in pulmonary and systemic inflammation (10, 46, 69). While pulmonary inflammation was associated with structural alterations, it improved pulmonary function due to an increased surfactant lipid pool as well as an enlarged lung gas volume (69-71). Furthermore, ovine and primate models indicated that intra-uterine inflammation was associated with decreased numbers of regulatory T cells

(Tregs) in several lymphatic organs, including the lung-associated mediastinal lymph node (46, 72-74). As Tregs are important for the regulation of inflammatory responses (75), we modulated the pulmonary inflammatory response in the fetus by clonal expansion of Tregs using IL-2, as described in **chapter 3**. Fetal sheep were instrumented and received prophylactic administration of IL-2, followed by intra-amniotic LPS exposure. Inflammatory parameters were assessed for the lungs and in the systemic circulation. Furthermore, we assessed pulmonary parameters associated with morphology, lung volume and surfactant concentration.

Although it is known that intra-uterine inflammation can affect pulmonary and vascular development at 120-124 days of gestational age in fetal sheep, which corresponds to the saccular stage (42, 64, 68, 69, 76), there is almost no knowledge regarding the effect of intra-uterine inflammation at a much more immature stage. Because of the inverse relation between the incidence of histological chorioamnionitis and the gestational age of the preterm infant at birth, the presence of histological chorioamnionitis is more common in extremely preterm infants (18, 77). Extremely preterm infants born during the canalicular stage are in an essential phase of pulmonary vascular development (50, 53). Therefore, in **chapter 4**, we assessed the inflammatory and vascular effects of intra-uterine inflammation at the canalicular stage (94 days) in the ovine fetus (68). Intra-amniotic exposure to LPS or *Ureaplasma parvum*, which is one of the most frequently found microorganisms in amniotic fluid of patients with PPROM (78), were used to induce intra-uterine inflammation. Furthermore, we assessed the effects of combined exposure to these two inflammatory stimuli, to determine whether chronic pre-exposure to *Ureaplasma parvum* modulated the subsequent LPS-induced pulmonary response.

The major findings of this thesis, its clinical application and future perspectives are discussed in **chapter 5**.

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Chapter 2

Altered canonical Wingless-Int signaling in the ovine fetal lung after exposure to intra-amniotic lipopolysaccharide and antenatal betamethasone

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Abstract

Background: Antenatal inflammation and maternal corticosteroids induce fetal lung maturation but interfere with late lung development. Canonical Wingless-Int (Wnt) signaling directs lung development and repair. We showed that intra-amniotic (IA) lipopolysaccharide (LPS) exposure disrupted developmental signaling pathways in the preterm lamb lungs. Therefore, we hypothesized that pulmonary Wnt signaling was altered by exposure to IA LPS and/or antenatal corticosteroids.

Methods: Ovine fetuses were exposed to IA LPS, maternal intramuscular betamethasone, a control saline injection, or a combination thereof at 107 and/or 114 d gestational age (term = 150 d gestational age) before delivery at 121 d gestational age.

Results: IA LPS exposure decreased the lung expression of lymphoid enhancer-binding factor 1 (LEF1), a major Wnt pathway effector. *WNT1*, *WNT4*, and downstream messenger β -catenin decreased after LPS exposure. *WNT7b* mRNA increased fourfold 14 d post-LPS exposure. Betamethasone treatment 7 d before LPS exposure prevented the reduction in LEF1 expression, whereas betamethasone administration after LPS normalized the LPS-induced increase in *Wnt7b* mRNA.

Conclusion: IA LPS exposure decreased canonical Wnt signaling in the developing lung. Antenatal corticosteroids before or after IA inflammation had different effects on pulmonary Wnt signaling. This study provides new insights into possible mechanisms by which prenatal inflammation affects lung development and how corticosteroid can be beneficial in this setting.

Introduction

Preterm birth is frequently initiated by chorioamnionitis, an intrauterine inflammation of the chorioamniotic membranes (1). Exposure to antenatal inflammation may contribute to adverse neonatal outcomes in preterm infants including lung injury (2). As a result, bronchopulmonary dysplasia (BPD) can develop, which is characterized by fewer and larger alveoli due to an arrested development of alveolar septation and impaired pulmonary microvascular development (3). The corticosteroid betamethasone is routinely administered to mothers at risk for preterm delivery to improve neonatal survival (4). Although corticosteroids accelerate lung maturation, they also inhibit the outgrowth of secondary septa and therefore can interfere with late lung development (5).

Wingless-Int (Wnt) signaling plays a role in lung development by regulating epithelial and mesenchymal interactions in an autocrine and paracrine manner (6,7). The canonical Wnt pathway signals through β -catenin (6). In the absence of Wnt ligands, β -catenin is phosphorylated by glycogen synthase kinase (GSK)-3 β , ubiquitinated, and degraded. Upon activation of the Wnt cascade, GSK-3 β becomes phosphorylated and inhibited (8). Consequently, β -catenin phosphorylation is prevented, and the amount of cytosolic β -catenin increases (8). The accumulated β -catenin translocates to the nucleus and regulates gene transcription via formation of an active transcription complex with T-cell-specific transcription factor/ lymphoid enhancer-binding factor (LEF) family members (9). The importance of Wnt signaling during lung development was demonstrated in several transgenic models of different Wnt isoforms. Wnt2^{-/-} mice have severe lung hypoplasia and a poorly developed lung mesenchyme at birth (10). Similarly, knockout of Wnt7b in mice results in lung hypoplasia and perinatal death due to respiratory failure (11). Reduced pulmonary Wnt7b expression impairs mesenchymal growth and vascular development (11). Wnt1 and Wnt4 are associated with the expression of matrix metalloproteinases, which are known to be involved in lung epithelial repair processes and pulmonary fibrosis (12,13).

Previously, we showed that exposure to intra-amniotic (IA) lipopolysaccharide (LPS) induced pulmonary inflammation in fetal lambs and influenced lung developmental pathways (14,15). IA LPS exposure altered the expression of the lung structural proteins elastin and collagen, leading to a simplified lung structure as seen in BPD patients (16). Betamethasone pre or posttreatment partially counteracted these effects (14). However, little is known about the molecular signaling cascades by which perinatal events affect late lung development. Rodent models of neonatal hyperoxia-induced lung injury have recently related perturbations in the Wnt cascade with developmental alterations in postnatal lung development, similar to BPD (17). In this

study, we investigated the effects of intrauterine inflammation, which is implicated in the pathogenesis of BPD, on canonical Wnt signaling in a preterm ovine model in which the pulmonary development closely resembles the early alveolarization stage in extremely preterm infants (18). We hypothesized that Wnt signaling was altered in ovine fetal lungs exposed to *in utero* inflammation and/or antenatal corticosteroids. For this purpose, we examined different Wnt isoforms and downstream effectors of the canonical Wnt pathway in the lungs of fetal sheep that were exposed to IA LPS and/or antenatal corticosteroids.

Materials & Methods

Animal Model and Sampling Protocol

All animal experiments were approved by the Animal Ethics Committees of Cincinnati Children’s Hospital Medical Center and the University of Western Australia (animal ethics protocol RA/3/100/830). Time-mated Merino ewes with singleton fetuses were randomly divided into six treatment groups (*n* = 5–8 per group). The ewes received either: an IA injection of 10 mg LPS (*Escherichia coli*, 055:B5; Sigma-Aldrich, St Louis, MO), an intramuscular injection of the corticosteroid betamethasone (Celestone Soluspan 0.5mg/kg maternal weight; Schering-Plough, North Ryde, Australia), a control saline injection, or a combination thereof at 107 and/or 114 d gestational age (Figure 1). Because betamethasone treatment can induce preterm delivery in sheep, all ewes also received a single intramuscular injection of medroxyprogesterone acetate (Depo-Provera 150mg; Kenral, New South Wales, Australia) at 100 d gestational age to decrease the risk of preterm labor (19). Lambs were surgically delivered preterm at 121 d gestational age (term is 150 d gestational age) and were humanely killed. The lungs were removed, and samples of right caudal lobe (right lower lobe) tissue were snap frozen. The whole right cranial lobe (right upper lobe) was inflation fixed for 24h in 10% buffered formalin at a pressure of 30 cm H₂O and processed for paraffin embedding.

Figure 1. Study design. Ovine fetuses were exposed to intra-amniotic (IA) LPS, a maternal intramuscular (IM) betamethasone (Beta) injection, a control saline injection, or a combination thereof at 107 and/or 114 d gestational age (dGA) (term = 150 dGA). All ewes also received an intramuscular injection of medroxyprogesterone acetate (Depo-Provera) at 100 dGA to decrease the risk of preterm labor. Lambs were delivered preterm by cesarean section at 121dGA. LPS, lipopolysaccharide.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from snap-frozen right lower lobe tissue using the SV Total RNA Isolation System (Z3100, Promega, Madison, WI), according to the manufacturer's protocol. A DNase treatment was performed to eliminate possible genomic DNA contamination, using the RQ1 RNase-Free DNase kit (M6101; Promega). cDNA was synthesized with a Transcriptor First Strand cDNA Synthesis Kit using Anchored-oligo(dT)18 Primers (04379012001; Roche Applied Science, Penzberg, Germany), according to the manufacturer's protocol. Primers were created based on genome sequences of *Bos taurus* and *Ovis aries* (Table 1). Dilution experiments were performed to ensure proper PCR amplification efficiency of the primers. Quantitative PCR reactions were run in duplicate for each primer set at the appropriate melting temperature for 45 cycles with a LightCycler 480 Instrument (Roche Applied Science) and LightCycler 480 SYBR Green I Master mix (4707516001; Roche Applied Science). Quantitative PCR results were normalized to the housekeeping gene ovine ribosomal protein S15 (ovRPS15). Mean fold changes in mRNA expression, as compared with control animals, were calculated using the $\Delta\Delta C_t$ method (20).

Table 1. Primers used for RT-PCR

Gene		Sequence (5'-3')	Amplicon size (bp)	Temperature (°C)
<i>WNT1</i>	Fw	ATTTATCTTCGCCATCACCTC	123	64
	Rv	ATTCGATGGAGCCCTCTG		
<i>WNT2</i>	Fw	GGTGGCTGCAGTGATAACATTGAC	60	58
	Rv	ACCTCTTTACAGCCTTCCTGCC		
<i>WNT4</i>	Fw	GCTGGGCTCCAAGTACACC	241	60
	Rv	GGCTATCCTGACACACATGC		
<i>WNT7b</i>	Fw	TGCACTCCAGCTTCATGCGC	60	58
	Rv	ACCTGCACAACAACGAGGCG		
<i>CTNNB1</i>	Fw	CTATTGAAGCCGAGGGAG	200	60
	Rv	CAAGATCAGCAGTCTCATTCC		
<i>GSK3B</i>	Fw	CGAGACACACCTGCACTCTT	157	60
	Rv	CACGGTCTCCAGCATTAGCA		
<i>LEF1</i>	Fw	CAACTCCAAACAAGGCATGTCC	90	60
	Rv	GGTAATCTGTCCAACACCACC		
<i>ovRPS15</i>	Fw	CGAGATGGTGGGCAGCAT	93	60
	Rv	GCTTGATTCCACCTGGTTGA		

Fw, forward; RT-PCR, real-time PCR; Rv, reverse.

Immunohistochemistry

Paraffin-embedded right upper lobe lung tissue sections (4 μ m) were deparaffinized using a xylene and ethanol (100, 96, and 70%) series. Incubation in sub-boiling citrate buffer (10 mmol/l, pH 6.0) for 30 min was used for antigen retrieval. To block endogenous peroxidase activity, slides were incubated in phosphate-buffered saline (pH 7.4) with 0.5% hydrogen peroxide (H_2O_2) for 20 min. Nonspecific binding of antibodies was prevented by incubating the sections with 10% normal goat serum (NGS) in phosphate-buffered saline. Sections were stained overnight at 4°C with monoclonal rabbit anti-LEF1 antibody (1:100, 2230; Cell Signaling Technology, Danvers, MA). A negative control was included by incubating with 0.1% bovine serum albumin/phosphate-buffered saline instead of the primary antibody. After 1h of incubation with biotinylated polyclonal swine antirabbit secondary antibody (1:200, E0353; Dako, Glostrup, Denmark), the immunostaining was augmented with the addition of a peroxidase-labeled ABC complex (1:500; Vectastain Elite ABC kit PK-6200; Vector Laboratories, Burlingame, CA). Immunostaining was visualized with Nickel-enhanced diaminobenzidine, and the sections were counterstained with 0.1% nuclear fast red solution.

Staining of the sections was evaluated by light microscopy (DM2000; Leica Microsystems, Wetzlar, Germany). The area fraction (%) of LEF1 immunoreactivity was measured in six random images per section by a blinded observer by applying a standard threshold using a specifically designed algorithm in Leica QWin Pro V 3.5.1 software (Leica, Rijswijk, The Netherlands) and corrected for the total percentage of lung tissue measured.

Western Blot

For protein analysis, snap-frozen right lower lobe tissue was homogenized in ice-cold radioimmunoprecipitation assay buffer (R0278; Sigma-Aldrich) supplemented with 0.1% protease inhibitor cocktail (P9599; Sigma-Aldrich). Homogenates were centrifuged for 5min at 12× relative centrifugal force. To determine protein concentrations, a Micro Bicinchoninic Acid Protein Assay Kit (23235; Thermo Fisher Scientific, Waltham, MA) was used with bovine serum albumin as a standard, according to the manufacturer's instructions. Samples were diluted in 5× sample buffer (consisting of glycerol, sodium dodecyl sulfate (SDS), 0.5mol/l Tris-HCl (pH 6.8) + 0.4% sodium dodecyl sulfate, bromophenol blue, dithiothreitol, and Milli-Q) and denatured by heating at 95°C for 10 min. Equal amounts of protein (40 μ g per sample) were loaded on sodium dodecyl sulfate–polyacrylamide gels. Proteins were separated

within 1.5h (90V) by gel electrophoresis and transferred onto 0.2 μm pore size Protran BA83 nitrocellulose membranes (10402495; Whatman, Dassel, Germany) within 1h (350 mA). Membranes were blocked for 1h with a 1:1 mixture of ODYSSEY Infrared Imaging System Blocking Buffer (927-40000; LI-COR, Lincoln, NE) and Tris-buffered saline (2.5 mmol/l, pH 7.5) and incubated overnight at 4°C with the following primary antibodies: monoclonal rabbit anti- β -catenin (1:1000; 9582, Cell Signaling Technology), monoclonal rabbit anti-GSK-3 β (1:4000; 9315, Cell Signaling), or monoclonal mouse anti- β -actin (1:1000; A5441, Sigma-Aldrich). IRDye 800CW conjugated goat antirabbit (1:6000; 926-32211, LI-COR) or IRDye 680RD conjugated donkey antimouse (1:6000; 926-32222, LI-COR) were used as secondary antibodies (1h). Protein bands were detected and analyzed using a LI-COR Odyssey Infrared Imager System. Results were normalized to the housekeeping gene β -actin.

Data Analysis

Statistical analysis was conducted using GraphPad Prism v5.0 software (GraphPad Software, La Jolla, CA). One-way ANOVA with Tukey's test for post hoc analysis was used to compare the groups. All data are presented as means $\pm$ SEM. $P < 0.05$ was considered to be significant.

Results

Pulmonary LEF1 Expression

To investigate changes in pulmonary Wnt signaling, we determined the mRNA levels and immunoreactivity of LEF1, the major downstream effector of the Wnt pathway, the nuclear expression of which increases upon Wnt activation (21). Exposure to LPS 7 or 14 d before preterm delivery resulted in decreased *LEF1* mRNA levels (*Figure 2a*) and LEF1 protein immunoreactivity (*Figure 2b*). LEF1 immunoreactivity was mainly localized in the nucleus of the alveolar cells in control animals. LPS-exposed animals showed reduced nuclear LEF1 staining in the alveoli. Betamethasone treatment before the LPS exposure but not after LPS exposure prevented the decrease in LEF1. Representative images of positive LEF1 staining are shown for controls (*Figure 2c*) and 14-d LPS-exposed lungs (*Figure 2d*).

Figure 2. Pulmonary LEF1 expression. (a) *LEF1* mRNA: mRNA levels of *LEF1* were decreased 7 and 14 d after LPS exposure. Combined exposure to 14-d LPS and 7-d Beta also resulted in decreased *LEF1* mRNA levels. (b) LEF1 expression: the area fraction (%) of LEF1 immunoreactivity in the lung decreased after exposure to LPS 7 or 14 d before delivery. Beta treatment before the LPS exposure but not after LPS exposure prevented the decrease in LEF1. (c) LEF1 staining (black staining) in the fetal lung tissue (red counterstaining) was detected in the nucleus of the alveoli in control animals. (d) 14-d LPS-exposed animals showed reduced nuclear LEF1 staining. Scale bar = 50 μ m; scale bar, insert = 20 μ m. * P < 0.05 vs. controls and § P < 0.05 between experimental groups using a one-way ANOVA with Tukey's post hoc test. Beta, betamethasone; LPS, lipopolysaccharide.

Lung mRNA Levels of WNT Isoforms

The mRNA expression of different WNT isoforms was measured to characterize the upstream changes in Wnt signaling after IA LPS exposure. *WNT1* mRNA levels in the fetal lung decreased by 90% 7 d after the exposure to LPS as compared with controls (Figure 3a). Exposure to LPS at 14 d followed by betamethasone exposure at 7 d before delivery increased *WNT1* mRNA levels when compared with 14-d LPS exposure alone. *WNT2* mRNA decreased by 80% 14 d after LPS exposure irrespective of

betamethasone posttreatment (Figure 3b). *WNT4* mRNA levels were significantly decreased in all LPS-exposed treatment groups as compared with controls (Figure 3c). In contrast, *WNT7b* levels increased fourfold 14 d after LPS exposure as compared with controls (Figure 3d). Treatment with betamethasone 7 d after the LPS exposure normalized this increase. However, in this group, there was still a trend toward increased *WNT7b* expression.

Figure 3. Lung mRNA levels of *WNT* isoforms. (a) *Wnt1* mRNA: the mRNA levels of *WNT1* decreased ninefold 7 d after LPS exposure as compared with controls. (b) *Wnt2* mRNA: *WNT2* mRNA decreased after 14 d of LPS exposure irrespective of the betamethasone treatment. (c) *Wnt4* mRNA: levels of *WNT4* mRNA decreased in all LPS-exposed groups as compared with controls. (d) *Wnt7b* mRNA: mRNA levels of *WNT7b* increased nearly fourfold 14 d after LPS exposure as compared with controls. This increase was prevented if betamethasone (Beta) was administered 7 d after LPS exposure. * $P < 0.05$ vs. controls and § $P < 0.05$ between experimental groups using a one-way ANOVA with Tukey's post hoc test. LPS, lipopolysaccharide.

β -Catenin and GSK-3 β Expression in the Fetal Lung

LPS and/or betamethasone exposure did not change *CTNNB1* mRNA or *GSK3b* mRNA levels in the fetal lung (data not shown). β -Catenin protein expression decreased after the exposure to LPS 7 d before delivery and after exposure to LPS followed by betamethasone posttreatment (*Figure 4a*). GSK-3 β protein expression in the fetal lung did not change significantly in any of the experimental groups (*Figure 4b*).

Figure 4. β -Catenin and GSK-3 β expression in the fetal lung. (a) β -Catenin expression. β -Catenin protein expression decreased by two-thirds in fetal lungs exposed to LPS for 7 d and in lung exposed to 14-d LPS + 7-d Beta. (b) GSK-3 β expression: GSK-3 β protein expression did not significantly change in any of the experimental groups. * $P < 0.05$ vs. controls using a one-way ANOVA with Tukey's post hoc test. GSK, glycogen synthase kinase; LPS, lipopolysaccharide.

Discussion

Although epidemiological and experimental studies have demonstrated a clear correlation between prenatal inflammation and altered fetal lung development (22,23), little is known about the molecular pathways mediating these effects. We found that exposure to IA LPS downregulated the Wnt pathway as illustrated by decreased nuclear LEF1 immunoreactivity and decreased β -catenin protein expression. β -Catenin signaling during pulmonary development is necessary for growth and differentiation of pulmonary epithelial cells (24). Subsequently, inhibition of Wnt signaling can lead to impaired branching and defects in vascular development in the lung (11). Previously, we demonstrated reduced alveolar septation following LPS exposure in this animal model (14).

In this study, we show aberrant Wnt signaling in this model of disrupted lung morphology after exposure to IA inflammation. Of the four WNT isoforms we measured, *WNT1* and *WNT4* had similar patterns of decreases as LEF1 after IA LPS exposure, suggesting that these changes are responsible for the decreased LEF1 expression. However, differential expression patterns of these WNT isoforms and others occur both in lung development and in lung disease (25,26). The net outcome of these changes is dependent not only on the Wnt receptor and the affinity of the Wnt isoform for that receptor (27,28) but also on the cross talk of Wnt components with other signaling pathways such as the transforming growth factor- β pathway (15,29). Interestingly, 14 d after the exposure to LPS, *WNT7b* mRNA levels were upregulated. These relatively late effects may represent a response of the developing lung to repair the damage from IA LPS-induced inflammation. Wnt signaling can stimulate tissue remodeling, cell migration, and wound healing in the lung. Activation of the Wnt pathway in alveolar type II cells promotes epithelial survival and differentiation toward alveolar epithelial cells after lung injury (30). β -Catenin can induce fibroblast growth factor (FGF)-10 expression in parabronchial smooth muscle cell progenitors, which help to repair the damaged lung epithelium (31). We previously showed in this animal model that exposure to IA LPS was accompanied by increased levels of FGF10 in the fetal lung 14 d after the injection (14). Canonical Wnt signaling may stimulate FGF10 expression and subsequent myofibroblast differentiation in the ovine fetal lung after exposure to IA inflammation. Although canonical Wnt signaling plays a key role in the repair phase after lung injury, aberrant activation of the Wnt pathway can have deleterious consequences. Increased canonical Wnt signaling is seen in fibrotic diseases such as idiopathic fibrosis and ventilation-induced lung injury (26,32). It, however, remains to be determined if Wnt

signaling remains perturbed in this model, and if so, how persistently altered Wnt signaling affects lung development beyond the fetal environment.

Lung development is affected *in utero* by both chorioamnionitis and antenatal corticosteroids. Nevertheless, the interaction between both the exposures is less understood. We previously reported that corticosteroids given 7 d before the exposure to IA LPS inhibited pulmonary inflammation and injury in the ovine fetal lung (33). Here, we further demonstrate that corticosteroid treatment before the exposure to LPS attenuated the decrease in LEF1 expression, which is the main effector of the canonical Wnt pathway. A single dose of antenatal corticosteroid 7 d after the LPS exposure normalized some of the investigated Wnt signaling components in the fetal lung. Others have reported interactions that are consistent with these results. Stimulation of the glucocorticoid receptor can activate GSK-3 β , which in turn increases β -catenin breakdown and thus inhibits the Wnt pathway (34). In addition, the glucocorticoid receptor can directly decrease Wnt signaling by binding to the T-cell-specific transcription factor- β -catenin complex (35).

Chorioamnionitis and antenatal corticosteroids are common exposures for the preterm fetus. Our model allowed testing their interaction in a time-dependent manner, which is not possible in the clinical setting, where chorioamnionitis might be present but is usually clinically silent. A recent prospective cohort study demonstrated that antenatal corticosteroids reduced adverse outcomes in preterm infants with histological chorioamnionitis, irrespective of whether inflammation was present on the maternal side only or the fetus was involved (36), which was confirmed in a meta-analysis (37). Clinical timing of antenatal corticosteroid therapy is additionally influenced by the presence of premature rupture of membranes. In the setting of premature rupture of membranes, antenatal corticosteroids substantially reduce the risks of adverse outcomes, such as neonatal respiratory distress syndrome, without significantly increasing the risk of infection for mother or baby (38). However, premature rupture of membranes itself is strongly associated with intrauterine inflammation, and the rate of histological chorioamnionitis and funisitis appears to increase with prolonged latency between premature rupture of membranes and delivery (39). Therefore, we speculate that this group of preterm infants might benefit from antenatal corticosteroids because of its positive influence on subsequent inflammation.

This is the first report to show altered Wnt signaling after antenatal inflammation in the fetal lung. However, future *in vitro* and *in vivo* studies are needed to demonstrate a causal link between the presence of inflammation and disturbances in this pathway and subsequently late lung development. Although we were not able to perform an

in-depth analysis of the investigated pathway due to lack of specific reagents, these initial observations of changes in Wnt signaling do provide interesting options for possible intervention strategies because redirecting Wnt signaling in the fetal lungs may provide beneficial outcomes for the premature lung. Inhibition of GSK3- β by a pharmacological modulator SB216763 resulted in reduced pulmonary inflammation and improved alveolarization in a hyperoxic rodent model of BPD (40). Future studies will be needed to show if and how pharmacological modulation of the Wnt pathway can redirect late lung development after exposure to chorioamnionitis. In conclusion, our results demonstrate that fetal lung exposure to LPS can decrease Wnt signaling and that maternal corticosteroid administration can partially prevent these changes. Thereby this study helps to provide insight into the beneficial effects of antenatal corticosteroid treatment on the fetal lung in the setting of intrauterine inflammation.

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Chapter 3

Systemic interleukin-2 administration improves lung function and modulates chorioamnionitis-induced pulmonary inflammation in the ovine fetus

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Abstract

Chorioamnionitis, an inflammatory reaction of the fetal membranes to microbes, is an important cause of preterm birth and associated with inflammation-driven lung injury. However, inflammation *in utero* overcomes immaturity of the premature lung by inducing surfactant lipids and lung gas volume. Previously, we found that lipopolysaccharide (LPS)-induced chorioamnionitis resulted in pulmonary inflammation with increased effector T cells and decreased regulatory T cell (Treg) numbers. Because Tregs are crucial for immune regulation, we assessed the effects of interleukin (IL)-2-driven selective Treg expansion on the fetal lung in an ovine chorioamnionitis model. Instrumented fetuses received systemic prophylactic IL-2 treatment [118 days gestational age (dGA)] with or without subsequent exposure to intra-amniotic LPS (122 dGA). Following delivery at 129 dGA (term 147 dGA), pulmonary and systemic inflammation, morphological changes, lung gas volume, and phospholipid concentration were assessed. IL-2 pretreatment increased the FoxP3⁺/CD3⁺ ratio, which was associated with reduced CD3-positive cells in the fetal lungs of LPS-exposed animals. Prophylactic IL-2 treatment did not prevent pulmonary accumulation of myeloperoxidase- and PU.1-positive cells or elevation of bronchoalveolar lavage fluid IL-8 and systemic IL-6 concentrations in LPS-exposed animals. Unexpectedly, IL-2 treatment improved fetal lung function of control lambs as indicated by increased disaturated phospholipids and improved lung gas volume. In conclusion, systemic IL-2 treatment *in utero* preferentially expanded Tregs and improved lung gas volume and disaturated phospholipids. These beneficial effects on lung function were maintained despite the moderate immunomodulatory effects of prophylactic IL-2 in the course of chorioamnionitis.

Introduction

Chorioamnionitis, which is an inflammation of the chorion, amnion, and placenta, is a frequent cause of preterm birth (1). Pulmonary inflammation can be induced by translocation of cytokines from the circulation in the fetal lungs and by direct exposure to contaminated amniotic fluid following breathing movements (1-6). Whereas histological chorioamnionitis is associated with reduced incidences and severity of respiratory distress syndrome (RDS) (7), several postnatal events such as mechanical ventilation can amplify and prolong pulmonary inflammation, thereby increasing the risk for bronchopulmonary dysplasia (BPD) (5, 6, 8).

Insight into the role of intrauterine inflammation on pulmonary pathologies has been gained in translational ovine chorioamnionitis models. This work revealed that intra-amniotic delivery of proinflammatory stimuli resulted not only in pulmonary inflammation but also in systemic and multiorgan inflammation (1, 9, 10). Although pulmonary inflammation was associated with structural changes, it also improved lung function by increasing surfactant lipids and lung volume (10-12). Importantly, increased CD3-positive cell numbers were reported in the lungs and the mediastinal lymph node within days (2, 9, 13). Numerous reports in ovine and primate models show that within this time frame chorioamnionitis is followed by a rapid transient decrease in regulatory T cell (Treg) numbers in several lymphatic organs, including the mediastinal lymph node, and the gut (9, 14-16).

Tregs are important in regulating inflammatory responses and maintaining immune homeostasis (17, 18). The forkhead transcription factor FoxP3 is expressed in CD4⁺CD25⁺ Tregs (19, 20) and is essential for development and function of these cells (19). FoxP3 gene mutations result in fatal autoimmune pathology affecting multiple organs, including the lungs (21, 22). Because Treg activity in chorioamnionitis-exposed preterm neonates may not be adequate to control excessive inflammation and since broad immunosuppression is undesirable in vulnerable target groups (23, 24), boosting of Tregs in these infants may modulate chorioamnionitis-induced inflammatory responses. Previously, we observed that prophylactic administration of the immunoregulatory cytokine interleukin (IL)-2, necessary for growth, clonal expansion, and suppressor function of Tregs (25-27), prevented fetal chorioamnionitis-induced intestinal inflammation and injury (28). In this study we assessed the effects of IL-2-driven selective Treg expansion on the fetal lung in an ovine chorioamnionitis model.

Materials & Methods

Animal model

The animal study was approved by the Animal Ethics Committee of Maastricht University, The Netherlands, and executed as one large animal experiment. Animals were randomly allocated to the following groups: control, IL-2, lipopolysaccharide (LPS), or IL-2 + LPS. All experimental procedures and the study design (7 days exposure) were published previously (28).

Sampling protocol

Fetal blood samples were taken at 24 h or 3, 5, or 7 days (end of experiment) after intra-amniotic LPS or saline administration using heparin-containing blood-collecting tubes. Plasma was prepared by centrifugation at 4,000 revolutions/min. Body weight of the fetus was recorded upon delivery. The thoracic cavity was opened, and a static pulmonary pressure-volume curve was performed. Lung gas volumes were recorded for every pressure and were corrected for body weight. Lungs were weighed, and the left lung was subjected to bronchoalveolar lavage. The right upper lobe (RUL) of the lungs was inflation fixated using 10% buffered formalin and paraffin embedded. Right lower lobe (RLL) tissue was embedded in optimal cutting temperature compound (OCT).

Immunohistochemistry

Paraffin-embedded RUL tissues (4 μm) were stained for CD3 (T cells), FoxP3 (Tregs), myeloperoxidase (MPO; activated neutrophils and monocytes), and PU.1 (maturation marker for myeloid cells). Protocols and information about antibodies are published previously (28, 29).

OCT-embedded RLL tissues (5 μm) were stained for CD25 [IL-2 receptor- α (IL-2R α) (20)]. Slides were fixated in cold acetone, and endogenous peroxidase activity was blocked by 0.3% H_2O_2 in methanol. The rest of the protocol was the same as described previously for FoxP3 (28), with the exception that anti-CD25 (MCA2218; AbD Serotec, Kidlington, UK) was used.

CD3-, MPO-, and PU.1-positive cells were counted in five random representative images (x200), corrected for lung tissue percentage per image using ImageJ 1.45s software and averaged per animal. Sections were semiquantitatively scored for FoxP3-positive cells as follows: 0, no cells; 1, a few cells; 2, medium amount of cells; and 3, large amount of cells. To calculate the pulmonary FoxP3⁺/CD3⁺ ratio, we normalized the number of CD3-positive cells in all groups to controls and normalized the relative FoxP3 expression to controls. We then divided the normalized FoxP3 expression by the normalized CD3 expression for FoxP3⁺/CD3⁺ ratios. Observers were blinded.

Histology

Paraffin-embedded RUL tissue sections were stained with hematoxylin and eosin (H&E) and evaluated by two lung pathologists, blinded, to assess lung morphology and presence of neutrophils and lymphocytes.

Enzyme-linked immunosorbent assay

Protein concentrations of IL-8 in bronchoalveolar lavage fluid (BALF) and IL-8 and IL-6 in fetal plasma were measured using enzyme-linked immunosorbent assays according to the manufacturer's instructions, with anti-ovine IL-6 (MAB1004; Merck Millipore, Billerica, MA) or IL-8 (MAB1044; Merck Millipore) as capture antibodies; recombinant ovine IL-6 (6495; ImmunoChemistry Technologies, Bloomington, MN) or IL-8 protein (6542; ImmunoChemistry Technologies) for standards; and anti-ovine IL-6 (AB1839; Merck Millipore) or IL-8 (AB1840; Merck Millipore) as detection antibodies.

Disaturated phospholipid measurement

Disaturated phospholipid (DSPL) concentration was determined in BALF as described previously (30). Concentrations were corrected for collected BALF volume, body weight, and left and right lung weight.

Statistical analysis

Data are shown as means $\pm$ SE. For statistical analysis, GraphPad Prism version 6.01 software was used. To compare groups, the nonparametric Kruskal-Wallis test with Dunn's Multiple Comparison Test was used. In case of a potential outlier, we tried to identify causes for aberrant results. If no obvious cause was found, the potential outlier was not excluded since it might be a legitimate observation and due to the occurrence of an extreme value at the end of a distribution (31). Instead we used nonparametric methods to analyze our data. These methods make weaker assumptions about underlying distributions than normal-theory methods, thereby minimizing the effect of potential outliers on the overall results (31, 32).

Considering the relative low number of animals per group, we have depicted the actual *P* values in *Figures 1-4*.

Results

Pulmonary inflammation

H&E-stained slides were evaluated for influx of inflammatory cells. LPS exposure caused influx of neutrophils and lymphocytes compared with controls. In IL-2-treated animals no increase of neutrophils or lymphocytes was observed. Prophylactic IL-2 treatment reduced the infiltrated lymphocytes, whereas the presence of neutrophils within alveolar tissues was not diminished (data not shown).

To characterize the different inflammatory cell subsets, lung slides were stained for CD3, FoxP3, MPO, and PU.1. Intra-amniotic LPS exposure increased the number of CD3-positive cells ($P = 0.0168$), which was prevented by prophylactic IL-2 treatment ($P = 0.0062$; *Figure 1A–D*). IL-2 treatment did not change the number of CD3-positive cells compared with control animals (*Figure 1D*). Exposure to IL-2, LPS, or the combination led to an increased amount of FoxP3-positive cells compared with controls ($P = 0.0292$, 0.0027 , or 0.0187 ; *Figure 1E and G–J*). The FoxP3⁺/CD3⁺ ratio increased after exposure to IL-2 ($P = 0.0400$) or the combination of IL-2 with LPS ($P = 0.0083$) compared with controls (*Figure 1F*). The FoxP3⁺/CD3⁺ ratio in the combined treatment group was also higher compared with the LPS group ($P = 0.0227$).

Numbers of MPO- and PU.1-positive cells were increased after LPS exposure compared with control animals ($P = 0.0090$ or 0.0496 ; *Figure 2A and B*). Prophylactic IL-2 treatment in these LPS-exposed animals did not prevent increased MPO and PU.1-expressing cell numbers (*Figure 2A and B*). Similarly, elevated cell numbers were present in the combined group compared with control ($P = 0.0273$ or 0.0127 ; *Figure 2C–D and E–F*) and IL-2 ($P = 0.0022$ or 0.0455) animals.

IL-8 protein concentrations in BALF are used as a prognostic marker for lung pathologies of preterm infants, including chronic lung disease (33). IL-2 treatment did not affect IL-8 levels in BALF, whereas LPS exposure increased the IL-8 concentration compared with control ($P = 0.0227$; *Figure 3A*). Prophylactic IL-2 treatment did not prevent this increase.

Figure 1. Characterization of CD3 (A–D)- and FoxP3 (E)–positive cells and their ratio (F) after exposure to lipopolysaccharide (LPS), interleukin (IL)-2, or the combination in the ovine fetal lungs. Shown are representative images of CD3-positive cells for control (A), LPS-treated (B), and IL-2 + LPS-treated (C) animals and representative images of FoxP3-positive cells for control (G), IL-2-treated (H), LPS-treated (I), and IL-2 + LPS-treated (J) animals. Black arrowheads indicate FoxP3-positive cells (H–J). Scale bars: 100 μ m. For statistical analysis the Kruskal-Wallis test was performed followed by the Dunn's Multiple Comparison Test. Animal nos./group are as follows: control $n = 6$, IL-2 $n = 6$, LPS $n = 6$, IL-2 + LPS $n = 7$ for CD3; control $n = 4$, IL-2 $n = 5$, LPS $n = 6$, IL-2 + LPS $n = 6$ for FoxP3 and the FoxP3⁺/CD3⁺ ratio.

Figure 2. Quantitation of myeloperoxidase (MPO, A)- and PU.1 (B)-positive cells after exposure to lipopolysaccharide (LPS) with or without IL-2 in the ovine fetal lungs. Representative images of MPO (C and D)- and PU.1 (E and F)-positive cells for control (C and E) and IL-2 + LPS-treated (D and F) animals are displayed. Scale bars: 100 μ m; scale bars in insets: 25 μ m. The Kruskal-Wallis test was used for statistical analysis followed by the Dunn's Multiple Comparison Test. Animal nos./group are as follows: control $n = 5$, IL-2 $n = 6$, LPS $n = 6$, IL-2 + LPS $n = 6$ for both MPO and PU.1.

Figure 3. Bronchoalveolar lavage fluid (BALF) interleukin (IL)-8 levels (A) and plasma IL-8 (B and C) or IL-6 (D) levels in the ovine fetus after exposure to IL-2 with or without lipopolysaccharide (LPS). Protein levels were measured 7 days (A and B) or 24 h (C and D) after intra-amniotic saline or LPS exposure. For statistical analyses, an arbitrary value of 1 pg/ml was assigned to values below the detection limit. Statistical analysis was performed by a Kruskal-Wallis test and Dunn's Multiple Comparison Test. Animal nos./group are as follows: control $n = 4$, IL-2 $n = 6$, LPS $n = 5$, IL-2 + LPS $n = 7$ for BALF IL-8; control $n = 4$, IL-2 $n = 6$, LPS $n = 5$, IL-2 + LPS $n = 6$ for plasma IL-8 7 days; control $n = 5$, IL-2 $n = 6$, LPS $n = 6$, IL-2 + LPS $n = 6$ for plasma IL-8 and IL-6 24 h.

Systemic inflammation

Although BALF IL-8 levels were changed, plasma IL-8 levels did not differ between groups at 24 h or 7 days post-LPS or saline exposure (*Figure 3B and C*). We measured IL-6 protein levels in fetal plasma at 24 h and 3, 5, and 7 days after intra-amniotic LPS or saline to assess a systemic fetal inflammatory response (34). Twenty-four hours after intra-amniotic LPS exposure, IL-6 plasma concentration increased ($P = 0.0057$), which was not prevented by prophylactic IL-2 treatment (*Figure 3D*). At 3, 5, and 7 days after intra-amniotic LPS exposure no increase of plasma IL-6 concentrations was detected (data not shown).

Morphology

Morphology was equivalent to the saccular/alveolar phase of lung development (35) and similar between groups according to two independent lung pathologists.

Lung gas volumes and disaturated phospholipids

IL-2 treatment increased the lung gas volume compared with control animals (P values ranged from 0.0856 at 10 mmHg to 0.0656 at 40 mmHg; *Figure 4A*). LPS (P values ranged between 0.0015 at 0 mmHg and 0.0008 at 40 mmHg) and the combination of IL-2 with LPS (P values ranged from 0.0020 at 0 mmHg to 0.0010 at 40 mmHg) increased lung gas volumes compared with controls.

In addition, we determined the concentration of DSPLs in the BALF. In controls, the concentration of DSPLs was below the detection limit (*Figure 4B*). IL-2 administration increased the DSPL concentration ($P = 0.0754$). Increased DSPL values were detected in LPS-exposed animals with or without IL-2 treatment ($P = 0.0007$ or 0.0004). Combined exposure also increased DSPLs compared with IL-2 alone ($P = 0.0269$).

Figure 4. Pulmonary pressure-volume curve (A) and disaturated phospholipid concentration (B) of the ovine fetus after exposure to interleukin (IL)-2, lipopolysaccharide (LPS), or the combination. *P* values are compared with the control group for 40 mmHg pressure (A). For statistical analyses, an arbitrary value of 1 $\mu\text{g/kg}$ was assigned to values below the detection limit (B). The Kruskal-Wallis test was performed followed by the Dunn's Multiple Comparison Test. Animal nos./group are as follows: control $n = 7$, IL-2 $n = 5$, LPS $n = 5$, IL-2 + LPS $n = 6$ for the pressure-volume curve; control $n = 6$, IL-2 $n = 6$, LPS $n = 4$, IL-2 + LPS $n = 6$ for the disaturated phospholipid concentration. BW, body wt.

Discussion

Inadequate resolution of inflammatory responses in infants exposed to chorioamnionitis increases the risk for adverse outcomes of the lung, including BPD (36), which emphasizes the importance of the regulation of pulmonary inflammation in the course of adverse fetal lung development. Because Tregs are important in regulating inflammatory responses and IL-2 is necessary for clonal expansion of Tregs (18, 25-27), we evaluated the effects of IL-2-driven selective Treg expansion on the fetal lung in an ovine chorioamnionitis model.

IL-2 treatment preferentially expanded Tregs by increasing the FoxP3⁺/CD3⁺ ratio in the fetal lungs. FoxP3 expression was restricted to infiltrated immune cells, which is inconsistent with earlier reports which showed that bronchial epithelial cells can express FoxP3 (37, 38). FoxP3 expression might eventually be too low to be detected in bronchiolar epithelium by an immunohistochemical staining on our fetal ovine tissue. D'Alessio et al. (39) demonstrated *in vivo* that Tregs are crucial in resolving LPS-induced lung inflammation. Interestingly, the increased FoxP3⁺/CD3⁺ ratio in our model was associated with a moderate immunomodulatory effect *in utero*. Although prophylactic IL-2 administration did prevent influx of pulmonary T cells following LPS exposure, it was not accompanied by inhibition of systemic immune activation, indicating that this immunomodulatory effect in the lung was not systemically driven. The scope of this study was limited to the fetal lung *in utero* and not the long-term postnatal outcome. Chorioamnionitis is identified as a risk factor for adverse pulmonary outcome, such as the development of BPD. BPD is a multifactorial disease in which disturbed immune regulation is considered to be an essential underlying cause (36, 40). Therefore, we can only speculate about the consequences of the enhanced FoxP3⁺/CD3⁺ ratio *in utero* for the long-term postnatal outcome. Previously, it was shown that antenatal LPS exposure enhanced subsequent inflammatory responses in ventilated preterm lungs. Accordingly, mild ventilation-induced indicators of injury were amplified in fetal lambs exposed to LPS prenatally (41). In the present study we did not observe morphological changes. The absence of such changes post-LPS exposure is in line with previous findings in which no morphological differences were found by computerized morphometric analyses (42), which are apparently not provoked in our study since animals were not ventilated (11, 12). Future studies will clarify whether enhancement of the pulmonary FoxP3⁺/CD3⁺ ratio prenatally will protect the lungs after birth. Postnatally, these vulnerable preterm infants are exposed to additional injurious events such as mechanical ventilation and sepsis, which are known to induce pulmonary and systemic inflammatory responses, including circulatory IL-8 levels (1, 43-45).

We for the first time show that systemic IL-2 treatment improved lung gas volume and DSPLs *in utero*. These beneficial effects on lung function maintained despite moderate immunomodulatory effects of IL-2 pretreatment in LPS-exposed animals. Because surfactant deficiency in premature infants predisposes to RDS (46), the surprising finding that IL-2 treatment alone increased surfactant lipids and lung gas volume *in utero* warrants further evaluation to also elucidate the possible mechanism. Notably, IL-2R α (CD25) distribution in the fetal ovine lung was restricted to pulmonary T cells (data not shown), suggesting that the epithelium responds indirectly to IL-2. However, we cannot rule out the possibility that CD25 expression in the respiratory epithelium is too low to detect by immunohistochemistry, since Lesur et al. (47) demonstrated CD25 protein and RNA transcript expression besides functionality of IL-2 receptors in primary cultures of rat type II cells.

Given the relative small animal numbers per group, we report actual *P* values and tend to interpret *P* values between 0.05 and 0.1 as biologically relevant. This assumption will decrease the chance of a false negative finding but increases the chance that one of these differences is a false positive result. Another limitation is that we can only study pulmonary changes *in utero* at a fixed time point. Nevertheless, we chose this species since ovine pulmonary development closely resembles human lung development (48), and the model enables us to perform complex interventions necessary to administer IL-2 treatment *in utero*. Furthermore, we cannot elucidate whether the increased number of pulmonary FoxP3-positive cells is the result of proliferation, migration, or a combination of these processes.

In conclusion, systemic IL-2 treatment *in utero* preferentially expanded Tregs and improved lung gas volume and DSPLs. These beneficial effects on pulmonary function were maintained despite the moderate immunomodulatory effects of IL-2 treatment in the setting of chorioamnionitis. Combined with the clinical potential of IL-2 in the fetal gut (28) and the fact that no adverse outcomes of IL-2 treatment were observed (such as vascular leakage syndrome, assessed by total protein in the BALF and absence of hypotension), IL-2 may be beneficial in the clinic where antenatal inflammation is often followed by a second postnatal inflammatory hit. Reducing the prenatal proinflammatory response via preferential Treg expansion may prevent extension of this inflammatory response by a secondary postnatal hit. This may ultimately decrease the risk for complications such as BPD, for which potential therapeutic strategies are still insufficient (49).

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Chapter 4

Pulmonary vascular changes in extremely preterm sheep after intra-amniotic exposure to *Ureaplasma parvum* and lipopolysaccharide

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In revision

Abstract

Background: Chorioamnionitis can induce pulmonary inflammation and promote bronchopulmonary dysplasia development, distinguished by alveolar simplification and impaired vascular growth. Chorioamnionitis is more common during the extremely preterm canalicular lung stage (crucial for vascular development); and increases the risk for subsequent sepsis. We hypothesized that single/combined exposure to chronic and/or acute inflammation induces pulmonary inflammatory responses and vascular changes.

Methods: Ovine fetuses were intra-amniotically exposed to chronic *Ureaplasma parvum* (UP) at 24 days (d) before extreme preterm delivery at 94d (term 147d) and/or to lipopolysaccharide (LPS) 7 or 2d before delivery. Pulmonary inflammation, vascular remodeling and angiogenic factors were assessed.

Results: LPS exposure increased CD3-positive and myeloperoxidase-positive cells. Combined UP-LPS exposure increased pulmonary inflammation compared with 2d LPS or UP groups. The UP+2d LPS group had an increased adventitial fibrosis score and raised wall-to-lumen ratio compared with UP alone. Exposure to UP+2d LPS reduced *VEGF* and *VEGFR-2* levels compared with 2d LPS-treated animals. Angiopoietin-1 (*Ang1*) and tunica interna endothelial cell kinase 2 (*Tie-2*) levels were decreased after UP+7d LPS as well as after 7d LPS, but not with UP alone.

Conclusion: Chronic UP and subsequent LPS exposure increased pulmonary inflammation and disturbed pulmonary angiogenic signaling and vascular remodeling when compared to single hit-exposed animals.

Introduction

Preterm birth is frequently associated with chorioamnionitis, an inflammation of the chorion, amnion and placenta (1, 2). Direct pulmonary contact with contaminated amniotic fluid can initiate pulmonary inflammation (3, 4). Dependent on the presence of additional postnatal factors such as ventilation and sepsis, a progressive pulmonary inflammatory response may be induced which promotes the development of bronchopulmonary dysplasia (BPD) (5). BPD is characterized by alveolar simplification and impaired vascular growth (6, 7). In addition, BPD patients can display structural vascular alterations and decreased expression of angiogenic growth factors and corresponding receptors, such as vascular endothelial growth factor (VEGF) (8, 9). Increasing evidence suggests that pulmonary vascular development is very important during lung growth (10). The vascular hypothesis implies that disruption of pulmonary angiogenesis during essential stages of fetal growth may impair alveolarization and ultimately contribute to the pulmonary hypoplasia that typifies BPD (10, 11). In neonatal rats it was demonstrated that anti-angiogenic agents including VEGF (receptor) inhibitors attenuated pulmonary vascular growth and decreased alveolarization, which can persist until adulthood (12, 13).

Intra-amniotic infection is often polymicrobial, with *Ureaplasma parvum* (*U. parvum*) as one of the most common microorganisms detected in amniotic fluid of women with preterm prelabor rupture of membranes (PROM) and in respiratory aspirates of preterm infants (14, 15). Respiratory colonization of *Ureaplasma* is associated with the development of BPD in neonates (16). In addition, *U. parvum* has been shown to selectively downregulate antimicrobial peptide expression, which may increase the susceptibility to additional microorganisms *in utero* (17). Viral infections can sensitize pregnant mice to a subsequent bacterial infection as well, resulting in preterm delivery and fetal death (18). In our translational ovine chorioamnionitis model, we have previously not only shown that antenatal inflammation can induce fetal pulmonary inflammation and vascular changes, but also that chronic intra-amniotic exposure to *U. parvum* suppressed fetal pulmonary responsiveness to a subsequent endotoxin stimulus and thus renders the fetus more susceptible to an additional inflammatory stimulus (19-21). Nevertheless, our studies were performed using fetal sheep born at 120-124 days of gestational age (dGA) which is comparable to approximately 30 weeks of human gestation (22). It is unknown how the fetal pulmonary system will respond to exposure to chronic *U. parvum* and subsequent endotoxin at a lower gestational age. The incidence of histologic chorioamnionitis is higher in infants born at a lower gestational age (reaching up to 66% at 20-24 weeks of gestation) and sepsis occurs more frequently in very low birth weight infants when

exposed to chorioamnionitis (23, 24). Furthermore, extremely preterm infants born during the canalicular stage of lung development are in a crucial phase of vascular lung development (6, 10). Consequently, the aim of this study was to determine whether intra-amniotic chronic *U. parvum* exposure or acute inflammation during canalicular lung development induced a pulmonary inflammatory response and concomitant vascular changes. In addition, we determined whether chronic pre-exposure to *U. parvum* modulated this pulmonary response in the presence of a subsequent acute inflammatory stimulus. We hypothesized that single or combined exposure to chronic *U. parvum* and/or acute inflammation provoked a pulmonary inflammatory response and induced vascular changes. We exposed extremely preterm sheep intra-amniotically to chronic *U. parvum* and/or lipopolysaccharide (LPS) and assessed inflammatory parameters, vascular remodeling and expression levels of several angiogenic growth factors and their receptors in the ovine fetal lungs at the canalicular stage. In the current study, we have shown that in extreme preterm sheep contamination of the amniotic fluid mounted a pulmonary inflammatory response with concomitant impairment of the vasculature. In addition, we have shown that combined exposure to *U. parvum* and LPS enhanced pulmonary inflammation, and disturbed pulmonary angiogenic signaling and vascular remodeling compared to single hit-exposed animals.

Materials & Methods

Animal model & sampling procedure

The study was carried out in accordance with the 'Animal Welfare Act (2002)' and the requirements of the 'Australian code for the care and use of animals for scientific purposes (8th edition, 2013)'. All animal experiments were approved by the Animal Ethics Committee of The University of Western Australia (reference number RA/3/100/312) and were performed at The University of Western Australia (Perth, Australia). The human endpoints for this study included irresponsive pain, shock and preterm labor. In case a human endpoint would be reached, the animal would be euthanized after consultation with a practicing veterinarian who is experienced in treating ruminants. However, during the time of the experiment, no animals died before reaching the end of the experiment. Animal wellbeing was daily monitored. To minimize suffering and distress, animals were kept in flock and ultrasound-guided injections were performed by staff experienced in delivering these injections.

Date-mated Merino ewes (*Ovis aries*) were randomized to receive ultrasound-guided intra-amniotic (IA) injections of 2×10^5 color-changing units (CCU) *U. parvum* serovar 3 at 24 days prior to preterm birth ('UP' group); or 10 mg LPS (*Escherichia coli* 055:B5; Sigma-Aldrich, St Louis, MO) diluted in 2 mL saline at 2 or 7 days before preterm delivery ('2d LPS' and '7d LPS' groups) (Figure 1). Control animals received media 24 days ('media' group) or saline 2 or 7 days ('saline' group) before preterm birth. To assess a potential effect of *U. parvum* on subsequent exposure to LPS, two separate groups of animals received IA *U. parvum* 24 days before delivery followed by IA LPS 2 days ('UP+2d LPS' group) or 7 days ('UP+7d LPS' group) before preterm birth. All fetuses (n=6-8 per group) were prematurely delivered at 94 days of gestational age (dGA) by Caesarean section (term at ± 147 dGA). Following euthanasia of the ewe using intravenous pentobarbital, the fetus was delivered and euthanized. Lung tissue from the right lower lobe (RLL) was snap-frozen until further analysis. The right upper lobe (RUL) was inflation-fixated at 30 cm H₂O pressure in 10% buffered formalin for 24 hours and processed for paraffin embedding.

Figure 1. Experimental study design. Ovine fetuses were randomized and prematurely delivered at 94 days of gestational age (dGA) (term at ± 147 dGA). In the 'UP' group, animals received an intra-amniotic (IA) injection with *U. parvum* (UP) 24 days prior to preterm birth. In the '2d LPS' and '7d LPS' groups, animals were IA injected with lipopolysaccharide (LPS) 2 or 7 days before preterm delivery. Control animals received media 24 days ('Media' group) or saline 2 or 7 days ('Saline' group) before preterm birth. To assess a potential effect of *U. parvum* on subsequent exposure to LPS, two separate groups of animals received IA *U. parvum* 24 days before delivery followed by either IA LPS for 2 days ('UP+2d LPS' group) or for 7 days ('UP+7d LPS' group) before preterm birth.

Immunohistochemistry

Paraffin-embedded RUL tissues were cut (4 μ m) and stained for CD3 to identify T cells, for myeloperoxidase (MPO) to detect activated neutrophils and monocytes, and for α -smooth muscle actin (α -SMA) to visualize the tunica media of arterioles (20, 25). Information about antibodies and protocols were published previously for MPO (26) and CD3 (19). The protocol for α -SMA was comparable to CD3. Primary antibodies were diluted 1:500, 1:1000 and 1:5000 for MPO, CD3 and α -SMA, respectively. For CD3, 0.3% H_2O_2 and 5% bovine serum albumin were used for blocking of endogenous peroxidase activity and for non-specific binding, respectively, while for α -SMA 0.5% H_2O_2 and 2% normal goat serum were used. Sections were stained with monoclonal mouse anti- α -SMA primary (A5228, Sigma-Aldrich) and polyclonal biotin-conjugated goat anti-mouse secondary antibodies (E0433; Dako, Glostrup, Denmark).

The number of CD3-positive cell infiltrates was counted within each lung section. An infiltrate was defined as at least ten CD3-positive cells per 2500 μm^2 . CD3-stained slides were scanned (magnification x200) with a VENTANA iScan HT slide scanner (Ventana Medical Systems, Inc.; Roche Group, Tucson, AZ). Next, the total lung parenchyma surface area was measured per slide using Panoramic Viewer software (V1.15.4; 3DHISTECH Ltd., Budapest, Hungary). The total number of CD3-positive cell infiltrates was divided by this total lung parenchyma surface area for each tissue section.

In addition, CD3-positive cells were counted in ten random representative images without infiltrates (magnification x200), corrected for the percentage of lung tissue per image using ImageJ software (ImageJ 1.45s, W. Rasband, US National Institutes of Health, Bethesda, MD) and averaged per animal.

Infiltration of MPO-positive cells was semi-quantitatively scored within the whole section as follows: 0, no cells; 1, minor number of cells; 2, medium number of cells; 3, large number of cells; and 4, very large number of cells.

All arterioles per α -SMA-stained lung section which met the same criteria as previously described (20) were measured. Morphometric measurement of the wall-to-lumen ratio (media thickness divided by the radius of the lumen) was performed on the digital images (magnification 400x) using Leica Qwin software (V3.5.1; Leica Microsystems, Wetzlar, Germany).

During the evaluation, counting and quantification of stained slides, all observers were blinded to experimental groups.

Histology

For the detection of collagen, slides were incubated in 0.2% phosphomolybdic acid (HT153; Sigma-Aldrich) and stained for 90 minutes with 0.1% Sirius red (365548; Sigma-Aldrich) diluted in saturated picric acid (P6755, Sigma-Aldrich) in the dark. After incubation in 0.01N HCl, slides were dehydrated and coverslipped.

To assess adventitial fibrosis, all arterioles per lung section with the same criteria as previously described were semi-quantitatively scored by two blinded observers for the deposition of collagen around these arterioles: 1, mild; 2, moderate; 3, severe; and 4, very severe collagen deposition around arteriole (20).

RNA extraction and real-time PCR

RNA extraction and real-time PCR were performed for the following genes: Angiopoietin-1 (*Ang1*), *VEGF*, VEGF receptor 2 (*VEGFR-2*), tunica interna endothelial cell kinase 2 (*Tie-2*) and ovine ribosomal protein S15 (*ovRPS15*). In brief, total RNA was extracted from snap-frozen RLL lung tissue by Trizol (15596018; Invitrogen, Life Technologies, Carlsbad, CA)/chloroform extraction. Isolated RNA was DNase treated to remove possible contamination with genomic DNA by use of the RQ1 RNase-Free DNase kit (M6101; Promega, Madison, WI) and afterwards reverse transcribed into cDNA using oligo(dT)₁₂₋₁₈ primers (18418-012; Invitrogen) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (28025-021; Invitrogen). Real-time PCR reactions were performed in duplicate within a LightCycler 480 Instrument (Roche Applied Science, Basel, Switzerland) using the SensiMix™ SYBR® No-ROX Kit (QT650; Bioline, London, UK) for 45 cycles. Primer sequences (*Table 1*) were designed or used from literature (26, 27). Results were normalized to the house keeping gene *ovRPS15* and relative changes, over control values, were calculated.

Table 1. Primers used for Real-time PCR

Gene		Sequence (5'-3')	Reference
<i>Ang1</i>	Fw	TTGCCATAACCAGTCAGAG	(27)
	Rv	AACCACCAGCCTCCTGTTA	
<i>VEGF</i>	Fw	CATGCCAAGTGGTCCCAG	
	Rv	GAAGATGTCCACCAGGGTC	
<i>VEGFR-2</i>	Fw	CCCTGATTACACCACACC	
	Rv	TCTTTGCCATCCTGTTGAG	
<i>Tie-2</i>	Fw	CTATGGCGTGTACTATGGGAG	
	Rv	GAGATCATACACCTCGTCGTC	
<i>ovRPS15</i>	Fw	CGAGATGGTGGGCAGCAT	(26)
	Rv	GCTTGATTCCACCTGGTTGA	

Fw, forward; Rv, reverse; *Ang1*, Angiopoietin-1; *VEGF*, vascular endothelial growth factor; *VEGFR-2*, VEGF receptor 2; *Tie-2*, tunica interna endothelial cell kinase 2; *ovRPS15*, ovine ribosomal protein S15.

Statistical analysis

GraphPad Prism v6.01 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Experimental groups were compared using the non-parametric Kruskal-Wallis test, followed by Dunn's Multiple Comparison Test for post-hoc analysis. All data are displayed as mean ± standard error of mean (SEM) and significance was considered as $p < 0.05$.

Results

Pulmonary inflammation

First, we addressed whether the extreme premature lung mounted an inflammatory response following an intra-amniotic inflammatory stimulus. We observed that CD3-positive cells specifically clustered into infiltrates around vascular and bronchiolar structures after exposure to an intra-amniotic inflammatory stimulus. Therefore, we quantified the number of CD3-positive cell infiltrates in the ovine fetal lungs (*Figure 2A*) and found that chronic *U. parvum* exposure did not significantly raise the infiltrate number compared with the media group. The CD3-positive cell infiltrate count increased in the 2d LPS- and 7d LPS-treated animals compared with the saline group (*Figure 2A, E-F*). Infiltrate numbers were also increased in the UP+2d LPS and UP+7d LPS groups compared with UP- and/or media-treated animals (*Figure 2A, C-D, G-H*). Furthermore, CD3-positive cell numbers were quantified in representative lung pictures without infiltrates, to assess whether the overall spread of CD3-positive cells within the lung tissue was different between experimental groups. No differences were found between UP- and media-treated animals (*Figure 2B-D*). CD3-positive cell numbers were also elevated in the UP+2d LPS and UP+7d LPS groups compared with UP- and/or media-treated animals, which is in line with the CD3-positive cell infiltrates of *Figure 2A*. In addition, the UP+2d LPS group showed higher CD3-positive cell numbers compared to the 2d LPS group. These combined data suggest an overall increase in the number of CD3-positive cells after exposure to LPS alone or to the combination of *U. parvum* with LPS compared with control animals (media and saline groups).

Intra-amniotic administration of *U. parvum* did not affect the amount of MPO-positive cells in the pulmonary tissue compared with the media group (*Figure 3A-C*). The amount of MPO-positive cells did not change within the 2d LPS group, while exposure to 7 days of LPS increased the accumulation of MPO-positive cells compared with the saline and 2d LPS groups (*Figure 3A, D-F*). MPO-positive cell infiltration was enhanced in the UP+2d LPS group compared with media- and 2d LPS- treated animals (*Figure 3A-B, E, G*). UP+7d LPS-treated animals had an increased amount of MPO-positive cells compared with media- and UP-treated animals (*Figure 3A-C, H*).

Figure 2. CD3-positive cell infiltrate number (A) and quantification of CD3-positive cells (B) in preterm ovine lungs. Representative pictures of lung sections, immunohistochemically stained for CD3, are shown for media- (C), *Ureaplasma parvum* (UP)- (D), saline- (E), 7d lipopolysaccharide (LPS)- (F), UP+2d LPS- (G) and UP+7d LPS-treated animals (H). Pictures show CD3-positive cell infiltrates (F-H) and singular CD3-positive cells not belonging to cell infiltrates (C-E). Scale bars: 100 μ m. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between experimental groups using the non-parametric Kruskal-Wallis test, followed by Dunn's Multiple Comparison Test for post-hoc analysis.

Figure 3. Semi-quantitative scoring of myeloperoxidase (MPO)-positive cells in the extreme preterm ovine lung. Lung sections were immunohistochemically stained for MPO. Representative images of MPO-positive cells are shown for media- (B), *Ureaplasma parvum* (UP)- (C), saline- (D), 2d lipopolysaccharide (LPS)- (E), 7d LPS- (F), UP+2d LPS- (G) and UP+7d LPS-treated (H) animals. Inserts in the left lower corner show a higher magnification of the squared area. Scale bars: 100 μ m; scale bars in inserts: 25 μ m. * p <0.05 and ** p <0.01 between experimental groups, analyzed with the non-parametric Kruskal-Wallis test and Dunn's Multiple Comparison Test for post-hoc analysis.

Pulmonary arteriolar remodeling

Since antenatal inflammation is known to induce vascular remodeling of small pulmonary arteries in the preterm lung at 122 dGA (20), we determined whether intra-amniotic exposure to *U. parvum* and/or LPS at an extreme preterm age also led to vascular remodeling. Vascular remodeling is defined as a structural change in the lumen diameter which can result in a changed wall-to-lumen ratio (28, 29). To assess vascular remodeling, we therefore defined the wall-to-lumen ratio of pulmonary arterioles. The ratio was unchanged in the UP group compared with the media group (Figure 4). Animals exposed to LPS for 7 days had a decreased wall-to-lumen ratio compared with 2d LPS-treated animals. Compared with single exposure to UP alone, combined exposure to UP+2d LPS increased the wall-to-lumen ratio with approaching significance ($p=0.06$). Within the UP+7d LPS group, the ratio was unaffected.

Figure 4. Wall-to-lumen ratio of pulmonary arterioles in *Ureaplasma parvum* (UP)- and/or lipopolysaccharide (LPS)-treated extreme preterm sheep. * p <0.05 between experimental groups.

Furthermore, we assessed the accumulation of collagen in the adventitia of pulmonary arterioles, as adventitial matrix deposition is common during pulmonary artery remodeling (30). Although a single exposure to either UP, 2d LPS or 7d of LPS had no effect compared with control animals, dual exposure to UP+2d LPS increased the adventitial fibrosis score compared with single exposure to UP (Figure 5A-D). No effect on adventitial fibrosis was found in the UP+7d LPS group.

Figure 5. Adventitial fibrosis score of pulmonary arterioles in *Ureaplasma parvum* (UP)- and/or lipopolysaccharide (LPS)-treated preterm sheep. Representative pictures of lung sections histologically stained for collagen are shown for media- (B), UP- (C) and UP+2d LPS-treated animals (D). Scale bars: 50 μ m. * p <0.05 between experimental groups using the non-parametric Kruskal-Wallis test, followed by Dunn's Multiple Comparison Test for post-hoc analysis.

Expression of angiogenic growth factors & receptors

Since VEGF and VEGF receptors are essential for pulmonary vascular growth (12, 13), we determined lung mRNA levels of *VEGF* and *VEGFR-2* after antenatal inflammation. Intra-amniotic exposure to *U. parvum* or LPS alone did not affect *VEGF* mRNA levels compared with control animals (Figure 6A). Dual exposure to UP+2d LPS decreased *VEGF* mRNA levels compared with single exposure to 2d LPS. Similar results were found for *VEGFR-2* mRNA levels (Figure 6B). Intra-amniotic administration of *U. parvum* did not affect *VEGFR-2* mRNA levels, while *VEGFR-2* levels were decreased after combined exposure to UP+2d LPS compared with single exposure to 2d LPS. In addition, exposure to LPS for 7 days decreased *VEGFR-2* mRNA levels compared with exposure to 2 days of LPS.

Since Angiopoietin-1 and its receptor Tie-2 are necessary for vessel maturation and stability (9, 13), their expression levels were determined in the ovine fetal lungs. *Ang1* mRNA levels were unchanged in the UP group, compared with the media group (Figure 6C). 7d LPS-treated animals had reduced *Ang1* mRNA levels compared with saline animals (Figure 6C). Reduced *Ang1* mRNA levels were also found in UP+7d LPS exposed animals, when compared with UP-treated animals. Similar findings were observed for *Tie-2* mRNA levels (Figure 6D). *Tie-2* mRNA levels remained unaltered after exposure to *U. parvum*, while exposure to 7d of LPS attenuated *Tie-2* levels. UP+2d LPS- and UP+7d LPS-treated animals had decreased *Tie-2* mRNA levels compared with the UP group.

Figure 6. Relative mRNA levels of angiogenic growth factors and receptors in the extreme preterm ovine lung. Pulmonary mRNA levels of vascular endothelial growth factor (VEGF) (A), VEGF receptor 2 (VEGFR-2) (B), Angiopoietin-1 (Ang1) (C) and tunica interna endothelial cell kinase 2 (Tie-2) (D) are shown for *Ureaplasma parvum* (UP)- and/or lipopolysaccharide (LPS)-treated preterm sheep. Results were obtained using real-time PCR reactions. Data were normalized to the house keeping gene ovRPS15. * $p < 0.05$ and ** $p < 0.01$ between experimental groups with the non-parametric Kruskal-Wallis test and Dunn's Multiple Comparison Test for post-hoc analysis.

Discussion

Since the incidence of histologic chorioamnionitis is inversely correlated with gestational age at preterm birth and because extremely preterm infants are often born during critical stages of pulmonary vascular development (6, 10, 23, 24), we evaluated in this study whether exposure to intra-uterine inflammatory stimuli resulted in pulmonary inflammation and vascular changes in extreme premature fetuses at the canalicular stage.

Preterm infants have an immature immune system which may be further compromised by additional factors related to preterm birth (31). Correa-Rocha et al. (32) showed that preterm infants have lower absolute values of all immune cell subsets (lymphocytes, monocytes, granulocytes) compared with term infants. Furthermore, there appears to be a positive correlation between gestational age and absolute immune cell numbers (32), indicating that immunological properties are age-dependent (33). Since priming of adaptive immune responses is limited due to restricted exposure to antigens *in utero*, fetuses primarily rely on innate immune responses, which however, can also still be immature (33). Whereas our group previously demonstrated that intra-amniotic LPS stimulated fetal lung and skin inflammation in extreme preterm sheep (34), we determined in this study how the extremely premature sheep lung responded to chronic *U. parvum* exposure. Contrary to LPS, chronic exposure to *U. parvum* did not provoke a pulmonary inflammatory response. Previous research with ovine fetuses born at a more mature age already indicated that acute or chronic *U. parvum* exposure was only responsible for mild lung inflammation due to low virulence (19, 21).

Previously, it was demonstrated that chronic intra-amniotic pre-exposure to *U. parvum* decreased the pulmonary immune response to subsequent contact with LPS, suggesting that chronic intra-uterine inflammation attenuates fetal immune responses and thereby increases the risk for a following nosocomial infection (21). Postnatal nosocomial infections are quite common in preterm infants due to extended stay within a hospital (31). Nevertheless, our early gestational age study indicates that chronic exposure to *U. parvum* did not diminish subsequent LPS responsiveness within the fetal lungs. The presence of CD3- and MPO-positive cells even increased after dual *U. parvum* and LPS exposure compared with 2d LPS or *U. parvum* alone. Levy et al. (35) and Netea et al. (36) already suggested that stimulation of the innate immune system of the neonate may alter the innate immunity set point and leads to immunological memory of the innate response (so-called 'trained immunity'). This may result in a heightened immune response towards a following challenge with the same or a different microorganism (35, 36). Although trained immunity may be

beneficial for the vulnerable neonate while adaptive immune responses are still limited, it may also be harmful in the sense that enhanced immune responses can result in concomitant neonatal morbidities (37). Our study and that of Kallapur et al. (21) furthermore suggest that the gestational age at exposure to the first stimulus (chronic *U. parvum*) as well as the gestational age at preterm birth are both important and clearly influence the pulmonary inflammatory response and the response to a second inflammatory stimulus.

Intra-amniotic LPS alone or the combined exposure of LPS with *U. parvum* decreased the expression of angiogenic factors and their receptors. In addition, combined exposure was associated with increased adventitial collagen deposition and an increased wall-to-lumen ratio. These findings suggest that multiple inflammatory stimuli have synergistic deteriorating effects on vascular development, compared with exposure to a single hit. In preterm sheep with a gestational age of 122 days, Kallapur et al. (20) already showed that intra-amniotic endotoxin decreased the expression of VEGF, VEGFR-2 and Tie-2, which was followed by vascular remodeling. In a rat model of chorioamnionitis, intra-amniotic administration of endotoxin during the late canalicular phase of lung development also decreased VEGF and VEGFR-2 lung protein expression at birth and led to pulmonary vascular remodeling and pulmonary hypertension at postnatal day 14 (38). In addition, human studies indicated that BPD patients have decreased *VEGF* and *Tie-2* mRNA levels within their lungs (9). In the pathophysiology of BPD it is thought that numerous prenatal (such as chorioamnionitis) and postnatal factors can impair angiogenic signaling, which disrupts vascular growth and causes abnormal vascular function. Increased pulmonary vascular resistance can alter vasoreactivity and causes vascular remodeling. All these steps may eventually contribute to the development of pulmonary hypertension in the setting of BPD (39). Therefore, the disturbed expression of angiogenic factors and vascular remodeling as observed in our study may potentially contribute to the pathogenesis of BPD and BPD-associated pulmonary hypertension.

According to the vascular hypothesis, disrupted pulmonary vascular development during essential stages of development can lead to impaired alveolarization and contributes to pulmonary hypoplasia (10, 11). In neonatal rats it was already revealed that anti-angiogenic agents not only reduced pulmonary vascular growth but also impaired alveolarization (12, 13). We were not able to assess parameters involved with alveolarization, due to the fact that alveolar development has not yet started at 94 dGA (22). In addition, pulmonary function could not be evaluated, since the lungs were too fragile at this stage. Therefore, we can only speculate about the consequences of the observed vascular changes for the alveolar development in our model. Previously, chronic endotoxin exposure starting at 80 dGA and using an intra-

amniotic osmotic pump, resulted in decreased vascular proteins and a reduced saccular number within the ovine fetal lungs at 100 dGA. However, within the same model no residual vascular and morphometric effects of this chronic endotoxin exposure were observed at 138 dGA (40). A second chronic exposure model using multiple endotoxin injections from 100 dGA onwards caused small vascular changes in sheep delivered at 130 dGA or 145 dGA (near-term) and tended to lower alveolar numbers with 30% in the 145 dGA animals (40). Kallapur et al. (40) postulated that the ovine fetal lungs recover and develop rather normal in spite of exposure to chronic endotoxin, when examined closer to term. Persistent progressive structural alterations may not be present within the lungs close to term regardless of this prolonged intra-amniotic endotoxin exposure (40). Nevertheless, the study designs of these two chronic endotoxin exposure studies (40) are different from ours considering timing and model of exposure and the age at preterm birth. Therefore, further research is needed to explore whether vascular changes at an extreme preterm age (94 dGA), as induced by exposure to single or combined intra-amniotic *U. parvum* and LPS, will remain and will be accompanied by impaired alveolar development at an older gestational age (e.g. near-term) or after exposure to additional postnatal inflammatory stimuli.

In conclusion, we have shown that contamination of the amniotic fluid is capable to mount an inflammatory response in the lungs of extreme preterm fetuses with concomitant impairment of the vasculature. Chronic pre-exposure to *U. parvum* and subsequent LPS enhanced the pulmonary inflammatory response compared to exposure with LPS or *U. parvum* alone. In addition, combined exposure to *U. parvum* and LPS resulted in disturbed pulmonary angiogenic signaling and vascular remodeling compared with single exposure. This study contributes to our understanding regarding the effects of antenatal inflammation on vascular lung development, which has become increasingly important for normal pulmonary development during the last years. Consequently, modulation of vascular development may be potentially beneficial in the treatment of inflammation-associated lung diseases, such as BPD.

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Chapter 5

General Discussion

Chorioamnionitis, which is defined as an intra-uterine infection or inflammation of the chorion, the amnion and the placenta, is considered to be a multi-organ disease which can affect multiple fetal organs upon exposure *in utero* (1-4). This thesis focused on the effects of exposure to intra-uterine inflammation on the fetal lungs, since the lungs are directly exposed to contaminated amniotic fluid as the fetus practices breathing movements *in utero* (1, 5). The resulting pulmonary inflammatory response is associated with a disturbed fetal lung structure, but matured lung function (6-8). Therefore, in this thesis pulmonary inflammation and concomitant effects on structural, functional and developmental parameters were assessed in the setting of intra-uterine inflammation. Furthermore, the potential of modulating this pulmonary inflammatory response by different treatments was evaluated within the fetal lungs. We used an established translational ovine model for this, as lung development in sheep is very similar to human lung development (9).

Pulmonary inflammation upon exposure to intra-amniotic lipopolysaccharide or *Ureaplasma parvum*

In this thesis, the intra-amniotic administration of *Escherichia coli*-derived lipopolysaccharide (LPS) (**chapter 2 and 3**) or of live *Ureaplasma parvum* (*U. parvum*; **chapter 4**) was used as a model for chorioamnionitis to induce and study intra-uterine inflammation.

LPS is a reliable and reproducible pro-inflammatory stimulus which can be easily administered *in utero*. The use of LPS is clinically relevant since clinical and histological chorioamnionitis are associated with elevated LPS levels in cord blood plasma of preterm infants (9, 10). Intra-amniotic (IA) injection of LPS already induced pulmonary inflammation within the fetal lungs at a gestational age of 94 days (**chapter 4**). In addition, a pulmonary inflammatory response was induced by antenatal IA inflammation at a higher gestational age of 121 or 129 days ((11) and **chapter 3**, respectively). It should be noted that the pulmonary inflammation part of the 121 days of gestational age (dGA) study is not a chapter within this thesis, but is discussed since the Wingless-Int (Wnt)/ β -catenin part of the same animal study is included within this thesis (**chapter 2**).

Since term delivery is at approximately 147 days of gestational age (dGA), the data in **chapter 3 and 4** indicated that the fetus can mount a pulmonary inflammatory response at an extreme early age (94 dGA); and that this ability to respond to intra-uterine inflammation was maintained throughout gestation (129 dGA). In addition, pulmonary inflammation was rapidly initiated within 2 days (**chapter 4**) and

detectable for at least 14 days (11) after IA LPS administration. Previously, our research group demonstrated that the fetal lungs already responded within 5 hours (h) after IA endotoxin injection by induction of pro-inflammatory cytokines within the fetal lung tissue, or influx of inflammatory cells within the alveolar lavage fluid (12, 13). These data indicate that the fetal lungs are one of the first organs to respond to IA LPS.

While IA LPS is a good model to mimic intra-uterine inflammation, the use of live microorganisms is even more closely related to the clinical situation. Although intra-amniotic infection is regularly polymicrobial (14-17), we chose *U. parvum* to induce intra-uterine inflammation because it is one of the most frequently detected microorganisms in amniotic fluid of women with preterm premature rupture of membranes and in respiratory secretions of preterm infants (18-20). The findings described in **chapter 4** demonstrated that chronic IA *U. parvum* exposure failed to induce a robust pulmonary pro-inflammatory response at a gestational age of 94 days, while LPS did. These distinctive, different fetal pulmonary responses between LPS and *U. parvum* could potentially be explained by the fact that LPS is a potent immuno-inflammatory stimulus, while the virulence of *U. parvum* is low (21-24). In addition, a possible explanation could be that *U. parvum* and LPS signal via different toll-like receptors (TLRs). TLRs are pattern recognition receptors, which are used by innate immune cells within the lungs to recognize microbial pathogens. Downstream signaling pathways are then triggered to induce specific immune responses against these pathogens (25, 26). LPS is a component found on the outer membrane of Gram-negative bacteria and is known to stimulate TLR4 (27). In contrast, *U. parvum* lacks a cell wall and *U. parvum*-derived lipoproteins signal via TLR1, TLR2 and TLR6 to activate nuclear factor κ B (NF- κ B) (20, 28). Distinct TLR activation may lead to activation of different signaling pathways and consequently specific immune responses.

It should be noted that at an older gestational age (approximately 124 days), acute or chronic *U. parvum* exposure did induce pulmonary inflammation. Nevertheless, the observed inflammatory response within the lungs was mild, probably due to the low virulence of *U. parvum* (22-24). The pulmonary inflammatory response was also shown to be time-dependent when a double IA inflammatory hit model was used. Fetal sheep born at 124 dGA and chronically exposed to *U. parvum* for 70 days demonstrated a suppressed pulmonary inflammatory response upon subsequent exposure to LPS (24). On the contrary, in **chapter 4** we demonstrated that chronic pre-exposure to *U. parvum* for 24 days and subsequent exposure to LPS even increased the presence of inflammatory cells within the fetal lungs at a gestational age of 94 days, compared with exposure to *U. parvum* or LPS alone. These time- and gestation-dependent findings in fetal sheep strongly suggest that the timing of exposure to

inflammatory stimuli (e.g. gestational age at start of exposure, duration of the exposure and gestational age at time of preterm birth) is very important for the outcome of the pulmonary inflammatory response. This timing aspect will be discussed in more detail later in this chapter.

While the fetal pulmonary immune responses differed after exposure to LPS or *U. parvum*, exposure to one or the combination of these stimuli was associated with pulmonary developmental, structural or functional changes; which will be further discussed in the upcoming sections. Interestingly, pulmonary exposure to IA LPS can also result in fetal gut inflammation and injury, as well as in a systemic inflammatory response (29). The importance of the initial site of exposure to a pro-inflammatory stimulus has been studied in fetal sheep with the help of surgical interventions that isolated the different organs (lungs, gut and chorioamnion/skin) from direct contact with the amniotic fluid (29). These surgical experiments indicated that the consequences of inducing a pulmonary inflammatory response are not limited to the fetal lungs.

Wingless-Int/ β -catenin signaling & its potential as a target for therapy

The data reported in **chapter 2** indicated that IA contact with LPS disrupted pulmonary canonical Wingless-Int (Wnt)/ β -catenin signaling, which is considered to be the most important Wnt signaling pathway (30). Members of the Wnt family are growth factors that are essential during lung development. Expression levels of Wnts and the downstream messenger β -catenin are therefore tightly regulated (30). Nevertheless, LPS decreased the expression of multiple Wnt members, β -catenin and lymphoid enhancer-binding factor 1 (LEF1) transcription factor. Nuclear β -catenin co-operates with members of the LEF1/T cell factor (TCF) family of transcription factors to regulate the transcription of Wnt target genes (30, 31). Previously, β -catenin was suggested to be critical for the formation of alveoli. Deletion of β -catenin during mice lung development was shown to block peripheral epithelial cell differentiation, resulting in lungs which were primarily composed of conducting airways and in which the formation of terminal alveolar saccules was reduced (32, 33). Hyperactivation of β -catenin was not beneficial either, since it resulted in enlarged air spaces (33). Although the LPS-induced reduction in β -catenin protein expression in our study was not associated with differences in lung morphometric analyses, we did find less elastin foci and increased collagen deposition within alveolar structures within the same study (11, 34). These two structural proteins are involved in alveolar development and

are abnormally distributed in BPD patients (34-39). Due to the fact that BPD patients show impaired alveolarization and due to the involvement of β -catenin in alveolar formation (32, 33, 40), disruption of Wnt/ β -catenin signaling possibly contributes to the development of BPD. Recently, Li et al. (41) identified genes involved in BPD using neonatal blood spot samples of BPD patients and their unaffected twin siblings. They found that regulation of the Wnt pathway was highly involved in the development of BPD, indicating that the Wnt pathway is possibly associated with this disease (41). We therefore speculate that the observed LPS-induced aberrant Wnt/ β -catenin signaling in our study, in the combination with postnatal stimuli such as mechanical ventilation, potentially contributes to the development of BPD (42).

Given the above, β -catenin is suggested as a potential target for treatment of BPD. In a hyperoxia-induced BPD neonatal rat model, increased β -catenin nuclear translocation was associated with increased expression of β -catenin target genes, impaired alveolarization, reduced vascular density and increased muscularization of pulmonary peripheral arterioles (43). Pharmacological inhibition of β -catenin by ICG001 downregulated the expression of these β -catenin target genes, improved alveolar and vascular development and decreased vascular remodeling within the lungs, indicating the potential of β -catenin signaling modulators for the treatment of BPD (43). Similar results were observed with the use of other therapeutic strategies involving inhibition of Wnt/ β -catenin signaling activation (44, 45). Connective tissue growth factor (CTGF) interacts with the Wnt receptor complex by binding to co-receptors of Wnt signaling (46). A neutralizing antibody against CTGF blocked activation of β -catenin signaling and improved alveolar and vascular development in a comparable neonatal rat model of hyperoxia (44). In addition, hyperoxia-induced upregulation of Wnt/ β -catenin signaling, inflammation and arrested alveolarization were prevented by rosiglitazone, an agonist of peroxisome proliferator-activated receptor (PPAR)- γ (45).

Although inhibition of β -catenin seems promising in the context of hyperoxia-induced BPD, other signaling pathways than the Wnt/ β -catenin pathway can be affected as well when β -catenin is targeted. In the rosiglitazone study which was just discussed, Dasgupta et al. (45) showed that in parallel to Wnt/ β -catenin signaling also transforming growth factor (TGF)- β signaling was activated by hyperoxia and prevented by rosiglitazone treatment. TGF- β is involved in lung development, while the nuclear receptor PPAR- γ has multiple functions including adipogenesis (45, 47). In addition, PPAR- γ determines the phenotype of lipofibroblasts, which support proliferation and differentiation of alveolar type II cells (48, 49). Physical interactions between members of the Wnt/ β -catenin, TGF- β and PPAR- γ signaling pathways have been reported, suggesting a potential mechanism of how rosiglitazone prevented the

hyperoxia-induced activation of both pathways (45, 50, 51). Similarly, besides changes in the Wnt/ β -catenin pathway as described in **chapter 2**, we also found LPS-induced pulmonary changes in the TGF- β pathway within the same study (52). This could suggest that also within our model crosstalk between different signaling pathways may be present.

Another link was found between Wnt/ β -catenin and angiopoietin/Tie-2 signaling pathways, which are both involved in pulmonary vascular development (53). All these different types of crosstalk between Wnt/ β -catenin and other signaling pathways within the lungs should be kept in mind when Wnt/ β -catenin signaling is considered as a target for treatment of BPD or after chorioamnionitis. Furthermore, Wnt/ β -catenin signaling is not limited to the lungs during fetal development, as nuclear β -catenin is expressed in human fetal cartilage, adrenal and kidney tissues, and other organs (54). Therefore, caution is needed when the Wnt/ β -catenin pathway is targeted using local or systemic treatment, since other signaling pathways and non-pulmonary organs can be affected during fetal development as well.

Vascular changes & prospective therapies

The results in **chapter 4** demonstrated that IA LPS alone and the dual exposure together with *U. parvum* decreased the pulmonary expression of fetal angiogenic growth factors and their associated receptors at an extremely preterm age. This disturbed expression was associated with enhanced deposition of adventitial collagen and an elevated wall-to-lumen ratio after combined exposure. These data indicated that multiple inflammatory hits had synergistic deteriorating effects on the fetal pulmonary vascular development, compared with a single intra-uterine inflammatory hit. In line with this, a rat model of chorioamnionitis demonstrated that IA LPS administration combined with postnatal hyperoxia exposure decreased the pulmonary expression of vascular endothelial growth factor (VEGF) and its receptor, compared to animals which were only exposed to either endotoxin or hyperoxia (55).

Since disrupted pulmonary vascular growth during fetal development does not only contribute to impaired alveolarization and lung hypoplasia, but also tends to be involved in the development of pulmonary hypertension in a context of BPD (56-58), therapies directing lung vascular development may be potentially beneficial for chorioamnionitis-exposed infants and BPD patients. In a neonatal rat hyperoxia-induced model of BPD, recombinant human VEGF therapy enhanced vessel growth and alveolarization when provided during or after hyperoxia (59, 60). Within a comparable model, postnatal intratracheal delivery of adenovirus-mediated VEGF

gene therapy improved survival, promoted pulmonary capillary formation and preserved alveolarization (61). However, capillaries were fenestrated and exhibited an immature and leaky phenotype (61). Since the angiogenic growth factor Angiopoietin 1 is necessary for vessel maturation and stability, the authors combined VEGF and Angiopoietin 1 gene transfer and showed that the combination resulted in more mature and less permeable capillaries (61, 62). These studies show the potential of therapies directing vascular development to not only enhance vascular growth but also alveolar development.

Modulating pulmonary inflammation and subsequent lung development & function

The data described in **chapters 2 and 4** indicated that IA inflammation can dysregulate pathways involved in lung development and can disturb vascular lung structure. Previous studies from our research group already demonstrated that pulmonary inflammation, induced by an IA inflammatory stimulus, was associated with lung structural changes on the one hand, but increased pulmonary surfactant lipids and improved lung gas volume on the other (6, 7, 63, 64).

Antenatal corticosteroids

Also maternal glucocorticoids can accelerate lung maturation and surfactant production, but they prevent inflammation (65, 66). Therefore, a single course of maternal antenatal corticosteroids is recommended as a standard treatment in case of an anticipated preterm delivery to decrease respiratory distress syndrome (RDS) and neonatal death (65). Regularly, fetuses are exposed *in utero* to both microorganisms and antenatal corticosteroids (67). Therefore, the interactive effects between chorioamnionitis and antenatal corticosteroids on pulmonary inflammation and maturation were previously studied in our ovine sheep model (11). It was shown that betamethasone suppressed LPS-induced pulmonary inflammation when it was maternally and intramuscularly administered before IA LPS. In contrast, if betamethasone was given after IA LPS, it did not prevent inflammation but increased lung maturation as observed by high expression of surfactant components and enlarged lung gas volumes (11).

Considering these interactions between LPS and betamethasone on lung inflammatory outcome, we questioned whether LPS and betamethasone would also

interact on lung developmental signaling pathways. We assessed the net result of maternal betamethasone administration on LPS-induced disturbed expression of components of the Wnt/ β -catenin signaling pathway in the fetus (as already discussed earlier in this chapter). In **chapter 2** we found that maternal corticosteroid exposure before or after LPS had different effects on Wnt/ β -catenin signaling. Corticosteroid administration only partially prevented LPS-induced disturbed Wnt/ β -catenin signaling in the fetus. As an example, betamethasone administration before IA LPS prevented decreased LEF1 expression, while betamethasone treatment after IA LPS did not. The results reported in **chapter 2** and the inflammatory data previously described by Kuypers et al. (11) suggest that the interaction between LPS and corticosteroids on pulmonary outcome can be complex. This is supported by the finding that the consecutive order of exposure to betamethasone and LPS matters, which will be discussed in more detail later on in this chapter. Furthermore, it is likely that not only the pulmonary immune system is involved in the outcome of the Wnt/ β -catenin signaling pathway after combined exposure to corticosteroids and intra-uterine inflammation. Other signaling pathways, such as the TGF- β and PPAR- γ pathways, may contribute as well, considering their before-mentioned interactions with the Wnt/ β -catenin pathway (45, 50, 51).

Although the combined effects of corticosteroids and intra-uterine inflammation seem to be complex, a meta-analysis by Been et al. (67) indicated that antenatal steroids reduce adverse neonatal outcomes such as mortality and RDS in preterm infants born after chorioamnionitis. However, data regarding the outcome after the neonatal period are scarce, demanding the need for long-term follow-up studies (67). Currently, the use of corticosteroids is indispensable in the neonatal clinic, but it is shown that glucocorticoids can also be detrimental for alveolarization (7, 66). Therefore, the search for new and improved treatments should be encouraged. This may possibly lead to new therapies with the same beneficial effects as corticosteroids but lacking the harmful effects on neonatal pulmonary outcome.

Prophylactic interleukin 2

In **chapter 3** we tested the potential of such a possible future treatment in our ovine model of chorioamnionitis. Systemic interleukin (IL)-2 was prophylactically administered to the fetus *in utero* before the onset of LPS-induced chorioamnionitis. This immunoregulatory cytokine is required for clonal expansion, growth and suppressor function of regulatory T cells (Tregs) (68-70). Tregs are essential for the regulation of inflammatory responses and the maintenance of immune homeostasis (71). Previously, increased CD3-positive cells were detected within days within the

lungs and the mediastinal lymph node in the ovine model (22, 24, 72). Several studies with primate and ovine models indicated that within this time frame, chorioamnionitis was followed by a rapid transient reduction in Tregs in various lymphatic organs, including the lung-related mediastinal lymph node (29, 72-74). Because of this decrease in Tregs and the finding that the activity of Tregs in preterm neonates was reduced after chorioamnionitis (75), we wanted to preferentially expand these Tregs to modulate chorioamnionitis-induced inflammatory responses. Therefore, in **chapter 3** we evaluated the effects of IL-2-driven selective Treg expansion on the fetal lungs in our ovine model of chorioamnionitis. It was demonstrated that systemic IL-2 treatment had moderate immunomodulatory effects in the lung when preterm ovine fetuses were subsequently exposed to IA LPS. Despite these immunomodulatory effects of prophylactic IL-2, the beneficial effects on lung function (increased disaturated phospholipids (DSPLs) and improved lung gas volume) were maintained in LPS-exposed animals.

As far as we know, we are the first to report the clinical potential of prophylactic IL-2 therapy in *in vivo* experiments in the fetal gut (76) and lungs (**chapter 3**) in the setting of chorioamnionitis. Future studies are necessary to investigate the potential of prophylactic IL-2 when intra-uterine inflammation is followed postnatally by a second inflammatory hit, such as sepsis or mechanical ventilation. These second hits can amplify and prolong the pulmonary inflammatory response and thereby increase the risk for BPD (42, 77). Reduction of the pro-inflammatory response after antenatal inflammation, via IL-2-driven Treg expansion, possibly prevents amplification of this inflammatory response upon encounter with a second postnatal hit.

High-dose (HD) intravenous IL-2 (600,000-720,000 IU/kg per dose every 8 hours) is already an approved clinical therapy by the US Food and Drug Administration for the treatment of metastatic melanoma and renal cell carcinoma (78-80). A potential drawback for using HD IL-2 is the development of side effects, which can be quite severe (79). The majority of these side effects are due to the development of the vascular leakage syndrome, which is caused by increased vascular permeability and results in fluid extravasation and hypotension (79). However, Schwartzenruber et al. (80) emphasized that the variation in toxicities experienced by patients receiving HD IL-2 is large, and that some patients only have few side effects. In addition, most of these side effects quickly disappear on termination of HD IL-2 treatment (79, 80). In our ovine model, we observed no signs of vascular leakage syndrome or other adverse outcomes. This can be explained by the fact that we used a clinically relevant and moderate dose (250,000 IU/kg per day) instead of the HD IL-2, since the occurrence of vascular leakage syndrome is dependent on the dose of IL-2 (76, 79, 81).

Chapter 3 and the study by Nikiforou et al. (76) both were a proof of principle that prophylactic IL-2 treatment *in utero* preferentially expanded Tregs, which has the potential to be used in the clinic in the setting of intra-uterine inflammation. Future studies are needed to examine whether IL-2, besides as a prophylactic approach, can be used as a treatment after the induction of intra-uterine inflammation. Although in our prophylactic model of IL-2 treatment we did not find changes in the number of CD8-positive cells in the gut-associated mesenteric lymph node or spleen after IL-2 treatment alone or in the combination with IA LPS exposure (unpublished data), IL-2 is known to expand CD8-positive cells and natural killer (NK) cells besides Tregs (82). In addition, it was demonstrated that viral intra-uterine infection can also increase the NK cell count in fetal cord blood (83). Therefore, IL-2 treatment should possibly, when administered after initiation of intra-uterine inflammation, be combined with inhibitory monoclonal antibodies to prevent further NK cell stimulation. These inhibitory antibodies form specific complexes with IL-2, ensuring exclusive Treg stimulation without influencing the proliferation of CD8-positive cells or NK cells (79). Future studies will identify the possible need for inhibitory antibodies in combination with IL-2 in the setting of chorioamnionitis.

Furthermore, **chapter 3** demonstrated that systemic IL-2 treatment alone increased the pulmonary DSPL concentration and improved lung gas volume as well. This finding can be beneficial in the context of RDS, which may develop in premature infants due to surfactant deficiency (84). The exact mechanism how IL-2 improves lung function in our ovine model is largely unknown. The lung epithelium in our study did not stain positive for CD25 (IL-2 receptor- α), although Lesur et al. (85) found that primary cultures of rat pulmonary type II cells do express CD25. One possibility is that the epithelial expression of CD25 was too low to detect by immunohistochemistry in our study. Another option could be that the fetal lung epithelium responded indirectly to IL-2. Further research should clarify the exact mechanism how IL-2 improved lung function in the ovine fetus.

Timing of intra-uterine exposure is crucial

Throughout this whole thesis it was indicated that the timing of exposure to intra-uterine stimuli is a crucial aspect for fetal pulmonary outcome. One of the most prominent examples was the observation in **chapter 2** and in (11) that the order of exposure to antenatal corticosteroids and intra-amniotic inflammation influenced the pulmonary inflammatory response and subsequent Wnt/ β -catenin signaling. Dependent on whether betamethasone was administered before or after IA LPS,

pulmonary inflammation was prevented, lung maturation was increased or disturbances in the Wnt/ β -catenin signaling pathway were averted.

Another example of a timing effect was found when pulmonary inflammation was compared between different gestational ages. **Chapter 4** demonstrated that the fetus exerts a different pulmonary inflammatory response to a double inflammatory hit model at 94 versus 124 dGA at birth: an increased versus a suppressed response, respectively. Probably the gestational age at the start of exposure, the duration of the exposure, and the gestational age at the time of preterm birth are all important. It should be further investigated whether fetuses experience different lung inflammatory responses and associated adverse outcomes postnatally and later on in life, when exposed to intra-uterine inflammation at different time points.

Future directions & conclusion

Nevertheless, to identify the fetal gestational age at the start of exposure and to determine the duration of exposure to intra-uterine inflammation, non-invasive and specific biomarkers are needed to detect intra-uterine inflammation. These biomarkers are also essential to evaluate whether and when therapy can be started *in utero*. Already many articles have been published about potential biomarkers to detect intra-uterine inflammation (86-88). Although some biomarkers seem to be promising for the implementation in the clinic, the clinical value of other biomarkers appears to be limited (86-88). Therefore, biomarker research should remain a point of focus to make early detection of chorioamnionitis possible.

In children born prematurely, an association has been found between chorioamnionitis and wheezing or physician-diagnosed asthma when these children were older (89-91). In addition, wheezing was more frequently reported in school children born extremely premature and with a history of BPD, compared to children who were only born extremely preterm and had no prior BPD (92). These data indicate that the consequences of chorioamnionitis and BPD can persist into childhood. Therefore, further research should identify more precisely the long-term pulmonary consequences of BPD and exposure to chorioamnionitis.

Another point of particular interest for the future is the search for a treatment for chorioamnionitis and its associated adverse outcomes, either by curing these adverse effects or by preventing these effects to occur. Using an ovine model of chorioamnionitis to mimic the situation in the clinic as closely as possible, this thesis focused on the consequences of exposure to intra-uterine inflammation on the fetal

lungs. In specific, pulmonary disturbances in the Wnt/ β -catenin signaling pathway, inflammatory and vascular effects, and lung function were discussed after one or multiple intra-amniotic inflammatory hits. In addition, it was considered how these specific effects can be used as potential targets for treatment. These potential targets need to be further explored in future studies using translational models to verify their efficacy, safety and feasibility as a possible treatment in the setting of chorioamnionitis.

Although much more research needs to be conducted, the data presented in this thesis contribute to our knowledge regarding chorioamnionitis and its lung-associated consequences. Implementation of the newly acquired information from this thesis and from future research may eventually contribute to treatment options for chorioamnionitis and its pulmonary adverse outcomes.

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Summary

Chorioamnionitis is defined as an intra-uterine inflammation or infection of the chorion, amnion and placenta. This thesis focused on the outcome of intra-uterine inflammation on the fetal lungs, as the development of the lungs into functional and healthy organs is essential for postnatal life. Because the fetal lungs are in direct contact with amniotic fluid due to the practice of breathing movements *in utero*, chorioamnionitis can result in pulmonary inflammation. Hence, within this thesis the pulmonary inflammatory response and its associated outcomes on fetal lung function, structure and developmental parameters are evaluated. Additionally, the potential of several therapies to modulate pulmonary inflammation and its outcomes is assessed in the setting of intra-uterine inflammation. A well-established and translational ovine model is used to study these effects, as ovine lung development closely resembles human lung development.

Chapter 1 provides a general overview of normal lung development and intra-uterine inflammation. It is described how intra-uterine inflammation can result in adverse lung development and eventually in bronchopulmonary dysplasia, a chronic lung disease. Furthermore, the use and potential benefits of corticosteroids in the setting of antenatal inflammation are described. This chapter concludes with the outline and aim of this thesis.

In **chapter 2**, the interaction between and the outcome of the order of exposure to chorioamnionitis and antenatal corticosteroids on fetal lung development are assessed. The fetus is regularly exposed to both of these stimuli, which can induce fetal lung maturation but can also negatively affect fetal pulmonary development. However, the order of exposure *in utero* can vary and is not always known, as chorioamnionitis is often clinically silent. To investigate the interaction and the effect of the order of exposure on the lung developmental Wntless (Wnt)-Int pathway, intra-uterine inflammation was induced by injection of lipopolysaccharide (LPS) into the ovine intra-amniotic fluid. Antenatal corticosteroids (betamethasone) were administered intra-muscularly to the ewe, either prior to or after LPS injection. In this chapter, it was demonstrated that LPS exposure decreased the expression of multiple members of the Wnt signaling pathway in the fetal lungs. Betamethasone exposure before or after LPS resulted in different effects on the Wnt pathway, and LPS-induced changes were only partially prevented by betamethasone treatment prior to or after LPS exposure. These findings indicate that the order of exposure to LPS and betamethasone is important. In addition, this study contributes to our knowledge how intra-uterine inflammation can affect fetal lung development and how antenatal corticosteroids may be beneficial within this setting.

Chapter 3 describes the effects of selective expansion of regulatory T cells (Tregs), using interleukin (IL)-2, on the fetal lungs in the setting of chorioamnionitis. Previous studies have shown that although lung inflammation was associated with pulmonary structural changes, it improved lung function by an increased surfactant lipid pool and an enlarged lung gas volume. In addition, intra-uterine inflammation resulted in a decreased number of Tregs in the lung-associated mediastinal lymph node. As Tregs are essential in regulating inflammatory responses, these cells were selectively expanded by use of IL-2 within this study. Ovine fetuses were instrumented and received intravenous prophylactic IL-2, followed by intra-amniotic exposure to LPS. IL-2 treatment preferentially expanded Tregs in the fetal lungs. Unexpectedly, IL-2 treatment beneficially affected lung function by increasing the disaturated phospholipid concentration and by improving the lung gas volume. These beneficial effects on fetal lung function were maintained regardless of the moderate immune modulating effects of IL-2 treatment in the course of chorioamnionitis.

In **chapter 4**, the inflammatory and vascular effects of antenatal inflammation were evaluated during the canalicular stage, which is a crucial stage for lung vascular development. Due to the inverse relation between gestational age and the incidence of chorioamnionitis, the incidence is higher in infants who are born extremely preterm during the canalicular stage of lung development. In an ovine model, intra-uterine inflammation was induced by chronic intra-amniotic exposure to *Ureaplasma parvum* (*U. parvum*) or by intra-amniotic LPS injection. Combined exposure to both stimuli was used to assess whether chronic pre-exposure with *U. parvum* modified the fetal pulmonary response induced by LPS. It was hypothesized that single or combined exposure to chronic *U. parvum* and/or acute inflammation induced a fetal pulmonary inflammatory response and vascular changes. Within this chapter it was demonstrated that contamination of the amniotic fluid was able to induce a fetal pulmonary inflammatory response and vascular changes at an extremely preterm age. In addition, combined exposure to *U. parvum* and LPS increased the pulmonary inflammatory response and resulted in disrupted angiogenic signaling and vascular remodeling in comparison to single exposure with *U. parvum* or LPS. This study provides new insights about the effects of intra-uterine inflammation on the pulmonary vascular development, which has been increasingly appreciated for normal lung development during the last years.

In summary, within this thesis it was demonstrated that intra-uterine inflammation is associated with a fetal lung inflammatory response, an impaired pulmonary development and a disrupted lung structure, but also that the timing of exposure is crucial for the fetal pulmonary outcome. In addition, the beneficial effects and clinical potential of several therapeutic strategies for the fetal lungs were evaluated in the

setting of antenatal inflammation, such as the finding that maternal corticosteroid administration can partially prevent decreased fetal pulmonary Wnt signaling. Furthermore, it was demonstrated that IL-2 treatment improved the fetal lung function and that this beneficial effect was maintained despite the moderate immunomodulatory effect of IL-2 treatment in the course of chorioamnionitis. In **Chapter 5**, these major conclusions and novel findings are discussed in more detail, together with the clinical potential and future directions. The results reported in this thesis contribute to our knowledge about antenatal inflammation and the fetal pulmonary outcome. Although additional research is required, the described findings may in time support the development of new pulmonary treatment options in the setting of intra-uterine inflammation.

Samenvatting

Chorioamnionitis is gedefinieerd als een intra-uteriene inflammatie of infectie van het chorion, het amnion en de placenta. De focus van dit proefschrift was het effect van intra-uteriene inflammatie op de foetale longen, aangezien de ontwikkeling van de longen tot functionele en gezonde organen essentieel is voor het leven na de geboorte. Omdat de longen ten gevolge van de foetale ademhalingsbewegingen in direct contact staan met de amniotische vloeistof, kan chorioamnionitis leiden tot longinflammatie. Om deze reden zijn in deze thesis de pulmonale inflammatoire reactie en de daarmee geassocieerde gevolgen voor de foetale longfunctie, –structuur en –ontwikkelingsparameters onderzocht. Daarnaast is bestudeerd of verschillende therapieën het potentieel hebben om longinflammatie en zijn gevolgen te moduleren in de context van intra-uteriene inflammatie. Er is gebruik gemaakt van een erkend en translationeel schaapmodel om deze effecten te bestuderen, aangezien de longontwikkeling bij schapen zeer sterk lijkt op de humane longontwikkeling.

Hoofdstuk 1 geeft een algemeen overzicht van de normale longontwikkeling en intra-uteriene inflammatie. Er is beschreven hoe intra-uteriene inflammatie de longontwikkeling nadelig kan beïnvloeden en hoe het kan leiden tot de chronische longziekte bronchopulmonale dysplasie. Daarnaast wordt in dit hoofdstuk het nut en het gebruik van corticosteroïden in de context van antenatale inflammatie omschreven. Het hoofdstuk eindigt met de hoofdlijnen en het doel van dit proefschrift.

In **hoofdstuk 2** werd de interactie tussen, en het resultaat van de volgorde van blootstelling aan chorioamnionitis en antenatale corticosteroïden op de foetale longontwikkeling onderzocht. Het komt regelmatig voor dat de foetus aan beide stimuli blootgesteld wordt, die zowel foetale longmaturing kunnen induceren, maar ook een negatief effect kunnen hebben op de foetale longontwikkeling. Echter, de volgorde van blootstelling binnen de baarmoeder kan variëren en is niet altijd bekend, aangezien chorioamnionitis vaak geen klinische symptomen vertoont. Om deze interactie en het effect van de volgorde van blootstelling op de Wingless-Int (Wnt) longontwikkeling pathway te onderzoeken, werd intra-uteriene inflammatie geïnduceerd door middel van een injectie met lipopolysacchariden (LPS) in het vruchtwater van het schaap. Voor of na de injectie met LPS werden corticosteroïden (betamethason) intramusculair toegediend aan de ooi. In dit hoofdstuk is aangetoond dat blootstelling aan LPS de expressie van meerdere factoren van de Wnt signaleringspathway verminderde in de foetale longen. Blootstelling aan betamethason, voor of na blootstelling aan LPS, resulteerde in verschillende effecten binnen de Wnt pathway. Daarnaast kon betamethason blootstelling, voor of na blootstelling aan LPS, maar gedeeltelijk de LPS-geïnduceerde veranderingen voorkomen. Deze bevindingen laten zien dat de volgorde van blootstelling aan LPS en

betamethason belangrijk is. Daarnaast draagt deze studie bij aan onze kennis hoe intra-uteriene inflammatie invloed kan hebben op de foetale longontwikkeling en hoe antenatale corticosteroïden hierop een gunstige invloed kunnen uitoefenen.

Hoofdstuk 3 beschrijft de effecten van interleukine (IL)-2 geïnduceerde selectieve expansie van regulatoire T-cellen (Tregs) op de foetale longen in de context van chorioamnionitis. Hoewel eerdere studies hebben aangetoond dat longinflammatie geassocieerd is met veranderingen in de longstructuur, kan het ook de longfunctie verbeteren door meer surfactant lipiden en een verhoogd longgasvolume. Daarnaast resulteerde intra-uteriene inflammatie in een verlaagd aantal Tregs in de mediastinale lymfeknoop, die geassocieerd is met de longen. Aangezien Tregs essentieel zijn bij de regulatie van inflammatoire reacties, is er in deze studie selectieve expansie van Tregs toegepast door middel van IL-2. Schapefoetussen werden geïstrumenteerd en kregen intraveneus profylactisch IL-2 toegediend, gevolgd door een injectie met LPS in het vruchtwater. IL-2 therapie zorgde voor een selectieve expansie van Tregs in de foetale longen. Een onverwachte uitkomst was dat de behandeling met IL-2 de longfunctie voordelig beïnvloedde door middel van een toename van de gedissatureerde fosfolipiden concentratie en een verhoogd longgasvolume. Deze gunstige effecten op de longfunctie bleven behouden ondanks de gematigde immuunmodulerende effecten van IL-2 therapie in de context van chorioamnionitis.

In **hoofdstuk 4** werden de inflammatoire en vasculaire effecten van antenatale inflammatie onderzocht tijdens de canaliculaire fase, een cruciale fase voor de ontwikkeling van de longvasculatuur. Door het omgekeerde verband tussen de zwangerschapsduur en de incidentie van chorioamnionitis, is de incidentie van chorioamnionitis hoger in baby's die extreem vroeggeboren zijn tijdens de canaliculaire longontwikkelingsfase. In een schape-model, werd intra-uteriene inflammatie geïnduceerd door middel van chronische blootstelling van het vruchtwater aan *Ureaplasma parvum* (*U. parvum*) of door injectie van LPS in het vruchtwater. Een gecombineerde blootstelling van beide stimuli werd gebruikt om te onderzoeken of chronische blootstelling aan *U. parvum* invloed had op de foetale longreactie die door LPS werd geïnduceerd. De hypothese was dat een enkele of gecombineerde blootstelling aan chronisch *U. parvum* en/of acute inflammatie een foetale pulmonale inflammatoire reactie en vasculaire veranderingen veroorzaakte. In dit hoofdstuk is aangetoond dat contaminatie van het vruchtwater kan leiden tot een foetale pulmonale inflammatoire reactie en vasculaire veranderingen op een extreem vroeggeboren leeftijd. Daarnaast kan een gecombineerde blootstelling aan *U. parvum* met LPS leiden tot een verhoogde pulmonale inflammatoire reactie, verstoorde angiogene signalering en vasculaire vervorming (remodeling), in vergelijking met een enkele blootstelling aan ofwel *U. parvum* of LPS. Deze studie draagt bij aan nieuwe

inzichten in het effect van intra-uteriene inflammatie op de vasculaire longontwikkeling, die tijdens de afgelopen jaren zeer belangrijk is gebleken voor de normale longontwikkeling.

Samengevat, is er in deze thesis aangetoond dat intra-uteriene inflammatie geassocieerd is met een foetale inflammatoire reactie in de longen en een verstoorde longontwikkeling en –structuur. Hierbij is de timing van de blootstelling cruciaal voor het effect op de foetale longen. Daarnaast zijn in dit proefschrift de voordelige effecten en het potentieel van verschillende therapieën voor de foetale longen beschreven in de context van intra-uteriene inflammatie, zoals de bevinding dat toediening van maternale corticosteroïden een verlaagde Wnt signalering in de foetale longen gedeeltelijk kan voorkomen. Ook is er aangetoond dat IL-2 therapie de foetale longfunctie verbetert en dat dit voordelige effect behouden blijft ondanks het gematigde immuun-modulerende effect van IL-2 in de setting van chorioamnionitis.

Hoofdstuk 5 bevat een uitgebreide uiteenzetting van deze belangrijkste conclusies en nieuwe bevindingen, samen met de klinische relevantie en toekomstperspectieven. De resultaten die in dit proefschrift staan beschreven dragen bij aan onze kennis over antenatale inflammatie en de gevolgen hiervan voor de foetale longen. Hoewel verder onderzoek vereist is, kunnen de bevindingen van dit proefschrift bijdragen aan de ontwikkeling van nieuwe behandelmethoden voor de longen, in de context van intra-uteriene inflammatie.

Valorization

Social & economic relevance

Preterm birth is defined by the World Health Organization (WHO) as birth before 37 weeks of gestation. It can have a tremendous impact on the health and life of the infant after birth (1). Globally, around 15 million infants are born preterm, which is more than 1 out of 10 births (1). In The Netherlands, in 2015 7.1% of all births were preterm (2). In absolute numbers, this accounted for 12,070 children out of a total number of 169,267 infants (2). Although within The Netherlands these numbers show a decrease compared to the year of 2013, globally still almost 1 million children die every year due to preterm birth-related complications (1, 3). Prematurity remains the main cause of death in children younger than five years (1). Even when preterm infants survive, they often will face a lifetime with disabilities and health problems (1). Therefore, the social and economic impact of preterm birth-related complications on families, society and the health care system remains high (2, 4, 5). The psychosocial and emotional impact on the family, such as psychological distress and maternal depression, mainly depends on the severity of the infant's health condition (4, 6). The economic costs related to preterm birth are large and include acute neonatal intensive care, ongoing long-term health care needs and the requirement for special education services (4). In The Netherlands, on average, the total number of days spent in the neonatal intensive care unit (NICU) and the hospital varied between 11 days (for a child born between 34.0-36.6 weeks) and 62 days (for a child born between 24.0-25.6 weeks) per child within the year of 2015 (2). This indicates the inverse relation between the gestational age at birth and the intensity of required neonatal care (7). In addition, prematurely born children have a higher chance to be re-hospitalized (6, 8). Preterm birth-related costs added up to \$26.2 billion in the United States in 2005; and the first-year medical costs for preterm infants (\$32,325) were on average roughly ten times higher than the costs for term infants (\$3,325) (4). Overall, these numbers indicate that preterm birth can be considered as a serious global health problem (1, 4).

Respiratory problems belong to the most common complications in preterm infants (9). The development of the lungs into healthy and functional organs is crucial for life after birth. As preterm infants are born before completion of the *in utero* stages of lung development, these infants have a higher risk to develop respiratory complications either directly after birth or at a later age (such as respiratory distress syndrome and wheezing disorders) (9-11). This is especially true when the preterm infant is additionally exposed to antenatal inflammation, like chorioamnionitis (9). Chorioamnionitis is an intra-uterine inflammation or infection of the chorion and the amnion (fetal membranes), and the placenta (12-14). Intra-uterine inflammation is

one of the most essential risk factors of preterm birth, as it is involved in up to 50% of the preterm births <28 weeks (12, 15). Prematurely born infants who were exposed to chorioamnionitis had an increased risk of asthma compared with gestational age-matched infants who were not exposed to chorioamnionitis, indicating the association between exposure to chorioamnionitis and childhood asthma (16). The highest risk of asthma (odds ratio [OR] 4.4; 95% CI: 2.2-8.7) and wheezing (OR 4.0; 95% CI: 2.0-8.0) was observed in former preterm infants who were exposed to chorioamnionitis and who were born at a lower gestational age (16, 17). In addition, antenatal infection or inflammation is associated with a higher risk for chronic lung disease if preterm infants are postnatally exposed to extended mechanical ventilation or sepsis (18). These findings suggest that the consequences of intra-uterine inflammation are not limited to life directly after birth and that they can persist into childhood.

Factors that have contributed to an increased survival rate of preterm infants are administration of antenatal corticosteroids to increase pulmonary maturation and advancements within mechanical ventilation techniques (5). To realize such new advances and to improve current medical technology and neonatal care, extensive research is essential and indispensable. As intra-uterine inflammation is such a critical risk factor for respiratory complications, this thesis focused on the effects of exposure to intra-uterine inflammation on the fetal lungs. Furthermore, in this thesis, the pulmonary effects of several therapies such as antenatal corticosteroids and interleukin-2 (IL-2) were evaluated and discussed within the setting of intra-uterine inflammation.

Novelty & future directions

Within this thesis, novel insights were presented. High-dose IL-2 therapy has already been approved by the US Food and Drug Administration as a treatment for metastatic melanoma and renal cell carcinoma (19). Nevertheless, as far as we know, we were the first to describe the clinical potential of prophylactic IL-2 treatment for the fetal lungs (**chapter 3**) and the gut (20) in an *in vivo* setting of intra-uterine inflammation. However, more research is required to investigate the use of IL-2 within this new indication of intra-uterine inflammation. Currently there are no adequate data available regarding the use of human recombinant IL-2 (Proleukin® [aldesleukin]) during pregnancy. No sufficient animal research was performed yet to assess the safety for the developing fetus, the course of gestation and the peri- and postnatal development (21, 22). Therefore, additional pre-clinical studies need to be conducted, to assess risks and benefits (23). Future pre-clinical studies will also reveal the effects of prophylactic IL-2 when chorioamnionitis is followed by a postnatal second hit (such

as mechanical ventilation or sepsis); and whether IL-2 can be used as a therapy *after* the induction of intra-uterine inflammation (instead of the prophylactic approach).

After sufficient and detailed information is known regarding animal fetal toxicity, safety and dosing of IL-2, pre-clinical studies can be followed by clinical studies. Clinical trials will be designed in compliance with Good Clinical Practice to test the fetal and maternal safety, the efficacy, the dosage and potential side-effects of IL-2 in humans (23).

This thesis also provides novel insights about the timing of exposure to intra-uterine inflammation. The data within this thesis clearly indicate that the timing of intra-uterine inflammation is crucial for the fetal pulmonary outcome. In **chapter 2** and in (24), it was shown that the order of exposure to intra-amniotic inflammation and antenatal corticosteroids influenced the pulmonary inflammatory response and the Wnt/ β -catenin signaling pathway. Furthermore, the fetal pulmonary inflammatory response was shown to be time-dependent and gestation-dependent. It is suggested that for the fetal pulmonary outcome multiple factors are crucial: the gestational age at the onset of exposure to intra-uterine inflammation, the gestational age at preterm birth and the duration of the exposure. This further emphasizes the need for the development of reliable biomarkers to detect the presence of intra-uterine inflammation during pregnancy.

Potential target groups & activities

Out of a scientific point of view, the data described within this thesis are of interest to scientists and researchers who are working in the field of intra-uterine inflammation, preterm birth or pediatric pulmonology. In addition to this scientific audience, the findings within this thesis will be of value to clinicians who are specialized in neonatal and perinatal care or come into contact with intra-uterine inflammation during their daily work. The clinical target group therefore consists –without limitation- of: neonatologists, neonatal nurses, pediatricians and (pediatric) pulmonologists. Although this thesis focused on the fetal outcomes, instead of the maternal effects, after exposure to intra-uterine inflammation, the described findings might also be of interest for clinicians who are also involved in maternal medicine, such as obstetricians and gynecologists.

An important non-scientific target group consists of patient associations or parent groups. Parent groups are crucial in raising awareness of the health problem and in improving the quality of care (4). In Europe, the European Foundation for the Care of Newborn Infants (EFCNI) is a network and organization which represents the interests

of preterm and ill neonatal infants and their families. It combines the power of all stakeholders, such as parents, policy makers, healthcare professionals and scientists (25). By doing so, it has successfully increased awareness, political attention and policy change regarding preterm birth across Europe (4). In addition, by promoting research, the EFCNI gathers and multiplies information to improve the quality of care for (ill) neonates (25). As this thesis focuses on the effects of antenatal inflammation on fetal lung development, it can be of interest to organizations such as the EFCNI to help raise awareness of this specific health problem within our society. Hopefully, raising awareness will eventually contribute to the development of new therapeutic strategies for this very vulnerable patient group.

Finally, the data within this thesis can be of interest to the pharmaceutical industry, as therapeutic interventions and strategies were discussed within this thesis. While the use of corticosteroids within the field of perinatology is not new, we are the first to present the potential of prophylactic IL-2 therapy for the lungs in a translational setting of intra-uterine inflammation (**chapter 3**). The pharmaceutical industry could be of help and support to further investigate the potential of IL-2 within the context of antenatal inflammation.

To reach these target groups and to make individuals within and outside of the scientific community aware of our findings, we published our research data in peer-reviewed scientific journals relevant to our field of research, such as *Pediatric Research* (**chapter 2**) and the *American Journal of Physiology Lung Cellular and Molecular Physiology* (**chapter 3**). Furthermore, the findings described within this thesis were presented to (inter)national audiences via oral and poster presentations at a conference, symposium, science day or research meeting.

To summarize, the data reported within this thesis are scientifically, socially and economically relevant. Therefore, the content of this thesis can be of interest to target groups within and outside of the scientific and clinical community. Although the results described within this thesis were obtained using a translational pre-clinical model, these data contribute to our knowledge regarding intra-uterine inflammation and its pulmonary effects; and may eventually lead to the set-up of novel clinical studies and the discovery of new medical advancements. Only innovations within the field of gynecology, neonatology and perinatology will in time lead to a reduction in the occurrence and severity of intra-uterine inflammation and the pulmonary complications associated with this indication. Reducing the prevalence and severity will also lower health care costs and the burden on the infant and its family, ultimately leading to a reduction in the economic and social impact of intra-uterine inflammation and its associated pulmonary effects.

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Curriculum Vitae

Monique Gerarda Mathieu Willems was born on January 5th 1989 in Sittard, the Netherlands. In 2007, after she graduated from secondary school at the Trevianum Gymnasium in Sittard, she started her bachelor study Biomedical Sciences at Maastricht University (Maastricht, The Netherlands). After she obtained her Bachelor's degree with distinction (cum laude) in 2010, she continued her education in Maastricht and enrolled into the Master program of Molecular Life Sciences, with a specialization in Oncology & Developmental Biology. During her Master education and the associated internship at the Department of Orthopedic Surgery, her enthusiasm for developmental biology increased even more. This interest led to a senior internship at the Department of Pediatrics at Maastricht University. After completion of her master thesis on the effects of intra-uterine inflammation on fetal lung development, she graduated in July 2012 for her Master's degree with distinction (cum laude).

In September 2012, she continued with her research at the Department of Pediatrics as a Junior Research Fellow. In February 2013, she became a PhD Student at the Department of Pediatrics and the Department of Biomedical Engineering within the GROW School for Oncology and Developmental Biology and under the supervision of Prof. Dr. B.W.W. Kramer, Prof. Dr. T. Delhaas and Dr. T.G.A.M. Wolfs. During her PhD, her research focused on the fetal pulmonary outcome after intra-uterine inflammation and how therapeutic interventions may beneficially affect this outcome. Main activities included to organize, execute and publish several research projects and to mentor and teach multiple Bachelor and Master students during their internships or tutor classes. Several conferences and symposia were attended, during which she presented her research via a poster or oral presentation. The findings of her doctoral research are described within this thesis.

As of March 2016, she is working as a Project Coordinator on medical device clinical trials at Medpace Medical Device B.V. in Vaals, The Netherlands.

Publications

1. Pulmonary vascular changes in extremely preterm sheep after intra-amniotic exposure to *Ureaplasma parvum* and lipopolysaccharide
Willems MG, Kemp MW, Fast LA, Wagemaker NM, Janssen LE, Newnham JP, Payne MS, Spiller OB, Kallapur SG, Jobe AH, Delhaas T, Kramer BW, Wolfs TG.
(In revision)
2. In vivo evaluation of the acute pulmonary response to poractant alfa and bovactant treatment in lung-lavaged adult rabbit and preterm lamb respiratory distress syndrome models.
Ricci F, Salomone F, Kuypers E, Ophelders DR, Nikiforou M, **Willems MG**, Hütten MC, Kramer BW, Bianco F.
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