Preterm birth after antenatal inflammation as underlying cause of bronchial hyperreactivity

Preterm birth after antenatal inflammation as underlying cause of bronchial hyperreactivity

Verena Anna Carolus Lambermont

The research presented in this thesis was conducted at the department Pediatrics of Maastricht University.

Financial support for the publication of this thesis was provided by: Stichting Kindergeneeskunde, Chiesi Pharmaceuticals B.V., Nutricia baby- en kindervoeding, AbbVie B.V. and GlaxoSmithKline B.V.

Cover Design: Frank Lambermont and Verena Lambermont. Picture was taken during a flight to a congress in New Orleans.

Printed by: Proefschriftmaken.nl | | BOXPress

Published by: Uitgeverij BOXPress, 's-Hertogenbosch

ISBN: 978-90-8891-991-6
NUR: 881

Preterm birth after antenatal inflammation as underlying cause of bronchial hyperreactivity

PROEFSCHRIFT

Ter verkrijging van de graad van doctor,
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. dr. L.L.G. Soete,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op dinsdag 9 december 2014 om 16:00 uur

door

Verena Anna Carolus Lambermont

Geboren op 16 oktober 1984
te Heerlen

Promotors

Prof. dr. Boris W.W. Kramer

Prof. dr. Luc J.I. Zimmermann

Co-promotor

Privatdozent dr. Christian Martin *(Institute for pharmacology, University of Aachen)*

Assessment Committee

Prof. dr. Edward D. Dompeling *(chairman)*

Prof. dr. Sidarto Bambang Oetomo *(Eindhoven University of Technology)*

Prof. dr. Irwin K.M. Reiss *(Erasmus Medical Centre Rotterdam)*

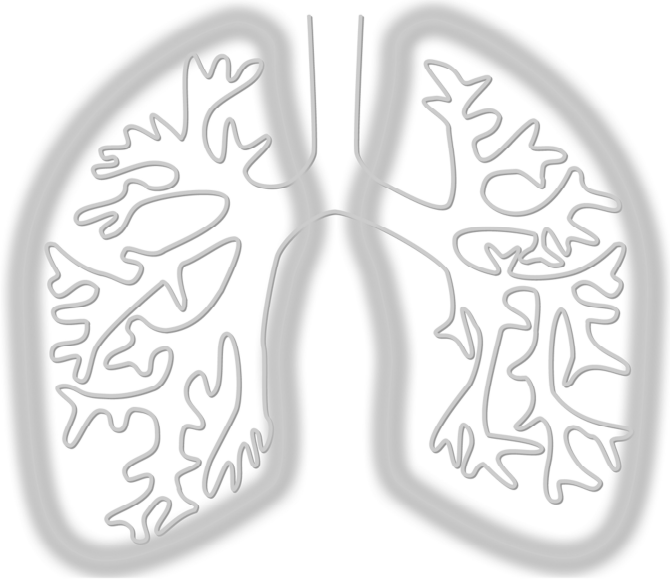
Prof. dr. Emiel F.M. Wouters

Prof. dr. Servaas A. Morré

Contents

CHAPTER 1

General introduction and outline of this thesis



Preterm birth

Preterm birth is defined as childbirth occurring before 37 completed weeks of gestation (1). It is estimated that preterm births occur in 5-13% of all pregnancies in the Western world with variations from country to country (2-4). The current rate of preterm birth in the Netherlands is for example 7.7% with 1% being born before the 32nd week of gestation (5, 6). Preterm birth is a significant public health burden, as it is associated with high risk of infant mortality, various morbidities in both the neonatal period and later in life, and has a significant societal economic impact (7-11). For example, preterm birth still account for up to 75% of all perinatal mortality and morbidity in the Western world, despite the advances in medical care (6).

Causes of preterm birth can be divided into two main categories: indicated preterm birth and spontaneous preterm birth. The first category consists mostly of infants delivered by caesarean section based on medical indications of maternal or fetal health (12). These include severe intrauterine growth retardation (IUGR), maternal pre-eclampsia and HELLP syndrome (haemolysis, elevated liver enzymes, low platelets) (13). The second category consists of preterm labor with intact membranes or with premature preterm rupture of membranes (PPROM) (12,14). This category is often associated with intrauterine infection, as bacterial infection of the intrauterine environment is present in many cases of preterm birth. The incidence increases with lower gestational age and reaches an incidence of 70% at 24 weeks of gestation (15-17).

Chorioamnionitis

Intrauterine infection often does not affect the mother and therefore remain clinically silent. Consistently, the duration of fetal exposure to an inflammatory stimulus is seldom known. Intrauterine infection may be diagnosed by histological examination of the placenta after birth (18,19). If both the chorionic and amniotic membranes are inflamed, this is referred to as chorioamnionitis. Chorioamnionitis exposes the fetus to proinflammatory mediators primarily by fetal breathing and swallowing of amniotic fluid, affecting the fetus in a multi-organ way (Figure 1). The exposure to chorioamnionitis is until now primarily attributed with alterations in lung development. Besides having effects on the lung, it is associated with increased risk for necrotizing enterocolitis (NEC), retinopathy, cerebral palsy (CP) and white matter damage (WMD) (20-22).

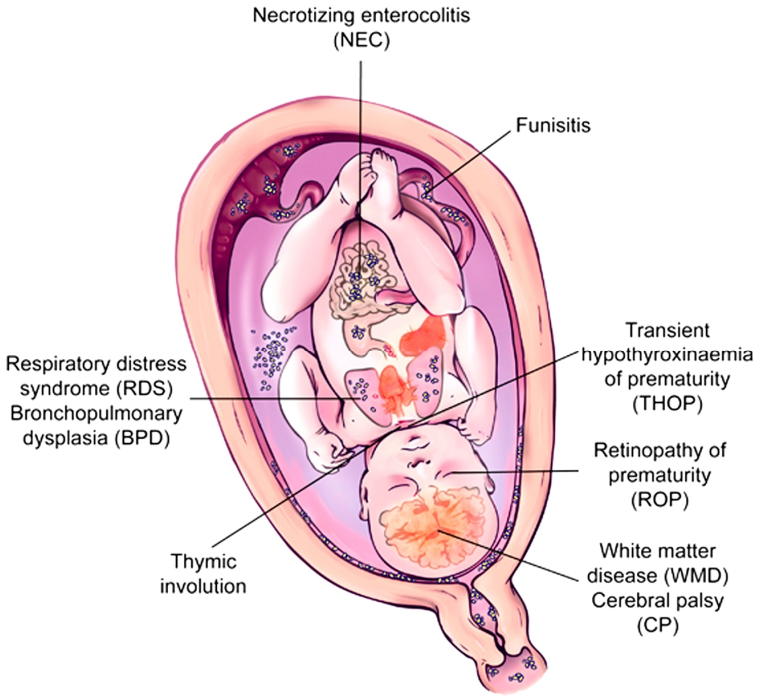


Figure 1: Organs known to be affected in preterm infants after prenatal exposure to inflammation (23).

The major factor limiting viability in extremely preterm infants is the development of the lung, because this is one of the last organs to mature in the womb (24). Extremely preterm birth is associated with an increase in the incidence of chronic lung disease of prematurity (bronchopulmonary dysplasia; BPD) with important long-term consequences on airway and lung function (25). In addition, gender differences for short and long-term outcomes after preterm birth are well recognized in the perinatal field. The lung development of female fetuses is for example faster than in male fetuses and is associated with an increased surfactant production (26-29). Male gender has almost invariably been associated with poor outcomes, including increased risk of developing respiratory distress syndrome (30,31), neurodevelopmental delay (32), abnormal cardiovascular development (33-35) and neonatal mortality as such (30,36). A gender difference in susceptibility for chorioamnionitis-associated adverse outcome may explain part of the discrepancy between male and female outcomes after preterm birth. However, there is still a lot of speculation about the underlying mechanisms for the female advantage in survival after preterm birth, since only a few studies have focused on gender differences as a major outcome and topic.

Lung development

Immaturity of the lung is the major factor limiting viability in extremely preterm infants. The threshold of viability is determined by gestational age since the preterm lung cannot facilitate proper gas exchange necessary for survival in the extrauterine environment. Gas exchange is regulated *in utero* by the placenta, from where oxygen is transported to the fetus through the umbilical cord. The lungs are not necessary for life inside the uterus and the fetal circulation partially bypasses the lung through the ductus arteriosus, which connects the pulmonary artery to the aorta. Although fetal breathing movements are present from the second trimester onwards, their function is to stimulate lung development but they do not affect or regulate gas exchange (37). In postnatal life, the lungs fulfill a crucial function in the development of other organs, as they supply the oxygen which the newborn needs to grow and develop into a healthy adult. The lung needs around 140 m^2 in the adult lungs for gas exchange to supply enough oxygen to the circulation. To achieve such a large gas exchange area, the lung is made up of branched airways in a tree-like structure.

Human lung morphogenesis starts around the 4^{th} week after conception and can be divided into various stages based on histological appearance (Figure 2): embryonic stage (4-9 weeks), pseudoglandular phase (7-17 weeks), canalicular phase (16-27 weeks), saccular phase (24-36 weeks), and alveolar phase (36 weeks-postnatal). These phases overlap somewhat, as the rostral side of the lungs develops slightly faster than the caudal side. Through successive stages of branching, the terminal bronchioles are formed in the pseudoglandular phase (38). Simultaneously, the pulmonary vasculature is formed through progressive branching from the pulmonary artery (39). The terminal bronchioles sprout several canaculi during the canalicular phase, which grow to become respiratory bronchiole and alveolar ducts. These structures will form the functional respiratory part of the lungs. During the last phase of lung development *in utero*, which is called the alveolar phase, functional alveoli are formed through a process of secondary septation which subsequently increases the surface area needed for optimal gas exchange (40).

This process is orchestrated by interactions between myofibroblasts, extracellular matrix proteins, the vascular endothelial cells and the airway epithelial cells. Pulmonary myofibroblasts produce focal deposits of elastin, which functions as a structural navigator for the formation of the growing alveolus (40). Mature alveoli are characterized by a dense structural organization and an extremely thin blood-air barrier, facilitating highly efficient gas-exchange (39). In addition, a low alveolar surface tension is essential to prevent alveolar collapse during expiration, which is accomplished by surfactant secretion through the alveolar type II cells.

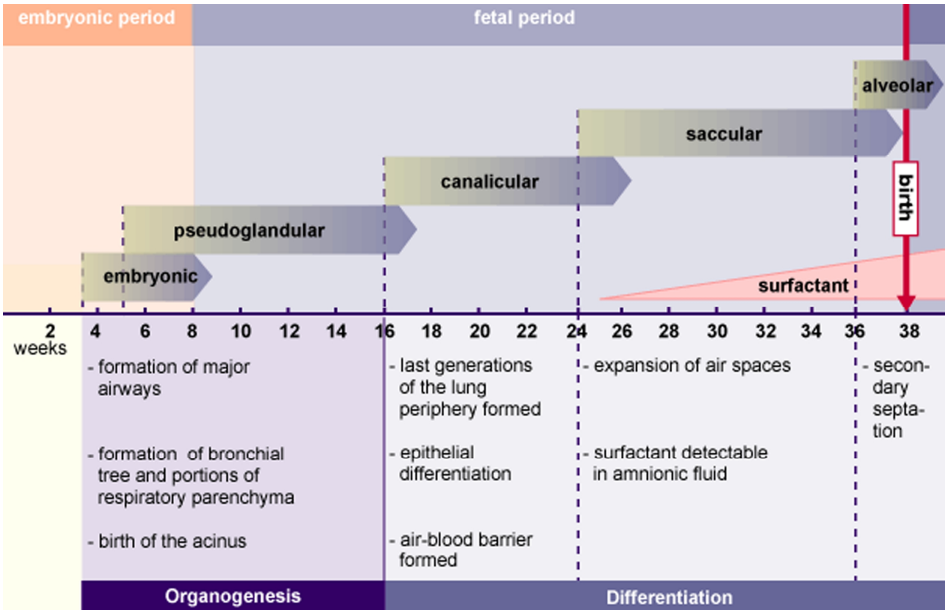


Figure 2: Stages of prenatal lung development (38).

Innate immune system

Fetal responses to infection must rely on innate immune signaling. Innate immune responses against microorganisms may have a significant impact on the success of pregnancy, as intrauterine infections have been shown to be strongly associated with certain complications of pregnancy. The innate immune system is the immunological first line of defense that provides an immediate response against invading pathogens (41,42). Activation of innate immunity is a critical step to the development of antigen-specific acquired immunity. Growing evidence shows that the innate immune system is activated at the maternal-fetal interface. For instance, innate immune cells such as natural killer (NK) cells, macrophages, and dendritic cells are known to infiltrate the decidua and accumulate around the invading trophoblasts (42-46). In addition, Toll-like receptor (TLR) 2 and TLR4 receptor levels in the chorioamnion are low and increase with preterm or term labor. Intra-amniotic lipopolysaccharide (LPS), which induces chorioamnionitis and lung injury, signals via TLR4 to initiate the innate immune response. That is the essential initial step for mobilizing inflammatory defenses and subsequently acquired immunity. The fetus is capable of sophisticated immune responses despite an immature immune system and the immune suppressive state of pregnancy. From animal studies it is known that the fetus responded to intra-amniotic LPS with a maximal inflammation at about 2 days and a partial resolution of inflammation by 7 days (47). Furthermore, the response to

chorioamnionitis induced a maturation of leukocyte cell lineages to alveolar macrophages that have increased inflammatory response potentials (48), but also cross-tolerance to different TLR-agonists (49). Thus, the naive fetal immune system is capable of complex modulations. However, the knowledge of the characteristics of these immune responses is limited and need more investigation, since inflammatory processes can affect organ function such as in asthma.

Bronchial hyperreactivity and childhood wheezing after preterm birth

In the treatment of preterm newborns, important advances have been made over the last decades. These include antenatal steroid administration and exogenous surfactant treatment, both of which have greatly improved survival and outcome after preterm birth (50,51). However, there are still major adverse outcomes associated with chorioamnionitis-induced preterm birth. Children who had been born preterm and developed BPD are frequently admitted to hospitals with respiratory infections (52,53). As these children grow older, they usually have poor lung function and more frequently develop a wheezing phenotype or asthma, compared to age-matched children born at term (52,54). Recently, an additional risk factor for asthma in preterm babies was identified. Chorioamnionitis is correlated with a 4.4-fold increased risk for developing physician-diagnosed asthma in very preterm infants (55).

Asthma is the most common chronic disease affecting children, causing constriction of the airways and air trapping. The most common triggers of asthma include cold air, exercise, allergens and some types of viral infections. Contact with these triggers causes overreactivity of the airways and leads to inflammation. This results in constriction of the airway muscles and swelling of bronchioles (bronchial hyperrreactivity); breathing passages are narrowed and breathing becomes very difficult. The outcome is a wheezing phenotype or childhood asthma which limits children's daily activities and affects their social activities. The link with preterm birth is of significant public health relevance because of the increasing incidence of both entities (56,57). Wheezing is common in young infants and toddlers with 27% of all children having at least one wheezing episode by the age of 9 years (58). For some of these children these asthmatic symptoms seem to remit with time, but many children develop asthmatic symptoms which persist throughout their life and are associated with more severe symptoms ending in the loss of lung function. About 15% of the wheezing infants develop persistent wheezing and clinical asthma later in life (59).

Specific risk factors during pregnancy can influence the risk of wheezing in early childhood. Maternal smoking, low maternal age, early bottle feeding, low birth weight and

prematurity are associated with lower respiratory tract illness in the first 2 years of life (60-68). Prematurity and low birth weight are considered significant risk factors for reduced lung function at school age and the development of childhood asthma (62,69,70). The underlying mechanisms by which adverse birth outcomes predispose to wheezing is not known. It seems to be in contrast to the "hygiene hypothesis". This hypothesis associates the exposure of young children to rural environments with high endotoxin levels and a subsequent decreased incidence of childhood asthma (71-74). The relationship between antenatal inflammatory exposures and postnatal abnormalities seems to be confounded by the fetal response to inflammation, which is difficult to study in patients and animal models. Therefore, hardly anything is known about the persistence of fetal immune modulations following a pro-inflammatory stimulus and how the duration of exposure to antenatal inflammation may affect the fetal response as gestation advances till near-term and the first years after birth. Knowledge of these processes is necessary for a better understanding of the development of childhood wheezing after preterm birth, and for the development of prevention and treatment options for childhood wheezing and asthma.

Sheep as a translational animal model of chorioamnionitis

In the last years translational research with various animal models has been helpful to answer some basic questions about the effect of various fetal exposures on different organs. However, the various animal models differ in their developmental biology compared to humans (75).

Development of the ovine lung is highly similar to that of humans (Figure 3). Sheep undergo the alveolar phase of lung development *in utero* and these animals are large enough to induce an intrauterine inflammation for example by LPS (76). The adolescence of sheep usually occurs at approximately 11 months of age and sheep with an age of 7 weeks are comparable to 4-5 years of age in humans.

Adult sheep have been used as a model of sensitization and airway reactivity frequently. Sheep have, in contrast to mice, a bronchial circulation, bronchial glands and mast cells that respond to stimuli similarly to the human lung with both early and late phase responses (77-79). In addition, sheep can be sensitized to house dust mite antigen which is a common human antigen in asthma. The sensitized sheep have allergen−specific IgE responses and acute eosinophil responses to allergen challenge (79). Recently, airway remodelling (increased airway collagen and smooth muscle with hyperplasia of goblet cells) following repeated airway exposures of sheep to house dust mite (HDM) was reported (80). Sheep respond to HDM sensitization and airway challenges with a complete

allergic/asthma response similar to that which occurs in humans. Therefore, preterm sheep are considered as an excellent translational model to study the effects of antenatal events on the developing fetus till adolescence in relation to the development of asthma.

Outline of this thesis

The main focus of this thesis was to give insight into how the lung development and airway function were influenced by antenatal inflammation as gestation advances and during the first period after birth. We used an animal model with highly similar lung development *in utero* (Figure 3) and allergy response as in human fetal lungs.

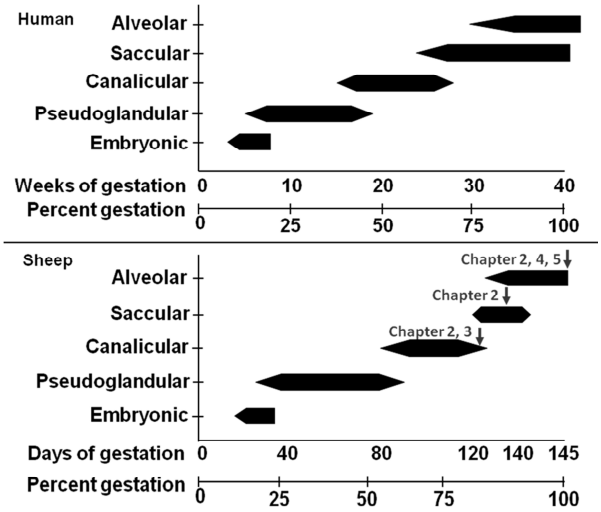


Figure 3: Human versus sheep lung development (76).

In **chapter 2** we studied how the lung was affected by inflammation as gestation advances. Fetal lambs were exposed to one or two doses of LPS into the amniotic fluid and the animals were delivered at three different gestational ages, to confirm that intra-amniotic LPS exposure would cause persistent fetal pulmonary responses as the lungs develop *in utero*.

In **chapter 3** we assessed how the lung maturational responses to inflammation *in utero* differ between male and female fetuses.

To study airway reactivity in sheep, a research model had to be developed to visualize and quantify airway contractility. In **chapter 4** a model of sheep precision-cut lung slices (PCLS) was established for the measurement of airway responses to early allergic response mediators and allergens in newborn and adult animals.

In **chapter 5** we assessed how the fetal innate immune responses to LPS-induced chorioamnionitis would alter postnatal systemic immune responsiveness and airway reactivity at the age of 7 weeks.

To conclude, **Chapter 6** provides a discussion of the presented research results, clinical perspectives and a recommendation for future research towards the understanding how the developing lung responds to inflammation and how this effects the development of bronchial hyperreactivity and asthma later in life.

References

1. Internal classification of diseases and related health problems. World Health Organization: Geneva 1992.
2. Bulletins--Obstetrics ACoP. ACOG practice bulletin. Management of preterm labor. Number 43, May 2003. Int J Gynaecol Obstet 2003;82:127-135.
3. American College of O, Gynecologists. ACOG Practice Bulletin. Assessment of risk factors for preterm birth. Clinical management guidelines for obstetrician-gynecologists. Number 31, October 2001. (Replaces Technical Bulletin number 206, June 1995; Committee Opinion number 172, May 1996; Committee Opinion number 187, September 1997; Committee Opinion number 198, February 1998; and Committee Opinion number 251, January 2001). Obstet Gynecol 2001;98:709-716.
4. Pawelec M, Palczynski B, Krzemieniewska J, et al. Initiation of preterm labor. Adv Clin Exp Med 2013;22:283-288.
5. Tamminga P, Rijninks-van Driel GC, Mohangoo AD, et al. Neonatale uitkomsten. NTOG 2009;122:83-87.
6. Stichting PRN. Stichting Perinatale Registratie Nederland. Perinatale zorg in Nederland 2008. Stichting PRN, Utrecht 2011.
7. Cole FS, Alleyne C, Barks JD, et al. NIH Consensus Development Conference statement: inhaled nitric-oxide therapy for premature infants. Pediatrics 2011;127:363-369.
8. Guellec I, Lapillonne A, Renolleau S, et al. Neurologic outcomes at school age in very preterm infants born with severe or mild growth restriction. Pediatrics 2011;127:e883-891.
9. Kochanek KD, Kirmeyer SE, Martin JA, et al. Annual summary of vital statistics: 2009. Pediatrics 2012;129:338-348.
10. Behrman RE, Butler AS. Preterm birth: causes, consequences, and prevention. National Academies Press, Washington, DC 2007.
11. Ferguson KK, O'Neill MS, Meeker JD. Environmental contaminant exposures and preterm birth: a comprehensive review. J Toxicol Environ Health B Crit Rev 2013;16:69-113.
12. Goldenberg RL, Culhane JF, Iams JD, et al. Epidemiology and causes of preterm birth. Lancet 2008;371:75-84.
13. Irminger-Finger I, Jastrow N, Irion O. Preeclampsia: a danger growing in disguise. Int J Biochem Cell Biol 2008;40:1979-1983.
14. Menon R. Spontaneous preterm birth, a clinical dilemma: etiologic, pathophysiologic and genetic heterogeneities and racial disparity. Acta Obstet Gynecol Scand 2008;87:590-600.
15. Lahra MM, Beeby PJ, Jeffery HE. Intrauterine inflammation, neonatal sepsis, and chronic lung disease: a 13-year hospital cohort study. Pediatrics 2009;123:1314-1319.
16. Lahra MM, Jeffery HE. A fetal response to chorioamnionitis is associated with early survival after preterm birth. Am J Obstet Gynecol 2004;190:147-151.

17. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med 2000;342:1500-1507.
18. Faye-Petersen OM. The placenta in preterm birth. J Clin Pathol 2008;61:1261-1275.
19. Redline RW. Inflammatory responses in the placenta and umbilical cord. Semin Fetal Neonatal Med 2006;11:296-301.
20. Robertson CM, Watt MJ, Dinu IA. Outcomes for the extremely premature infant: what is new? And where are we going? Pediatr Neurol 2009;40:189-196.
21. Moster D, Lie RT, Markestad T. Long-term medical and social consequences of preterm birth. N Engl J Med 2008;359:262-273.
22. Been JV, Lievense S, Zimmermann LJ, et al. Chorioamnionitis as a risk factor for necrotizing enterocolitis: a systematic review and meta-analysis. J Pediatr 2013;162:236-242 e232.
23. Gantert M, Been JV, Gavilanes AW, et al. Chorioamnionitis: a multiorgan disease of the fetus? J Perinatol 2010;30 Suppl:S21-30.
24. Rodriguez RJ. Management of respiratory distress syndrome: an update. Respir Care 2003;48:279-286; discussion 286-277.
25. Greenough A. Long-term pulmonary outcome in the preterm infant. Neonatology 2008;93:324-327.
26. Torday JS, Nielsen HC, Fencl Mde M, et al. Sex differences in fetal lung maturation. Am Rev Respir Dis 1981;123:205-208.
27. Torday JS, Nielsen HC. The sex difference in fetal lung surfactant production. Exp Lung Res 1987;12:1-19.
28. Fleisher B, Kulovich MV, Hallman M, et al. Lung profile: sex differences in normal pregnancy. Obstet Gynecol 1985;66:327-330.
29. Carey MA, Card JW, Voltz JW, et al. It's all about sex: gender, lung development and lung disease. Trends Endocrinol Metab 2007;18:308-313.
30. Khoury MJ, Marks JS, McCarthy BJ, et al. Factors affecting the sex differential in neonatal mortality: the role of respiratory distress syndrome. Am J Obstet Gynecol 1985;151:777-782.
31. Perelman RH, Palta M, Kirby R, et al. Discordance between male and female deaths due to the respiratory distress syndrome. Pediatrics 1986;78:238-244.
32. Teune MJ, van Wassenaer AG, van Dommelen P, et al. Perinatal risk indicators for long-term neurological morbidity among preterm neonates. Am J Obstet Gynecol 2011;204:396 e391-396 e314.
33. Sheiner E, Levy A, Katz M, et al. Gender does matter in perinatal medicine. Fetal Diagn Ther 2004;19:366-369.
34. Sheiner E, Hadar A, Hallak M, et al. Clinical significance of fetal heart rate tracings during the second stage of labor. Obstet Gynecol 2001;97:747-752.
35. Dawes NW, Dawes GS, Moulden M, et al. Fetal heart rate patterns in term labor vary with sex, gestational age, epidural analgesia, and fetal weight. Am J Obstet Gynecol 1999;180:181-187.
36. Binet ME, Bujold E, Lefebvre F, et al. Role of gender in morbidity and mortality of extremely premature neonates. Am J Perinatol 2012;29:159-166.
37. Harding R, Hooper SBl. Regulation of lung expansion and lung growth before birth. J Appl Physiol (1985) 1996;81:209-224.
38. Schönli-Affolter F, Dubuis-Grieder C, Strauch E. Module 18 Respiration tract. Human Embryology - Organogenesis. Embryology.ch.
39. Galambos C, Demello DE. Regulation of alveologenesis: clinical implications of impaired growth. Pathology 2008;40:124-140.
40. Burri PH. Structural aspects of postnatal lung development - alveolar formation and growth. Biol Neonate 2006;89:313-322.

41. Medzhitov R, Janeway CA, Jr. Decoding the patterns of self and nonself by the innate immune system. Science 2002;296:298-300.
42. Koga K, Izumi G, Mor G, et al. Toll-like Receptors at the Maternal-Fetal Interface in Normal Pregnancy and Pregnancy Complications. Am J Reprod Immunol 2014.
43. Bulmer JN, Morrison L, Longfellow M, et al. Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies. Hum Reprod 1991;6:791-798.
44. Bulmer JN, Pace D, Ritson A. Immunoregulatory cells in human decidua: morphology, immunohistochemistry and function. Reprod Nutr Dev 1988;28:1599-1613.
45. Kammerer U, Schoppet M, McLellan AD, et al. Human decidua contains potent immunostimulatory CD83(+) dendritic cells. Am J Pathol 2000;157:159-169.
46. Kammerer U, Eggert AO, Kapp M, et al. Unique appearance of proliferating antigen-presenting cells expressing DC-SIGN (CD209) in the decidua of early human pregnancy. Am J Pathol 2003;162:887-896.
47. Kallapur SG, Willet KE, Jobe AH, et al. Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. Am J Physiol Lung Cell Mol Physiol 2001;280:L527-536.
48. Kramer BW, Joshi SN, Moss TJ, et al. Endotoxin-induced maturation of monocytes in preterm fetal sheep lung. Am J Physiol Lung Cell Mol Physiol 2007;293:L345-353.
49. Kramer BW, Kallapur SG, Moss TJ, et al. Intra-amniotic LPS modulation of TLR signaling in lung and blood monocytes of fetal sheep. Innate Immun 2009;15:101-107.
50. Bonanno C, Wapner RJ. Antenatal corticosteroid treatment: what's happened since Drs Liggins and Howie? Am J Obstet Gynecol 2009;200:448-457.
51. Been JV, Zimmermann LJ. What's new in surfactant? A clinical view on recent developments in neonatology and paediatrics. Eur J Pediatr 2007;166:889-899.
52. Greenough A. Long term respiratory outcomes of very premature birth (<32 weeks). Semin Fetal Neonatal Med 2012;17:73-76.
53. Doyle LW, Ford G, Davis N. Health and hospitalistions after discharge in extremely low birth weight infants. Semin Neonatol 2003;8:137-145.
54. Fawke J, Lum S, Kirkby J, et al. Lung function and respiratory symptoms at 11 years in children born extremely preterm: the EPICure study. Am J Respir Crit Care Med 2010;182:237-245.
55. Kumar R, Yu Y, Story RE, et al. Prematurity, chorioamnionitis, and the development of recurrent wheezing: a prospective birth cohort study. J Allergy Clin Immunol 2008;121:878-884 e876.
56. Blencowe H, Cousens S, Oestergaard MZ, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet 1990;379:2162-2172.
57. Anandan C, Nurmatov U, van Schayck OC, et al. Is the prevalence of asthma declining? Systematic review of epidemiological studies. Allergy 2010;65:152-167.
58. Robison RG, Singh AM. Chapter 11: the infant and toddler with wheezing. Allergy Asthma Proc 2012;33 Suppl 1:S36-38.
59. Guilbert T, Krawiec M. Natural history of asthma. Pediatr Clin North Am 2003;50:523-538.
60. Speer CP, Silverman M. Issues relating to children born prematurely. Eur Respir J Suppl 1998;27:13s-16s.
61. Wjst M, Popescu M, Trepka MJ, et al. Pulmonary function in children with initial low birth weight. Pediatr Allergy Immunol 1998;9:80-90.
62. Patelarou E, Chochlidaki M, Vivilaki V, et al. Is there a link between wheezing in early childhood and adverse birth outcomes? A systematic review. Int J Environ Res Public Health 2009;6:2752-2761.
63. Schwartz J, Gold D, Dockery DW, et al. Predictors of asthma and persistent wheeze in a national sample of children in the United States. Association with social class, perinatal events, and race. Am Rev Respir Dis 1990;142:555-562.

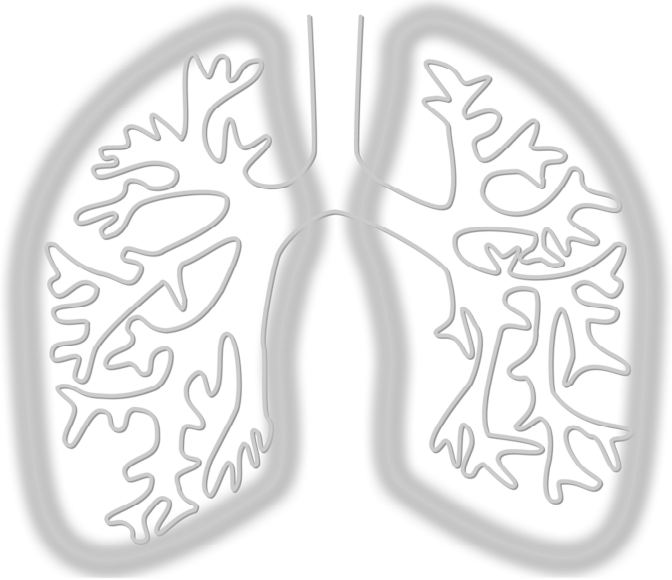
64. Martinez FD, Cline M, Burrows B. Increased incidence of asthma in children of smoking mothers. Pediatrics 1992;89:21-26.
65. Pattenden S, Antova T, Neuberger M, et al. Parental smoking and children's respiratory health: independent effects of prenatal and postnatal exposure. Tob Control 2006;15:294-301.
66. Elder DE, Hagan R, Evans SF, et al. Recurrent wheezing in very preterm infants. Arch Dis Child Fetal Neonatal Ed 1996;74:F165-171.
67. Rona RJ, Gulliford MC, Chinn S. Effects of prematurity and intrauterine growth on respiratory health and lung function in childhood. BMJ 1993;306:817-820.
68. von Mutius E, Nicolai T, Martinez FD. Prematurity as a risk factor for asthma in preadolescent children. J Pediatr 1993;123:223-229.
69. Parat S, Moriette G, Delaperche MF, et al. Long-term pulmonary functional outcome of bronchopulmonary dysplasia and premature birth. Pediatr Pulmonol 1995;20:289-296.
70. Giacoia GP, Venkataraman PS, West-Wilson KI, et al. Follow-up of school-age children with bronchopulmonary dysplasia. J Pediatr 1997;130:400-408.
71. Riedler J, Braun-Fahrlander C, Eder W, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 2001;358:1129-1133.
72. Riedler J, Eder W, Oberfeld G, et al. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. Clin Exp Allergy 2000;30:194-200.
73. Von Ehrenstein OS, Von Mutius E, Illi S, et al. Reduced risk of hay fever and asthma among children of farmers. Clin Exp Allergy 2000;30:187-193.
74. Braun-Fahrlander C, Gassner M, Grize L, et al. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. Clin Exp Allergy 1999;29:28-34.
75. Pringle KC. Human fetal lung development and related animal models. Clin Obstet Gynecol 1986;29:502-513.
76. Kramer BW. Chorioamnionitis - new ideas from experimental models. Neonatology 2011;99:320-325.
77. Bosse J, Boileau R, Begin R. Chronic allergic airway disease in the sheep model: functional and lung-lavage features. J Allergy Clin Immunol 1987;79:339-344.
78. Abraham WM, Delehunt JC, Yerger L, et al. Characterization of a late phase pulmonary response after antigen challenge in allergic sheep. Am Rev Respir Dis 1983;128:839-844.
79. Bischof RJ, Snibson K, Shaw R, et al. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. Clin Exp Allergy 2003;33:367-375.
80. Snibson KJ, Bischof RJ, Slocombe RF, et al. Airway remodelling and inflammation in sheep lungs after chronic airway challenge with house dust mite. Clin Exp Allergy 2005;35:146-152.

CHAPTER 2

Effects of intra-amniotic lipopolysaccharide exposure on the fetal lung as gestation advances

Verena A. Lambermont, Elke Kuypers, Jennifer J. Collins, Jane J. Pillow, John P. Newnham, Graeme R. Polglase, Ilias Nitsos, Matthew W. Kemp, Alan H. Jobe, Suhas G. Kallapur, Boris W. Kramer

Pediatr Res. 2014 Apr;75(4):500-6



Abstract

Background: Intra-amniotic lipopolysaccharide (LPS) exposure may affect neonatal outcome by altering fetal lung and immune system development. We hypothesized that intra-amniotic LPS exposure would cause persistent fetal pulmonary responses as the lungs develop *in utero*.

Methods: Fetal lambs were exposed to intra-amniotic LPS at 118 or at 118 and 123 days (d) of gestational age (GA) with delivery at 125, 133 or 140d (term = 147d). Immune responses, PU.1 expression, *Toll-like receptor (TLR)1,2,4,6* mRNA levels, mast cell levels, and pulmonary elastin deposition were evaluated.

Results: After a single dose of LPS, pulmonary inflammatory responses were observed with increases of (i) PU.1, *TLR1* at 125d GA and (ii) monocytes, lymphocytes, *TLR2* and *TLR6* at 133d GA. Repetitive LPS exposure resulted in (i) increases of neutrophils, monocytes, PU.1 and *TLR1* at 125d GA; (ii) increases of neutrophils, PU.1 and *TLR2* at 133d GA; and (iii) decreases of mast cells, elastin foci, *TLR4*, and *TLR6* at early gestation. At 140d GA, only PU.1 was increased after repetitive LPS exposure.

Conclusion: The preterm fetal lung can respond to a single exposure or repeated exposures from intra-amniotic LPS in multiple ways, but the absence of inflammatory and structural changes in LPS-exposed fetuses delivered near term suggest that the fetus can resolve an inflammatory stimulus *in utero* with time.

Introduction

Chorioamnionitis, an intrauterine inflammatory response of the chorioamniotic membranes to microorganisms, is a common antenatal exposure for very preterm infants that affects up to 70% of preterm deliveries before 30-weeks gestation (1). In chorioamnionitis, the fetus is exposed to inflammation through direct contact with amniotic fluid or via the placental-fetal circulation (2,3). Often, chorioamnionitis is identified only by histological examination of the placental and fetal membranes after birth. Consequently, the duration of fetal exposure to an inflammatory stimulus is seldom known.

Chorioamnionitis affects neonatal outcomes in an organ-dependent way (3), but is primarily associated with alterations in lung development (4). Chorioamnionitis is associated with a decrease in the incidence of respiratory distress syndrome (5), probably due to induced structural and functional maturity of the lung (6). Conversely, chorioamnionitis is associated with an increase in bronchopulmonary dysplasia (BPD) (4,7,8) due to inflammatory disruption of lung alveolar and vascular development (9,10). Chorioamnionitis may also negatively affect neonatal outcomes by modulating the fetal immune system (3,11,12). The fetal lung, gut and skin are directly exposed to the inflammation associated with chorioamnionitis, although other organs and the immune system, which are not in direct contact with the amniotic fluid, can also be affected adversely (3).

Neutrophils, monocytes and lymphocytes are increased in the alveolar lavages of preterm lambs after a single dose of lipopolysaccharide (LPS) (13). Additionally, intra-amniotic LPS exposure induces [i] maturation of monocytes to alveolar macrophages with increased inflammatory response potentials by inducing hematopoietic transcription factor PU.1 (14) and [ii] cross-tolerance to different Toll-like receptor (TLR) agonists (15). Thus, the naive fetal immune system is capable of complex modulations that can alter subsequent immune function (13). However, our knowledge of the characteristics of these immune responses is limited to short-term exposure to proinflammatory agonists. The persistence of fetal immune modulations following a proinflammatory stimulus and how the duration of exposure to antenatal inflammation may affect the fetal response as gestation advances till near term is poorly understood (13,16). Whether such changes in the immune system persist after a proinflammatory stimulus may enhance understanding of whether preterm/term infants are more susceptible to infections, sepsis and/or pneumonia later in life (17).

We used an ovine model of chorioamnionitis induced by intra-amniotic LPS to evaluate how exposure interval changed the lung as the fetus matured. Our hypothesis was that

intra-amniotic LPS exposures would cause persistent fetal pulmonary responses as the lungs develop *in utero.*

Materials and methods

Antenatal treatment

All animal procedures were approved by the Animal Ethics Committee of the University of Western Australia. Pregnant Merino ewes received an intra-amniotic (IA) injection with saline (sal, 2 mL; controls) or 10 mg LPS (*Escherichia coli* 055:B5; Sigma-Aldrich, St. Louis, MO) at 118 and 123 days (d) gestational age (GA) (Figure 1). The fetuses were delivered operatively at 125d (n=21), 133d (n=19) or 140d (n=20) gestation (term=150d). Each lamb was killed at delivery with an intravenous injection of pentobarbital (100 mg/kg, Valabarb, Jurox, Rutherford, NSW, Australia).

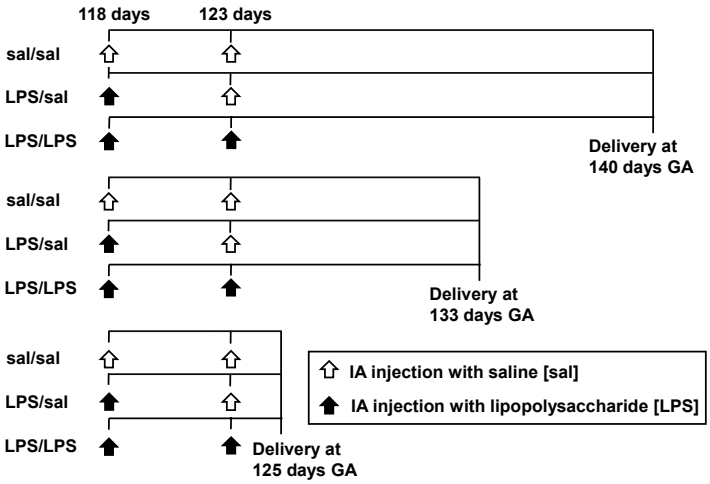


Figure 1: Animal model of intra-amniotic LPS exposure. All animals were exposed to saline (sal) or LPS at 118 and 123 d GA, resulting in three treatment groups: sal/sal (control), LPS/sal and LPS/LPS. Animals were delivered at three different GA's: 125, 133 and 140d. GA, gestational age; LPS, lipopolysaccharide.

Processing of the fetal lung

Lung compliance was assessed by measuring lung gas volumes from pressure-volume curves as a measure of lung maturation. The thorax was opened with a midline incision, and a tracheal tube was inserted and connected to a manometer (18). The maximal volume measured at a pressure of 40 cmH_2O was recorded as a measure of lung compliance. To collect bronchoalveolar lavage fluid (BALF), the left lung was lavaged three times with 0.9% NaCl (sal). The BALF samples were pooled and centrifuged at 500 rpm for 5 min. Differential cell counts were obtained on cytospin preparations after a Pappenheim

staining (May-Grünwald, Giemsa) (19). Tissue from the right lower lobe was snap-frozen for mRNA analyses. The bronchus to the right upper lobe was cannulated for airway fixation with 10% formalin for 24h at 30 cmH_2O pressure, followed by transfer into phosphate buffered saline (PBS).

RNA extraction and reverse transcription

Snap-frozen lung tissue from the right lower lobe was cut in 30-mg pieces. The tissue was transferred into lysis buffer (RLT buffer Qiagen, Hilden, Germany) for RNA extraction. After vortexing, the suspension was transferred to an RNeasy mini column (Qiagen) and processed according to the manufacturer's protocol. The purity and yield of the RNA were photometrically determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), and 1 µg total RNA was reverse transcribed by addition of 2 µL oligo (dT) primers (10 µmol/L), 1 µL RNase inhibitor (10 units/µL), 2 µL deoxynucleotide Mix (5 mM), and Omniscript transcriptase (0.2 U/µL; Qiagen) and incubation for 1h at 37°C. The cDNA was denatured at 93°C for 5 min.

Real-time PCR

The design of real-time PCR primers was based on published cDNA sequences (20,21). All PCRs were performed using 1 µg/µL cDNA per reaction in duplicates. Real-time PCR reactions were performed with the LightCycler 480 SYBR Green I Master mix (4707516001, Roche-Applied, Mannheim, Germany) on a LightCycler 480 Instrument according to the manufacturer's instructions. Real-time PCR results of *TLR1*, *TLR2*, *TLR4* and *TLR6* were normalized to the housekeeping gene ovine ribosomal protein S15 (*ovRPS15*), and mean fold changes in mRNA expression were calculated by the $\Delta\Delta C_t$ method (22).

Immunohistochemistry (PU.1 and Clara cell secretory protein)

Paraffin-embedded lung sections were deparaffinized in an ethanol series, and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in milli-Q water. Sections were incubated overnight at 4°C with the diluted primary antibody. Staining for hematopoietic transcription factor PU.1 (Santa Cruz Biotechnology, Santa Cruz, CA) was performed as described (14,16). After incubation with the appropriate secondary antibody, immunostaining was enhanced with the Vectastain ABC peroxidase Elite kit (Vector Laboratories, Burlingame, CA) and stained with nickel-diaminobenzidine (Ni-DAB). After counterstaining with Mayer's hematoxylin, the sections were washed and dehydrated. Sections were scored for PU.1 staining with a semi-quantitative scoring system: 1, little staining; 2, some staining; and 3, heavy staining. The analysis was performed in a blinded fashion.

A lung-specific Clara cell secretory protein (23) was detected in lung sections that were incubated overnight at 4°C with the diluted primary antibody (rabbit anti-uteroglobin, Abcam, Cambridge, UK). After incubation with the appropriate secondary antibody, immunostaining was enhanced as described above, stained with diaminobenzidine (DAB), and counterstained with Mayer's hematoxylin.

Mast cell staining

Mast cell staining was performed on paraffin-embedded lung sections (4 µm, transverse) from the right upper lobe (24). Briefly, the sections were stained with Weigert's iron hematoxylin working solution for 10 min. After rinsing with water, the sections were stained with 1% toluidine blue in 35% ethanol for 1 min. Subsequently, the sections were quickly dehydrated in ethanol and xylol. Microscopic images were taken at x100 and x200 magnifications using a Leica microscope and Leica Qwin Pro, version 3.4.0, software (Leica Microsystems, Mannheim, Germany). The circumference of all bronchi was measured at x100 magnification using ImageJ software, version 1.45 (National Institutes of Health, Bethesda, MD). Mast cells were counted at x200 magnification in six representative sections per animal. The analysis was performed in a blinded fashion.

Elastin staining

Sections of lung were incubated for 20 min in Weigert's resorcin-fuchsin (Chroma GmbH, Münster, Germany) at 60-70°C (24,25). After rinsing with water, the sections were incubated for 3 min in a tartrazine solution at room temperature. Subsequently, the sections were washed and dehydrated in ethanol and xylol. Microscopic images were taken at x200 magnification and elastin foci were counted in six representative sections per animal. The analysis was performed in a blinded fashion.

Data analysis

Results are given as mean ± standard error (SE). Comparisons between groups were performed by two-way analysis of variance (ANOVA) with Bonferroni *post-hoc* analysis (GraphPad Prism version 5, San Jose, CA). Significance was accepted at $P < 0.05$.

Results

Characterization of fetuses and lungs

The body weights and lung weights of LPS-exposed lambs were similar to those of the control group at each GA (Table 1), indicating similar growth. The cord blood pH, and pCO_2 were not different between groups.

As anticipated, the LPS-exposed lambs delivered at 125d GA had higher lung gas volumes compared with the control group (Table 1). Increased lung volume after LPS exposure persisted with delivery at 133d GA. At 140d GA, the control and the treatment groups had comparable pressure-volume deflation curves. The number of LPS injections did not influence lung gas volumes at any GA.

Table 1: Description of animals.

Treatment	No. of animals	BW (kg)	Lung weight (g/kg BW)	Cord blood pH	Cord blood pCO_2	V40 mL/kg BW
Delivered at 125d GA						
Control	7	3.0 ± 0.1	35.0 ± 0.9	7.28 ± 0.03	65 ± 3	12.1 ± 1.7
2d LPS	7	3.0 ± 0.1	31.9 ± 1.3	7.31 ± 0.02	62 ± 3	30.0 ± 2.3*
2+7d LPS	7	2.9 ± 0.1	38.5 ± 2.8	7.26 ± 0.03	67 ± 2	33.7 ± 2.5*
Delivered at 133d GA						
Control	7	4.0 ± 0.3	31.4 ± 1.0	7.20 ± 0.02	74 ± 4	27.8 ± 4.8
2d LPS	7	4.1 ± 0.1	34.3 ± 1.0	7.20 ± 0.01	74 ± 3	37.9 ± 1.8*
2+7d LPS	5	3.7 ± 0.1	32.0 ± 1.6	7.26 ± 0.02	68 ± 3	38.6 ± 1.1*
Delivered at 140d GA						
Control	7	5.0 ± 0.2	29.1 ± 0.9	7.25 ± 0.03	67 ± 4	40.9 ± 3.8
2d LPS	6	5.3 ± 0.1	35.2 ± 3.8	7.18 ± 0.04	80 ± 5	36.1 ± 3.4
2+7d LPS	7	5.0 ± 0.2	33.4 ± 1.8	7.24 ± 0.04	77 ± 6	37.7 ± 3.4

BW, body weight. V40 mL/kg BW, lung volume at 40 cmH_2O per kilogram body weight.

*P < 0.05 versus control group of the same gestational age.

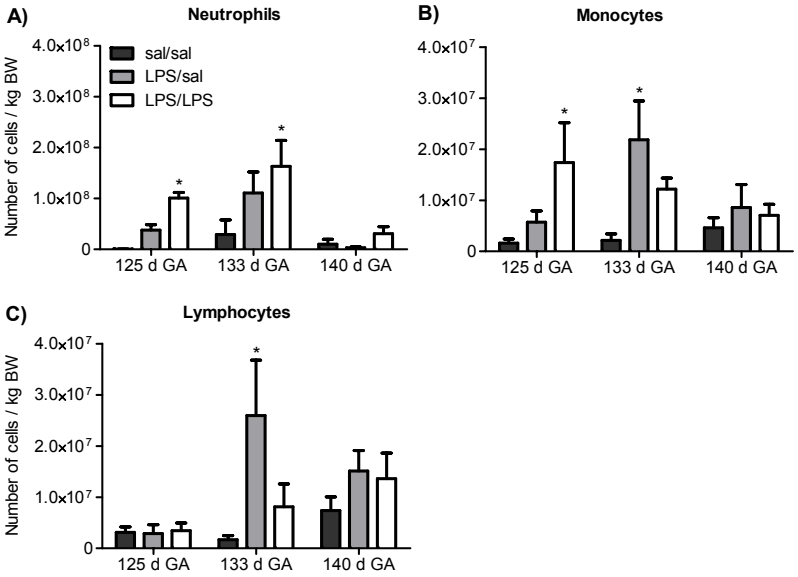


Figure 2: Pulmonary inflammation. Leukocytes were increased in BALF after LPS exposure at gestational ages of 125 and 133d, with a significant increase of **(A)** neutrophils and **(B)** monocytes at 125 and 133d GA. **(C)** Lymphocytes were increased after a single dose of LPS at 133d GA. In panels **(B)** and **(C)**, the y-axis was adjusted for the low number of monocytes and lymphocytes. **P* <0.05 versus control group of the same gestational age. BALF, bronchoalveolar lavage fluid; GA, gestational age; LPS, lipopolysaccharide; sal, saline.

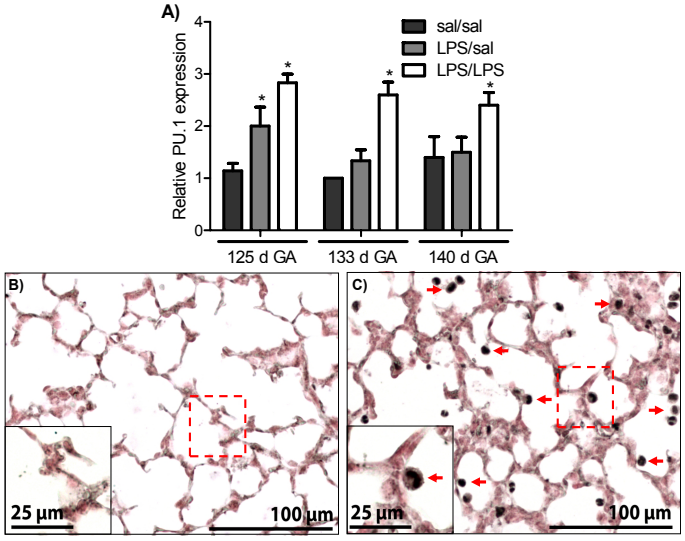


Figure 3: PU.1 expression in lung tissue. (A) The PU.1-positive cells in lung tissue exposed to LPS differed significantly from controls at each gestation. Representative staining (original magnification x200) is shown **(B)** for 125d GA control animals and **(C)** after two doses of LPS. The high-magnification insets (original magnification x400) illustrate the strong positivity of cells for PU.1 after exposure to LPS compared with that of controls. Red arrows indicate cells expressing PU.1. **P* <0.05 versus control group of the same gestational age. GA, gestational age; LPS, lipopolysaccharide; sal, saline.

Pulmonary inflammation

The total number of leukocytes increased after exposure to LPS at 125 and 133d GA. Neutrophils, in particular, increased significantly in the BALF after two injections of LPS (Figure 2A). Monocytes also increased at both 125 and 133d GA (Figure 2B). Lymphocytes increased only at 133d GA after a single LPS exposure (Figure 2C). No difference in the total number of neutrophils, monocytes and lymphocytes after LPS exposure were detected at 140d GA (Figure 2A, 2B and 2C).

PU.1-expressing cells in lung tissue

Cells expressing PU.1 increased in the lung tissue after two LPS injections at each gestation (Figure 3A). Compared with PU.1 expression in control lambs (Figure 3B), the expression of PU.1 was high in both alveolar and interstitial cells (Figure 3C). In contrast, a single LPS exposure resulted in only a transiently increased PU.1 expression: increased PU.1 level was evident at 125d GA, but PU.1 expression was lost at 133 and 140d GA (Figure 3A).

TLR mRNA in lung tissue

TLR1 mRNA levels increased after one and two doses of LPS at 125d GA (Figure 4A). mRNA levels of *TLR4* and *TLR6* decreased after the two exposures to LPS and subsequent delivery at 125d GA (Figure 4C and 4D). However, increased mRNA levels of *TLR2* and *TLR6* were detected at 133d GA (Figure 4B and 4D). No significant differences in *TLR* mRNA levels were detected at 140d GA.

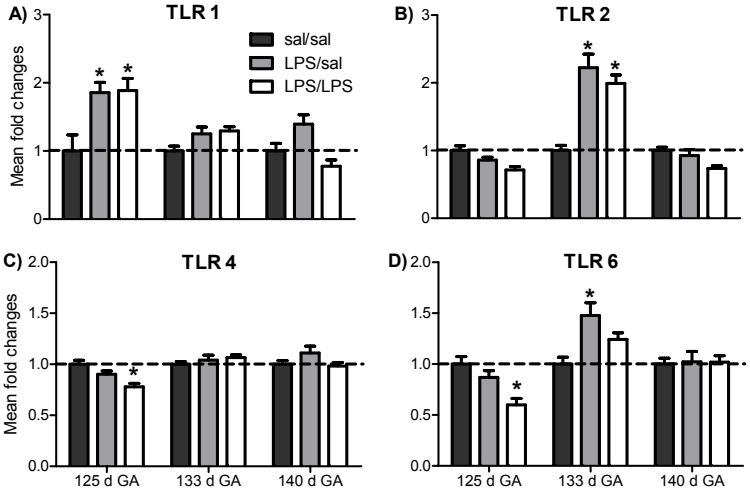


Figure 4: mRNA levels of *TLR 1, 2, 4* and *6*. (A) *TLR1* mRNA level was increased after a single dose and after double doses of LPS at 125d GA. At 125d GA, mRNA levels of **(C)** *TLR4* and **(D)** *TLR6* were decreased after a double dose of LPS. At 133d GA, increased mRNA levels of **(B)** *TLR2* and **(D)** *TLR6* were detected. No differences in mRNA levels were detected at 140d GA. **P* <0.05 versus control group of the same gestational age. GA, gestational age; LPS, lipopolysaccharide; sal, saline; TLR, Toll-like receptor.

Mast cells and Clara cells in lung tissue

Mast cells were present in the bronchial wall but not in other regions of lung tissue (Figure 5B and 5C). At 125d GA, there was a trend for a decreased number of mast cells with LPS (Figure 5A). The decrease in mast cells was significant after two doses of LPS at 133d GA. The number of mast cells increased as GA advanced (Figure 5A). A representative Clara cell staining (Figure 5D and 5E) was performed to demonstrate the specificity of the mast cell staining.

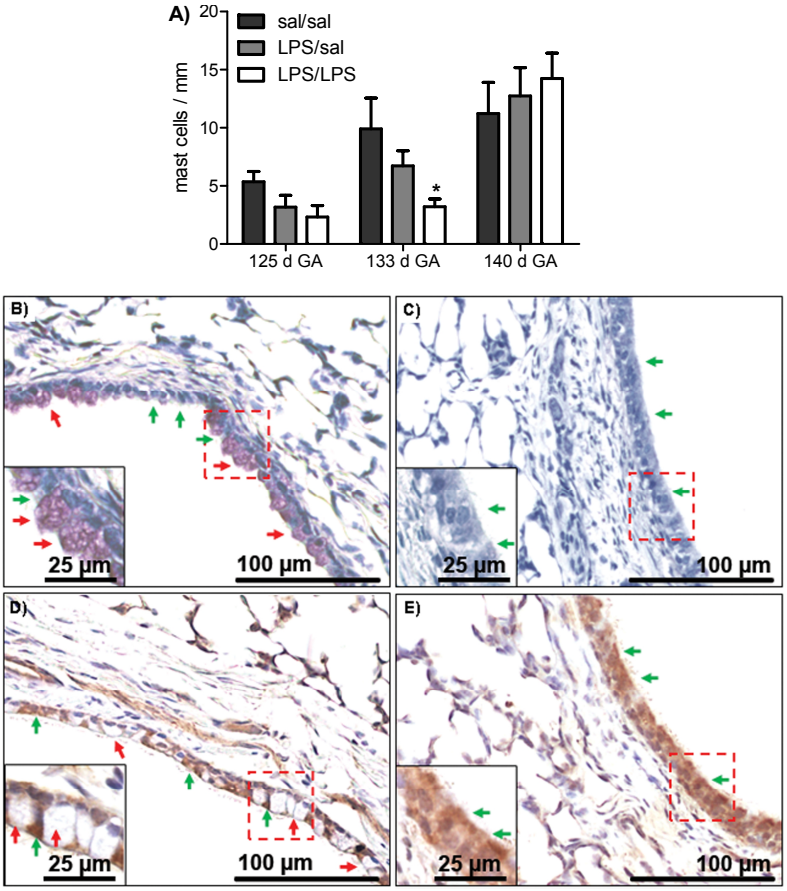


Figure 5: Representative staining and number of mast cells counted in the bronchial wall. (A) There were no changes in number of mast cells as a result of exposure to LPS with one or two doses compared with the control group at 125 and 140d GA. At 133d GA, the number mast cells decreased after repetitive LPS administration. Representative staining (original magnification x200) is shown in panel **(B)** for 133d GA control animals and in panel **(C)**, after two doses LPS. The high-magnification insets (original magnification x400) illustrate the loss of mast cells after exposure to LPS compared with the number in controls. Panels **(D)** and **(E)** represents the staining for Clara cells in the bronchial wall. Red arrows indicate mast cells, and green arrows indicate Clara cells. **P* <0.05 versus control group of the same gestational age. GA, gestational age; LPS, lipopolysaccharide; sal, saline.

Elastin expression in the lung

The elastin foci on alveolar septa were counted on sections from the right upper lobe (Figure 6A). Elastin foci were reduced in number after two LPS injections at 125d GA. The elastin was localized more diffusely in LPS-exposed lungs (Figure 6C), compared with control lungs, at 125d GA (Figure 6B). Elastin foci were not different in number or localization in lungs of LPS-exposed and control groups at 133 and 140d GA.

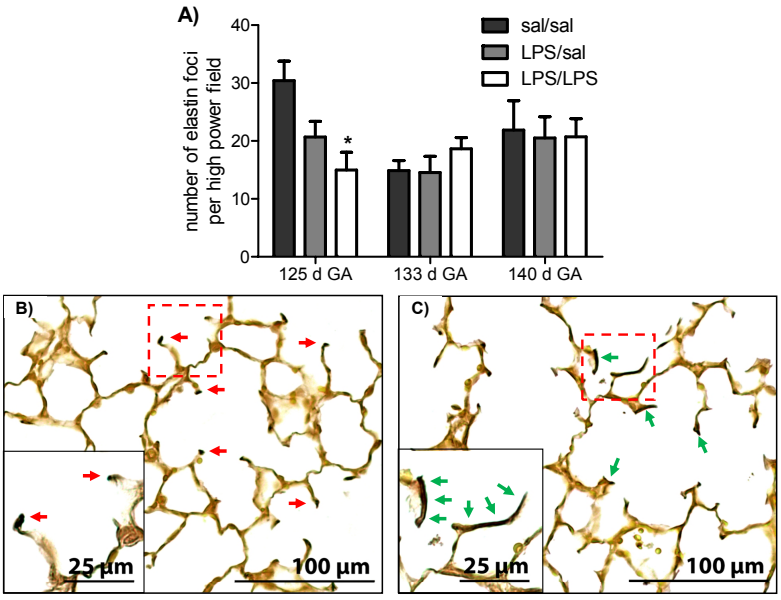


Figure 6: Expression of elastin foci on alveolar septa in lung tissue. (A) Decreased numbers of elastin foci were recorded at 125d GA after a double dose of LPS. At 133 and 140d GA, the lungs showed no differences after LPS exposure compared with control animal lungs. The high-magnification insets (original magnification x400) show that the focal depositions of elastin on the tips of alveolar septa, which are strongly present in **(B)** the lungs of controls at 125d GA, are diminished and diffusely expressed in **(C)** lungs after a double dose of LPS at 125d GA. Panels **(B)** and **(C)** are high-magnification images (original magnification x200). Red arrows indicate location of focal expression of elastin, and green arrows indicate diffusely expressed elastin. *P <0.05 versus control group of the same gestational age. GA, gestational age; LPS, lipopolysaccharide; sal, saline.

Discussion

To better characterize the effects of LPS on the fetal lung, we measured a number of variables that could contribute to longer-term adverse effects of fetal exposure to LPS. We used the single exposure at 118d and the two exposures at 118 and 123d to parallel our previous reports that demonstrated fetal immune tolerance to LPS (13,16,26). The deliveries at 2, 10, and 17d after the second exposure allowed evaluation of the persistence of responses during late fetal development.

LPS in the amniotic fluid has a half-life of 1.7d, with inflammation markers remaining measurable for at least 15d after administration (27). In this study, intra-amniotic LPS exposure resulted in changes in the fetal lung inflammatory responses. This is consistent with our previous findings at 125d GA regarding treatment after one or two doses of LPS (4,19,28). Our findings show that the increase in neutrophils at 133d was evidence of a persistent response to the initial inflammatory stimulus. Furthermore, two doses of LPS were required to induce significant differences in BALF neutrophil content at 125d GA. This finding suggests that the pulmonary immune response may be dependent on the intensity and/or persistence of the LPS exposure. The local immune system of the lung seems to remain active until 133d GA, with increased number of monocytes and lymphocytes. Interestingly a macrophage response was involved in the pulmonary inflammatory response to LPS in the course of gestation, shown by an increased PU.1 expression. Normally, the fetal lung contains few macrophages, and monocytes mature to macrophages with PU.1 expression after term birth (14,29,30). The lungs of fetal sheep exposed to intra-amniotic LPS expressed PU.1 within 1-2d, and vacuolated alveolar macrophages could be recovered by lavage several days later (14). We reported that PU.1 expression increased 7d after a single exposure to LPS but that expression was lost 15 and 22d later. In contrast, the second exposure 5d following the first exposure resulted in high PU.1 expression, which persisted until 140d GA. These observations demonstrate that monocyte to macrophage maturation in the fetal lung can be transient or persistent depending on the characteristics of the inflammatory exposure. *In vitro*, fetal alveolar macrophages responded to TLR agonists similarly to alveolar macrophages from the adult lung (15). The persistence of macrophages could promote inflammation in the fetal lung or in the lung following delivery after TLR stimulation and may lead to an increased susceptibility for neonatal sepsis and pneumonia (17).

TLR mRNA levels were low in the fetal sheep lung relative to the adult lung, and changes in expression are minimal during late gestation (20). Our results demonstrated that changes in *TLR* mRNA levels with LPS exposure differed by TLR, exposure, and interval from exposure. *TLR4* mRNA levels decreased 2d, but not 7d, following intra-amniotic injection of LPS. The delayed increases in *TLR2* and *TLR6* mRNA levels 15d after the initial exposure to the TLR4 agonist LPS might have clinical implications for the chronic infection and often polymicrobial organisms associated with chorioamnionitis (17). Interestingly, increased *TLR2* and *TLR6* mRNA levels seem to be related to increased monocytes numbers in BALF 15d after a single injection, indicating an early development of the monocytes (31). It is not clear why there was a resolution of TLR2 and TLR6 to term gestation.

The homeostasis of the airway is also maintained by mast cells. Mast cells in the lower airways contribute to the development of asthma and allergic diseases (32). Mast cells are

of interest because of a possible relation between chorioamnionitis, preterm birth, and the development of asthma later in life (33,34). Mast cell activation results in degranulation, leading to the rapid release of inflammatory mediators, such as histamine, proteoglycans, and cytokines, which stimulate the recruitment of other inflammatory cells (35). Mast cells are mostly present near epithelial surfaces such as mucosa of the lungs and the digestive tract (35). In this study, mast cells were found in the bronchial walls of preterm fetal lamb lungs. Although exposure to LPS resulted in a decrease of mast cells in the more immature lambs, mast cell numbers had returned to normal for lambs delivered near term. Our finding of reduced mast cell populations soon after LPS exposure contrasts with our hypothesis that LPS exposure would increase mast cells. However, repeated fetal exposures to LPS decreased airway responses to methacholine challenge in 7-week-old lambs (12). The relationship between prenatal inflammation and the mast cell response in the fetal airways has not been studied. Increased levels of mast cells were associated with the development of BPD in one autopsy study (36). Mast cell regulation is complex, depending on the timing of the fetal and postnatal stimuli. Our results demonstrated that fetal exposure to LPS could change mast cell numbers in the fetal lungs. However, the long-term effect of intrauterine exposure to inflammation on mast cell presence and function in postnatal life needs to be determined.

To examine lung structure, elastin foci on alveolar septa were counted on lung sections. Although a double LPS exposure resulted in more diffusely localized elastin foci in the more immature fetal lambs, a more normal distribution with localization of elastin foci at the tips of alveolar septa was reestablished near term, indicating recovery with advancing gestation. Whereas a single LPS exposure can induce structural maturation (4,37), a second LPS exposure may increase the abnormal elastin distribution and alveolar development. In addition, it was demonstrated that prenatal LPS exposure upregulated tropoelastin (soluble precursor of elastin) mRNA levels in lung tissue at the time of secondary septa development, resulting in a more diffuse elastin expression along the alveolar cell wall (38). This might contribute to the abnormal lung structure. The mechanism by which the fetal lung repairs the abnormal elastin response is unknown. Some of the variables in the “recovery” response, detected in part in this study, may be GA at assessment, GA at time of initial exposure (39), and magnitude of the inflammatory responses and/or antenatal steroids (40).

A limitation of our study was that no additional exposure to LPS was given immediately before delivery at the different GAs to challenge the fetal immune system. In human pregnancies affected by chorioamnionitis, fetuses are exposed to bacteria until delivery, which will challenge the immune system continuously. In our model, the fetus had a single or a second dose of LPS within a confined GA range, and the proinflammatory stimuli

might be less than those occurring in human infants. In this study, we focused on the lungs. From literature, it is known that chorioamnionitis is a multiorgan disease that affects the central nervous system, skin, and the gut in clinics and experimental models (3,41,42). The interaction of the different organs is not very well understood, but the role of the immune system of the fetus, as well as the role of endotoxins in human chorioamnionitis, warrants further research.

In summary, the fetal lung responded to intra-amniotic LPS with changes that might have long-term effects on lung and immune function. Although the abnormalities in elastin distribution did not persist, monocyte to macrophage maturation, selective TLR expression, and mast cell numbers could change at later times after the LPS exposure. How preterm delivery might interact with these changes remains to be determined.

Acknowledgement

We thank Richard Dalton, Andrea Lee, Carryn McLean, Masatoshi Saito, Clare Berry, Jennifer Henderson, Tim Wolfs, Nico Kloosterboer, and Lilian Kessels for excellent technical support.

References

1. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med 2000;342:1500–7.
2. Gotsch F, Romero R, Kusanovic JP, et al. The fetal inflammatory response syndrome. Clin Obstet Gynecol 2007;50:652–83.
3. Gantert M, Been JV, Gavilanes AW, et al. Chorioamnionitis: a multiorgan disease of the fetus? J Perinatol 2010;30 Suppl:S21–30.
4. Moss TJ, Nitsos I, Kramer BW, et al. Intra-amniotic endotoxin induces lung maturation by direct effects on the developing respiratory tract in preterm sheep. Am J Obstet Gynecol 2002;187:1059–65.
5. Watterberg KL, Demers LM, Scott SM, et al. Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. Pediatrics 1996;97:210–5.
6. Willet KE, Kramer BW, Kallapur SG, et al. Intra-amniotic injection of IL-1 induces inflammation and maturation in fetal sheep lung. Am J Physiol Lung Cell Mol Physiol 2002;282:L411–20.
7. Been JV, Zimmermann LJ. Histological chorioamnionitis and respiratory outcome in preterm infants. Arch Dis Child Fetal Neonatal Ed 2009;94:F218–25.
8. Hartling L, Liang Y, Lacaze-Masmonteil T. Chorioamnionitis as a risk factor for bronchopulmonary dysplasia: a systematic review and meta-analysis. Arch Dis Child Fetal Neonatal Ed 2012;97:F8–F17.
9. Speer CP. Chorioamnionitis, postnatal factors and proinflammatory response in the pathogenetic sequence of bronchopulmonary dysplasia. Neonatology 2009;95:353–61.
10. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med 2001;163:1723–9.

11. Wolfs TG, Jellema RK, Turrisi G, et al. Inflammation-induced immune suppression of the fetus: a potential link between chorioamnionitis and postnatal early onset sepsis. J Matern Fetal Neonatal Med 2012;25 Suppl 1:8–11.
12. Lee AJ, Lambermont VA, Pillow JJ, et al. Fetal responses to lipopolysaccharide-induced chorioamnionitis alter immune and airway responses in 7-week-old sheep. Am J Obstet Gynecol 2011;204:364.e17–24.
13. Kallapur SG, Jobe AH, Ball MK, et al. Pulmonary and systemic endotoxin tolerance in preterm fetal sheep exposed to chorioamnionitis. J Immunol 2007;179:8491–9.
14. Kramer BW, Joshi SN, Moss TJ, et al. Endotoxin-induced maturation of monocytes in preterm fetal sheep lung. Am J Physiol Lung Cell Mol Physiol 2007;293:L345–53.
15. Kramer BW, Kallapur SG, Moss TJ, et al. Intraamniotic LPS modulation of TLR signaling in lung and blood monocytes of fetal sheep. Innate Immun 2009;15:101–7.
16. Kramer BW, Ikegami M, Moss TJ, et al. Endotoxin-induced chorioamnionitis modulates innate immunity of monocytes in preterm sheep. Am J Respir Crit Care Med 2005;171:73–7.
17. Czikk MJ, McCarthy FP, Murphy KE. Chorioamnionitis: from pathogenesis to treatment. Clin Microbiol Infect 2011;17:1304–11.
18. Jobe AH, Kramer BW, Moss TJ, et al. Decreased indicators of lung injury with continuous positive expiratory pressure in preterm lambs. Pediatr Res 2002;52:387–92.
19. Kramer BW, Moss TJ, Willet KE, et al. Dose and time response after intraamniotic endotoxin in preterm lambs. Am J Respir Crit Care Med 2001;164:982–8.
20. Hillman NH, Moss TJ, Nitsos I, et al. Toll-like receptors and agonist responses in the developing fetal sheep lung. Pediatr Res 2008;63:388–93.
21. Seehase M, Gantert M, Ladenburger A, et al. Myocardial response in preterm fetal sheep exposed to systemic endotoxinaemia. Pediatr Res 2011;70:242–6.
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.
23. Singh G, Katyal SL. Clara cells and Clara cell 10 kD protein (CC10). Am J Respir Cell Mol Biol 1997;17:141–3.
24. Kramer BW, Albertine KH, Moss TJ, et al. All-trans retinoic acid and intraamniotic endotoxin-mediated effects on fetal sheep lung. Anat Rec (Hoboken) 2008;291:1271–7.
25. Collins JJ, Kallapur SG, Knox CL, et al. Inflammation in fetal sheep from intra-amniotic injection of Ureaplasma parvum. Am J Physiol Lung Cell Mol Physiol 2010;299:L852–60.
26. Kallapur SG, Nitsos I, Moss TJ, et al. Chronic endotoxin exposure does not cause sustained structural abnormalities in the fetal sheep lungs. Am J Physiol Lung Cell Mol Physiol 2005;288:L966–74.
27. Newnham JP, Kallapur SG, Kramer BW, et al. Betamethasone effects on chorioamnionitis induced by intra-amniotic endotoxin in sheep. Am J Obstet Gynecol 2003;189:1458–66.
28. Kallapur SG, Willet KE, Jobe AH, et al. Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. Am J Physiol Lung Cell Mol Physiol 2001;280:L527–36.
29. Shibata Y, Berclaz PY, Chroneos ZC, et al. GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. Immunity 2001;15:557–67.
30. Berclaz PY, Carey B, Fillipi MD, et al. GM-CSF regulates a PU.1-dependent transcriptional program determining the pulmonary response to LPS. Am J Respir Cell Mol Biol 2007;36:114–21.
31. Shen CM, Lin SC, Niu DM, et al. Development of monocyte Toll-like receptor 2 and Toll-like receptor 4 in preterm newborns during the first few months of life. Pediatr Res 2013;73:685–91.
32. Brown JM, Wilson TM, Metcalfe DD. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. Clin Exp Allergy 2008;38:4–18.
33. Kumar R, Yu Y, Story RE, et al. Prematurity, chorioamnionitis, and the development of recurrent wheezing: a prospective birth cohort study. J Allergy Clin Immunol 2008;121:878–84.e6.

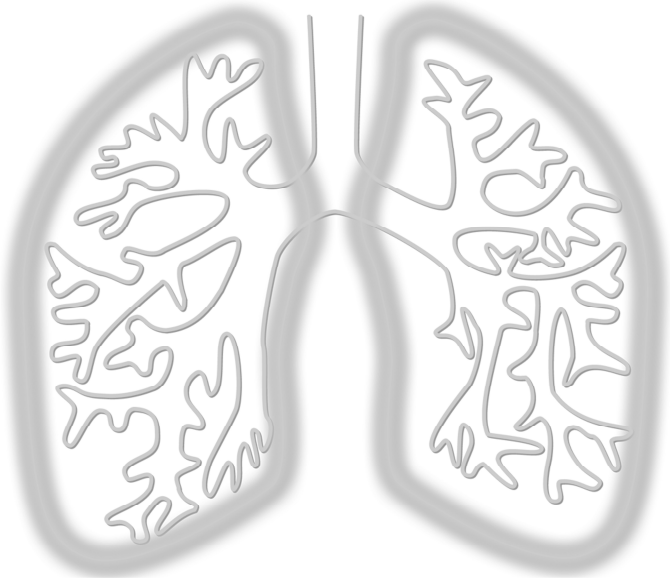
34. Getahun D, Strickland D, Zeiger RS, et al. Effect of chorioamnionitis on early childhood asthma. Arch Pediatr Adolesc Med 2010;164:187–92.
35. Prussin C, Metcalfe DD. 4. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol 2003;111(2 Suppl):S486–94.
36. Bhattacharya S, Go D, Krenitsky DL, et al. Genome-wide transcriptional profiling reveals connective tissue mast cell accumulation in bronchopulmonary dysplasia. Am J Respir Crit Care Med 2012;186:349–58.
37. Pillow JJ, Jobe AH, Collins RA, et al. Variability in preterm lamb lung mechanics after intra-amniotic endotoxin is associated with changes in surfactant pool size and morphometry. Am J Physiol Lung Cell Mol Physiol 2004;287:L992–8.
38. Cao L, Wang J, Tseu I, et al. Maternal exposure to endotoxin delays alveolarization during postnatal rat lung development. Am J Physiol Lung Cell Mol Physiol 2009;296:L726–37.
39. Moss TJ, Newnham JP, Willett KE, et al. Early gestational intra-amniotic endotoxin: lung function, surfactant, and morphometry. Am J Respir Crit Care Med 2002;165:805–11.
40. Kuypers E, Collins JJ, Kramer BW, et al. Intra-amniotic LPS and antenatal betamethasone: inflammation and maturation in preterm lamb lungs. Am J Physiol Lung Cell Mol Physiol 2012;302:L380–9.
41. Wolfs TG, Buurman WA, Zoer B, et al. Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep. PLoS ONE 2009;4:e5837.
42. Kemp MW, Kannan PS, Saito M, et al. Selective exposure of the fetal lung and skin/amnion (but not gastro-intestinal tract) to LPS elicits acute systemic inflammation in fetal sheep. PLoS ONE 2013;8:e63355

CHAPTER 3

Sex differences in lung gas volumes after lipopolysaccharide-induced chorioamnionitis in fetal sheep

Verena A. Lambermont, Jasper V. Been, Steffen Kunzmann, Sizzle F. Vanterpool, John P. Newnham, Suhas G. Kallapur, Alan H. Jobe, Boris W. Kramer

Gend Med. 2012 Aug;9(4):278-86



Abstract

Background: Preterm female infants have a survival advantage and enhanced lung development, which is an important determinant of preterm survival. Given the modulation of lung development by fetal exposure to infection/inflammation, we hypothesized that female fetuses have enhanced lung maturational responses to chorioamnionitis compared with male fetuses.

Methods: Time-pregnant ewes received intra-amniotic injections with saline (n=60) or lipopolysaccharide (LPS) at 2 days (n=30) or 7 days (n=45) before surgical delivery at 123 to 125 days of gestation (term: ~147 days). We assessed inflammatory responses in bronchoalveolar lavage fluid and cord blood, and lung maturation with pressure-volume curves, and lung structure.

Results: Lung gas volume showed differences between the sexes after 2 days LPS (male 4.6 ± 1.2 mL/kg, female 7.7 ± 4.4 mL/kg; $P = 0.02$) and 7 days LPS (male 20.5 ± 9.3 mL/kg, female 27.0 ± 7.0 mL/kg; $P = 0.01$). The control group was not different by sex (male 8.0 ± 3.6 mL/kg, female 8.9 ± 3.9 mL/kg; $P > 0.05$). No difference in lung structure and in pulmonary and systemic inflammatory response was evident by sex.

Conclusion: Preterm female sheep fetuses had increased lung gas volumes after exposure to LPS, without any detectable differences in fetal inflammatory responses.

Introduction

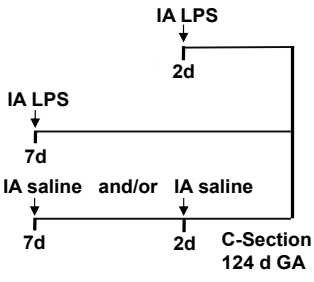
In the field of perinatology, sex differences for short- and long-term outcomes after preterm birth are well recognized. Male sex has almost invariably been associated with worse outcomes, including neonatal mortality (1-3), neurodevelopmental delay (4), and abnormal cardiovascular development (5-6). In line with these observations, preterm male infants have a higher respiratory morbidity and need more ventilatory support during the first week after birth than female infants (3,7). Male infants are intubated more frequently during the first 24 hours of life and receive the first dose of surfactant earlier than female infants (3). These preterm male infants are more likely to develop chronic lung disease (2). Although mothers carrying male infants received antenatal steroids more frequently compared with mothers carrying female infants, male infants are more often in need of supplemental oxygen at early gestational age (3). The underlying mechanisms to why male sex is associated with increased neonatal mortality and morbidity is still unknown. Previous studies have shown that surfactant increases earlier in gestation in female than in male neonatal lungs (8-11). This may contribute to the higher airflow rate and lower airway resistance in female neonatal lungs compared with male neonatal lungs (8). Although female lungs tend to be smaller and weigh less than male lungs, the number of alveoli per area do not differ (8,12). Animal studies support a role for sex hormones in the regulation of lung development (8,9). Androgens inhibit fetal lung surfactant production (9,13) whereas estrogens stimulate alveolar development (9,14).

The majority of extremely preterm births (24–26 weeks) are associated with chorioamnionitis (15,16), and it is mainly in this age group that sex differences are most evident. Chorioamnionitis is a well-known modulator of both short-term and long-term pulmonary outcomes after preterm birth (17-21). A sex difference in susceptibility for chorioamnionitis-associated adverse outcomes may explain part of the discrepancy between male and female outcomes after preterm birth. However, we are unaware of any clinical studies that have evaluated a possible interaction between chorioamnionitis and sex with regard to neonatal pulmonary outcome among preterm infants. We therefore hypothesized that chorioamnionitis would differentially affect lung injury as well as lung maturation based on sex. To test this hypothesis we evaluated the effect of experimental chorioamnionitis between male and female fetal sheep delivered prematurely for markers of inflammation in bronchoalveolar lavage fluid (BALF) and cord blood as well as pulmonary pressure-volume curves and lung histology as a proxy for lung maturation.

Methods

Animal Model

Pregnant ewes received intra-amniotic injections with 10 mg *Escherichia coli* 055:B5 endotoxin (lipopolysaccharide [LPS]) (Sigma, St Louis, Missouri) or saline (controls) at 2 or 7 days before surgical delivery (Figure 1). At 123 to 125 days of gestation (term is ~147 days) the lambs were killed without breathing with intravascular pentobarbital. These studies were performed during the period 2003 to 2010, and this report is a composite of animal groups previously reported (22-30).



# Male	# Female	# Total
17	13	30
18	27	45
32	28	60
67	68	135

Figure 1: Timing of fetal exposure to intra-amniotic (IA) lipopolysaccharide (LPS) indicating the time before surgical delivery and number (#) of animals per group. Surgical delivery was performed at 124 days (d) gestation.

Pressure-Volume Curve

After exsanguination of the lambs, the thorax was opened by a midline incision as previously described (31). An endotracheal tube was inserted and connected to a manometer. The deflation limb of a pressure volume curve was recorded from a maximum pressure of 40 cm water. All lung gas volumes are expressed as milliliters per kilogram body weight.

Lung Structure Histology

Paraffin-embedded lung tissue sections from the left upper lobe (4 µm, transverse) were stained with hematoxylin and eosin. Per section 5 representative microscopic images were made with a 10× magnification using a Leica microscope and Leica Qwin Pro version 3.4.0 software (Leica Microsystems, Mannheim, Germany). The images were uploaded in Matlab 6.0 software (The Mathworks, Inc, Natick, Massachusetts). This software calculated the alveolar wall thickness (AWT) and mean alveolar size (MAS) for analysis.

BALF

The left lung was lavaged 5 times with ice-cold saline (22). The BALF was pooled and centrifuged at 500 rpm for 5 minutes for cytospin preparations. After staining with Pappenheim-staining (May-Grünwald, Giemsa) differential cell counts were performed (22).

Cord Blood

Arterial cord blood samples were collected at delivery for complete white blood counts (22). Platelets were used as a proxy for systemic inflammation (32). The arterial pH was measured in an automated blood gas analyzer (Radiometer, Copenhagen, Denmark).

Statistical Analysis

Results are given as means (SD). For graphical display, SEM is used in the Figures. Comparisons between groups and sex were performed by 2-way ANOVA. Significance was accepted at probability value of <0.05. Analyses were performed using SPSS 16.0 (IBM, Amsterdam, the Netherlands) and GraphPad Prism 5 (GraphPad Software, Inc, California).

Results

General Characteristics

General characteristics for each group are shown in the Table. Mean gestational age (SD) was 124 ± 1 day. The body weights as well as the lung weights of male lambs were similar to that of females (see the Table), indicating similar growth and development. Measurement of pH in cord blood showed no differences between males and females.

Table: General physiology characteristics of the study groups.

Treatment*	Gestational Age (d)		Body Weight (kg)	
	Male	Female	Male	Female
Control	123.7 ± 1.1	124.2 ± 1.3	2.78 ± 0.45	2.70 ± 0.47
2d LPS	123.7 ± 1.0	123.6 ± 1.2	2.66 ± 0.35	2.51 ± 0.32
7d LPS	124.1 ± 1.1	124.0 ± 1.2	2.73 ± 0.38	2.61 ± 0.44
Treatment*	Lung weight† (g/kg BW)		pH in cord blood	
	Male	Female	Male	Female
Control	35.7 ± 5.4	36.5 ± 5.6	7.28 ± 0.09	7.26 ± 0.12
2d LPS	40.7 ± 5.4	41.5 ± 4.9	7.24 ± 0.12	7.23 ± 0.10
7d LPS	36.5 ± 3.8	38.5 ± 7.3	7.30 ± 0.12	7.23 ± 0.14

* There were no differences between males and females after 2d and 7d exposure to lipopolysaccharide

† Lung weight is standardized per kilogram body weight

Lung Compliance

Lung compliance was assessed with lung gas volumes from pressure volume curves as a measurement of lung maturation. In the control groups no differences in lung gas volumes were detected between male and female lambs (Figure 2A). LPS administered 2 days before birth significantly decreased lung gas volume in male lambs compared with controls, whereas females were unaffected (Figure 2B). Conversely, LPS administered 7 days before birth resulted in large increases in lung gas volumes in both sexes relative to controls; the females had higher lung gas volumes than the males (Figure 2C).

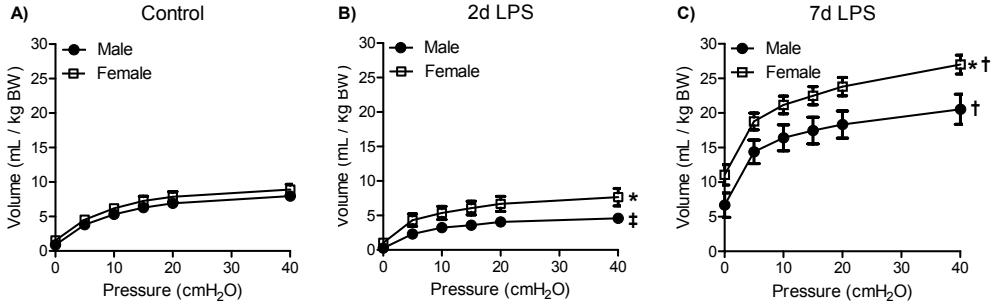


Figure 2: Pressure-volume curves of the lungs of (A) control subjects, (B) sheep fetuses at 2 days gestation, and (C) sheep fetuses at 7 days gestation. Significant differences between males and females were present for injections 2 days and 7 days of injections with lipopolysaccharide before deliveries. BW = body weight. *P <0.001 versus male; $^{\dagger}P$ <0.01 versus control; $^{\ddagger}P$ <0.001 versus control.

Lung Structure

Lung maturation was also assessed by the analysis of the lung structure (Figure 3). Lung structure was analyzed through measurements of AWT (Figure 3A) and MAS (Figure 3B). After 2 days LPS exposure differences were measured in female lungs with a decrease of MAS and an increase of AWT. Both parameters were not significantly different between sexes.

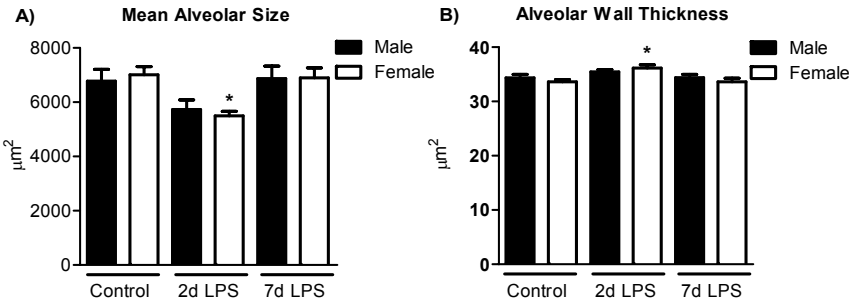


Figure 3: Evaluation of lung structure of preterm sheep fetuses. Two parameters were measured: **(A)** mean alveolar size and **(B)** alveolar wall thickness. No significant differences were detected by sex. LPS = lipopolysaccharide. *P <0.05 versus control.

Pulmonary Inflammation

Leukocytes were greatly increased in BALF after exposure to LPS at the different time points (Figure 4). LPS administered 2 days and 7 days before birth increased both total number of white blood cells and neutrophils compared with controls (Figures 4A and 4B). Monocytes were increased after 2-day LPS exposure (Figure 4C). No differences in lymphocytes were detected after LPS exposure compared with controls (Figure 4D). Although total white blood cell count, as well as the number of neutrophils and monocytes, tended to be higher in males after LPS, no significant differences were present.

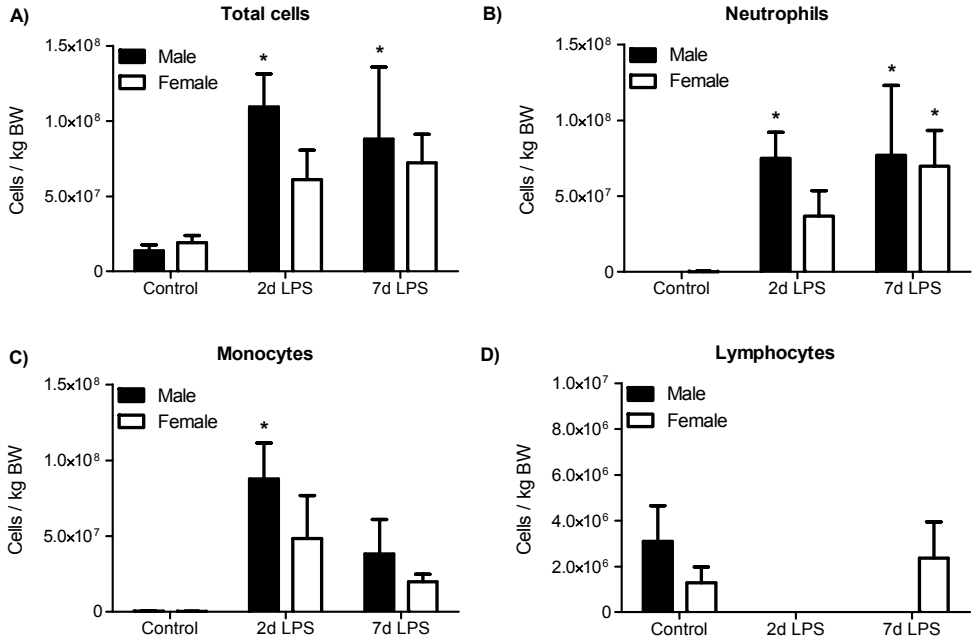


Figure 4: Inflammatory cells in bronchoalveolar lavage fluid, differentiated for **(A)** total cells, **(B)** neutrophils, **(C)** monocytes, and **(D)** lymphocytes (in this panel, the y-axis was adjusted for the low number of lymphocytes). No significant differences were detected by sex. LPS = lipopolysaccharide. **P* <0.05 versus control.

Blood Cells in Cord Blood

Platelets and total numbers of white blood cells increased significantly after 7 days of exposure to LPS in both sexes, but without any differences by sex (Figure 5A and 5B). Especially, cord blood neutrophils increased significantly 7 days after LPS exposure in both sexes, whereas monocytes were increased only in males (Figure 5C and 5D). No differences for neutrophils, monocytes, and lymphocytes were recorded between sexes after LPS exposure (Figures 5C, 5D, and 5E).

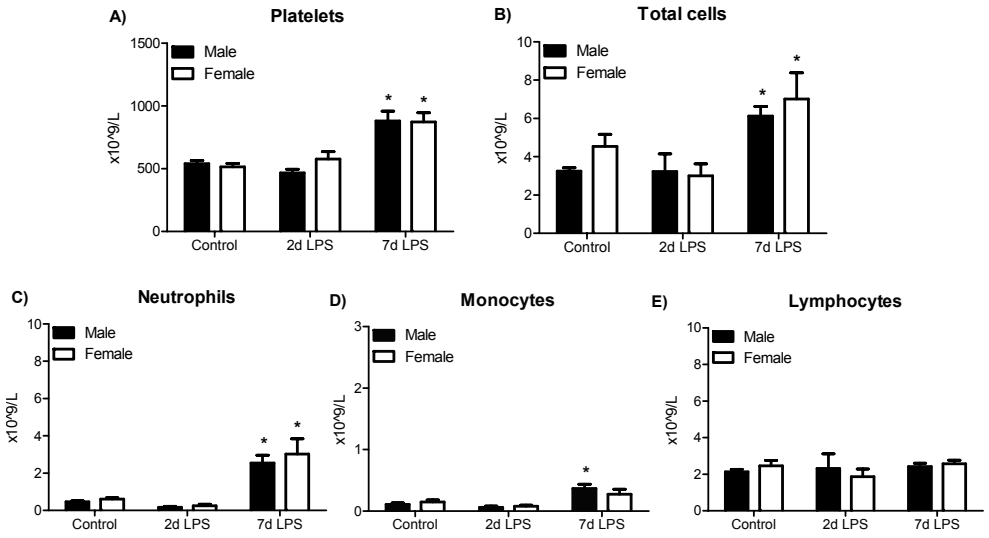


Figure 5: Systemic inflammation measured in cord blood, differentiated for **(A)** platelets, **(B)** total cells, **(C)** neutrophils, **(D)** monocytes (in this panel the y-axis was adjusted for the low number of monocytes), and **(E)** lymphocytes. No significant differences were detected by sex after lipopolysaccharide (LPS) exposure 2 days and 7 days before deliveries. **P* <0.05 versus control.

Discussion

Although many studies have focused on effects of antenatal exposure to inflammation on outcome after preterm birth, we are unaware of any previous studies evaluating a potential modulatory effect of sex. In our study we observed larger improvements in lung compliance after LPS-induced intrauterine inflammation among preterm female fetuses compared with males. We did not detect any potentially underlying differences in pulmonary and systemic markers of inflammation in these animals. No sex differences in either lung compliance or inflammatory responses were present in control animals delivered at the same gestational age.

We studied sex differences in response to chorioamnionitis at extremely preterm gestational age with severe surfactant deficiency because the female survival advantage is most evident at this stage for human beings (33). Furthermore, extremely preterm infants are at the highest risk of being exposed to chorioamnionitis (15,16). A maturational response of the lung to antenatal inflammation is well described in several animal models (34-36) and corresponds with the decrease in respiratory distress syndrome generally observed in chorioamnionitis-exposed infants (15,37). In our animal model a decrease in lung compliance was detectable 2 days after intrauterine LPS exposure only in male fetuses. Conversely, lung compliance was enhanced 7 days after LPS, when again lung compliance was better in female than in male fetuses. We propose that these differences may explain at least part of the female advantage in survival as well as in outcome among survivors after preterm birth. The finding that sex differences were only present after LPS exposure suggests that chorioamnionitis could well be an important modulator of this female advantage among preterm infants. To examine the mechanism behind this female advantage we analyzed the lung structure for differences in alveolar size and wall thickness. However no sex difference in MAS and AWT was noticed. This suggests that the female advantage in lung gas volume is not based on lung histology. This outcome contributes to the theory that not the lung structure but the surfactant deficiency in male lungs might play a key role in the female advantage after preterm birth. Further investigations in existing and future human cohorts are required to test this proposed mechanism between sexes.

In our study we assessed pulmonary as well as systemic markers of inflammation to determine their potential mechanistic role in the observed sex differences in lung compliance. The absence of major differences in these markers suggests that other mechanisms may primarily be involved.

Lung maturation as an effect of antenatal steroid exposure is well recognized, and fetal stress and cortisol release have been proposed as potential modulators of the pulmonary maturational response to inflammation (38,39). In previous studies (40,41) it was demonstrated that sex-specific differences in the cortisol stress response are indeed present before birth after a hypoxic event. However, the cortisol response was found to be much greater in male than female fetuses, theoretically putting males at an advantage with regard to lung maturation. Furthermore, we previously showed in fetal sheep that fetal plasma cortisol concentrations significantly increased after intra-amniotic LPS, but that this increase in fetal cortisol was not sufficient to cause preterm lung maturation (42). These findings do not support a primary role for differential corticosteroid-induced responses by sex in mediating pulmonary maturational differences. In addition, a study

about the number and binding affinity of lung glucocorticoid receptor showed no significant difference by sex (43).

Previous studies have pointed toward a potential role for sex-hormones in sex differences during lung development. Androgens inhibit fetal lung surfactant production by downregulation of epidermal growth factor receptor activity and upregulation of transforming growth factor-β receptor activity (9,13). Surfactant deficiency is a major contributor to the development of respiratory distress syndrome in preterm infants. Indeed surfactant production has been demonstrated to appear earlier in gestation in female compared with male neonatal lung (10,11).
In addition, interleukin 1 (IL-1) has a central role in the pathogenesis of chorioamnionitis-induced fetal inflammatory responses. Increased levels of cord IL-1 receptor antagonist (IL-1ra) were associated with adverse outcome in preterm infants (44). Interestingly, these levels were higher in female than in male fetuses. Although sex differences in IL-1ra concentrations may in part explain the lower susceptibility of female fetuses to infection (45), our model did not provide much evidence that a sex-specific immune response is involved in the pulmonary maturational response to chorioamnionitis.

To our knowledge no studies have investigated long-term effects of chorioamnionitis on lung maturation by sex. Recent investigation of the long-term effects of LPS injections *in utero* demonstrated recovery of structural changes and recovery of the immune system (35), but no analysis of sex effects was performed.
Our study has several limitations. We cannot rule out a modulatory effect of sex on inflammatory responses that could underlie pulmonary maturational responses, based on inflammatory cell numbers alone. Additional inflammatory markers may reveal differences. The findings reported here were from multiple experiments performed with other primary objectives, but performed with the same type and source of LPS, the same breed of sheep, and under similar experimental conditions. However, we cannot exclude variations between experiments occurred over this time frame. Strength of the study is the large animal population available for analyses.

Conclusions

Female sheep fetuses exposed to intra-amniotic LPS have increased lung gas volumes when compared with male sheep fetuses. These results highlight important sex differences in the pulmonary response to chorioamnionitis that may explain at least part of the female advantage in outcome after preterm birth.

Conflicts of Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

Acknowledgments

All authors contributed equally to the literature search, data interpretation, figure creation, and writing of the manuscript.

References

1. Binet ME, Bujold E, Lefebvre F, et al. Role of gender in morbidity and mortality of extremely premature neonates. Am J Perinatol. 2012;29:159 –166.
2. Vohr BR, Wright LL, Dusick AM, et al. Neurodevelopmental and functional outcomes of extremely low birth weight infants in the National Institute of Child Health and Human Development Neonatal Research Network, 1993–1994. Pediatrics. 2000;105:1216–1226.
3. Stevenson DK, Verter J, Fanaroff AA, et al. Sex differences in outcomes of very low birthweight infants: the newborn male disadvantage. Arch Dis Child Fetal Neonatal Ed. 2000;83:F182–F185.
4. Teune MJ, van Wassenaer AG, van Dommelen P, et al. Perinatal risk indicators for long-term neurological morbidity among preterm neonates. Am J Obstet Gynecol. 2011;204:396.e1–396.e14.
5. Sheiner E, Levy A, et al. Gender does matter in perinatal medicine. Fetal Diagn Ther. 2004;19:366 –369.
6. Sheiner E, Hadar A, Hallak M, et al. Clinical significance of fetal heart rate tracings during the second stage of labor. Obstet Gynecol. 2001;97(5 Pt 1):747–752.
7. Elsmen E, Hansen Pupp I, Hellstrom-Westas L. Preterm male infants need more initial respiratory and circulatory support than female infants. Acta Paediatr. 2004;93:529 –533.
8. Carey MA, Card JW, Voltz JW, et al. It's all about sex: gender, lung development and lung disease. Trends Endocrinol Metab. 2007;18:308 –313.
9. Carey MA, Card JW, Voltz JW, et al. The impact of sex and sex hormones on lung physiology and disease: lessons from animal studies. Am J Physiol Lung Cell Mol Physiol. 2007;293:L272–L278.
10. Torday JS, Nielsen HC. The sex difference in fetal lung surfactant production. Exp Lung Res. 1987;12:1–19.
11. Torday JS, Nielsen HC, Fencl Mde M, et al. Sex differences in fetal lung maturation. Am Rev Respir Dis. 1981;123:205–208.
12. Szpinda M. The normal growth of the pulmonary trunk in human foetuses. Folia Morphol (Warsz). 2007;66:126 –130.
13. Dammann CE, Ramadurai SM, McCants DD, et al. Androgen regulation of signaling pathways in late fetal mouse lung development. Endocrinology. 2000;141:2923–2929.
14. Massaro D, Massaro GD. Estrogen receptor regulation of pulmonary alveolar dimensions: alveolar sexual dimorphism in mice. Am J Physiol Lung Cell Mol Physiol. 2006;290:L866 –L870.
15. Lahra MM, Jeffery HE. A fetal response to chorioamnionitis is associated with early survival after preterm birth. Am J Obstet Gynecol. 2004;190:147–151.
16. Czikk MJ, McCarthy FP, Murphy KE. Chorioamnionitis: from pathogenesis to treatment. Clin Microbiol Infect. 2011;17:1304 –1311.
17. Gantert M, Been JV, Gavilanes AW, et al. Chorioamnionitis: a multiorgan disease of the fetus? J Perinatol. 2010;30(Suppl):S21–S30.

18. Getahun D, Strickland D, Zeiger RS, et al. Effect of chorioamnionitis on early childhood asthma. Arch Pediatr Adolesc Med. 2010;164:187–192.
19. Kumar R, Yu Y, Story RE, et al. Prematurity, chorioamnionitis, and the development of recurrent wheezing: a prospective birth cohort study. J Allergy Clin Immunol. 2008;121:878–884.
20. Been JV, Zimmermann LJ. Histological chorioamnionitis and respiratory outcome in preterm infants. Arch Dis Child Fetal Neonatal Ed. 2009;94:F218–F225.
21. Been JV, Rours IG, Kornelisse RF, et al. Chorioamnionitis alters the response to surfactant in preterm infants. J Pediatr. 2010;156:10 –15.
22. Kramer BW, Moss TJ, Willet KE, et al. Dose and time response after intraamniotic endotoxin in preterm lambs. Am J Respir Crit Care Med. 2001;164:982–988.
23. Kunzmann S, Glogger K, Been JV, et al. Thymic changes after chorioamnionitis induced by intraamniotic lipopolysaccharide in fetal sheep. Am J Obstet Gynecol. 2010;202:476.e1– e9.
24. Kallapur SG, Moss TJ, Auten RL Jr, et al. IL-8 signaling does not mediate intra-amniotic LPS-induced inflammation and maturation in preterm fetal lamb lung. Am J Physiol Lung Cell Mol Physiol. 2009;297:L512–L519.
25. Kramer BW, Kallapur SG, Moss TJ, et al. Intraamniotic LPS modulation of TLR signaling in lung and blood monocytes of fetal sheep. Innate Immun. 2009;15:101–107.
26. Sweet DG, Huggett MT, Warner JA, et al. Maternal betamethasone and chorioamnionitis induce different collagenases during lung maturation in fetal sheep. Neonatology. 2008;94:79–86.
27. Kallapur SG, Jobe AH, Ball MK, et al. Pulmonary and systemic endotoxin tolerance in preterm fetal sheep exposed to chorioamnionitis. J Immunol. 2007;179:8491– 8499.
28. Kramer BW, Ikegami M, Moss TJ, et al. Endotoxininduced chorioamnionitis modulates innate immunity of monocytes in preterm sheep. Am J Respir Crit Care Med. 2005;171:73–77.
29. Kramer BW, Joshi SN, Moss TJ, et al. Endotoxininduced maturation of monocytes in preterm fetal sheep lung. Am J Physiol Lung Cell Mol Physiol. 2007;293:L345–L353.
30. Kunzmann S, Speer CP, Jobe AH, et al. Antenatal inflammation induced TGF-beta1 but suppressed CTGF in preterm lungs. Am J Physiol Lung Cell Mol Physiol. 2007;292:L223–L231.
31. Jobe AH, Kramer BW, Moss TJ, et al. Decreased indicators of lung injury with continuous positive expiratory pressure in preterm lambs. Pediatr Res. 2002;52:387–392.
32. Smith TL, Weyrich AS. Platelets as central mediators of systemic inflammatory responses. Thromb Res. 2011;127:391–394.
33. Bader D, Kugelman A, Boyko V, et al. Risk factors and estimation tool for death among extremely premature infants: a national study. Pediatrics. 2010;125:696 –703.
34. Zhan CY, Yuan TM, et al. Early gestational intrauterine infection induces postnatal lung inflammation and arrests lung development in a rat model. J Matern Fetal Neonatal Med. 2011;24:213–222.
35. Lee AJ, Lambermont VA, Pillow JJ, et al. Fetal responses to lipopolysaccharide-induced chorioamnionitis alter immune and airway responses in 7-week-old sheep. Am J Obstet Gynecol. 2011;204:364.e17–24.
36. Bry K, Lappalainen U. Intra-amniotic endotoxin accelerates lung maturation in fetal rabbits. Acta Paediatr. 2001;90:74–80.
37. Been JV, Rours IG, Kornelisse RF, et al. Histologic chorioamnionitis, fetal involvement, and antenatal steroids: effects on neonatal outcome in preterm infants. Am J Obstet Gynecol. 2009;201:587 e581-588.
38. Kallapur SG, Kramer BW, Moss TJ, et al. Maternal glucocorticoids increase endotoxin-induced lung inflammation in preterm lambs. Am J Physiol Lung Cell Mol Physiol. 2003;284:L633–L642.
39. Jobe AH, Newnham JP, Moss TJ, et al. Differential effects of maternal betamethasone and cortisol on lung maturation and growth in fetal sheep. Am J Obstet Gynecol. 2003;188:22–28.

40. Turner AI, Hosking BJ, Parr RA, et al. A sex difference in the cortisol response to tail docking and ACTH develops between 1 and 8 weeks of age in lambs. J Endocrinol. 2006;188:443– 449.
41. Giussani DA, Fletcher AJ, Gardner DS. Sex differences in the ovine fetal cortisol response to stress. Pediatr Res. 2011;69:118 –122.
42. Nitsos I, Moss TJ, Cock ML, et al. Fetal responses to intra-amniotic endotoxin in sheep. J Soc Gynecol Investig. 2002;9:80–85.
43. Kovar J, Waddell BJ, Sly PD, et al. Sex differences in response to steroids in preterm sheep lungs are not explained by glucocorticoid receptor number or binding affinity. Pediatr Pulmonol. 2001;32:8 –13.
44. Elsmen E, Ley D, Cilio CM, et al. Umbilical cord levels of interleukin-1 receptor antagonist and neonatal outcome. Biol Neonate. 2006;89:220 –226.
45. Kallapur SG, Nitsos I, Moss TJ, et al. IL-1 mediates pulmonary and systemic inflammatory responses to chorioamnionitis induced by lipopolysaccharide. Am J Respir Crit Care Med. 2009;179:955–961.

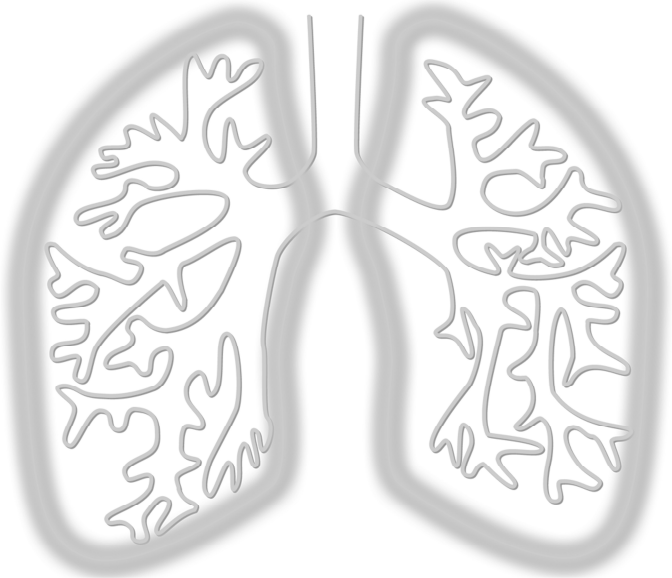
CHAPTER 4

Comparison of airway responses in sheep of different age in precision-cut lung slices (PCLS)

Verena A. Lambermont, Marco Schlepütz*, Constanze Dassow, Peter König, Stefan Uhlig, Boris W. Kramer, Christian Martin*

* both authors contributed equally

PLoS One. 2014 Sep;9(9):e97610



Abstract

Background: Animal models should display important characteristics of the human disease. Sheep have been considered particularly useful to study allergic airway responses to common natural antigens causing human asthma. A rationale of this study was to establish a model of ovine precision-cut lung slices (PCLS) for the *in vitro* measurement of airway responses in newborn and adult animals. We hypothesized that differences in airway reactivity in sheep are present at different ages.

Methods: Lambs were delivered spontaneously at term (~147 days) and adult sheep lived till 18 months. Viability of PCLS was confirmed by the MTT-test. To study airway provocations cumulative concentration-response curves were performed with different allergic response mediators and biogenic amines. In addition, electric field stimulation, passive sensitization with house dust mite (HDM) and mast cells staining were evaluated.

Results: PCLS from sheep were viable for at least three days. PCLS of newborn and adult sheep responded equally strong to methacholine and endothelin-1. The responses to serotonin, leukotriene D4 and U46619 differed with age. No airway contraction was evoked by histamine, except after cimetidine pretreatment. In response to EFS, airways in PCLS from adult and newborn sheep strongly contracted and these contractions were atropine sensitive. Passive sensitization with HDM evoked a weak early allergic response in PCLS from adult and newborn sheep, which notably was prolonged in airways from adult sheep. Only few mast cells were found in the lungs of non-sensitized sheep at both ages.

Conclusion: PCLS from sheep lungs represent a useful tool to study pharmacological airway responses for at least three days. Sheep seem well suited to study mechanisms of cholinergic airway contraction. The notable differences between newborn and adult sheep demonstrate the importance of age in such studies.

Introduction

The prevalence of asthma increased during the last decades (1,2) and may be related to Western lifestyle factors (2,3). However, the causal reasons and underlying mechanisms are not well understood. Several studies have shown that in most cases of persistent asthma, the initial asthma-like symptoms occur during the first years of life (4,5). It has been suggested that the child's environment plays an important role to develop asthma later in life. Children exposed to a farm environment had less asthma and atopy than children grown-up in a non-farming setting (6-9). The mechanism associated with this protective effect is unknown. It is suggested that the child's immune system may be stimulated along a Th1 pathway by early exposure to increased concentrations of bacterial components present in stables such as endotoxin (lipopolysaccharide [LPS]) (10). This theory is known as the hygiene hypothesis (11). For some of these children asthmatic symptoms seem to remit with time, but many children develop asthmatic symptoms which persist throughout their life and are associated with more severe symptoms ending in the loss of lung function. About 15% of the wheezing infants develop persistent wheezing and clinical asthma later in life (12).

Animal models of asthma should display the pathology of the human disease and have carefully to be selected. Several studies indicate for instance that the innervation of the lung differs considerably between and within species (13,14). Moreover, rodent airways do not or only weakly respond to leukotrienes (15), mediators that readily cause bronchoconstriction in humans (16) and guinea pigs (17). Sheep show a greater resemblance to humans concerning lung development compared to rodents. Rodents and guinea pigs undergo the alveolar phase of lung development postnatal whereas sheep and humans undergo this phase in the uterus (18-20). In addition sheep can, like rodents, be sensitized to house dust mite (HDM) antigen which is a common human antigen in asthma, and have allergen-specific IgE responses and acute eosinophil responses to allergen challenge (21,22). Therefore, sheep have been considered particularly useful as models to study allergic airway responses to common human natural antigens.

Airway responses can be visualized by precision-cut lung slices (PCLS), which are viable lung tissue slices of uniform thickness (≈250 µm). PCLS can easily be prepared from different species and are already established for many species including rat, mouse, guinea pig, non-human primates and humans (14,17,23-26). PCLS represent a highly useful model to study bronchial and pulmonary vascular responses by videomicroscopy (23,27). The responses in pulmonary vessels strips of newborn and adult sheep have shown interesting differences in reactivity (28). The diameter of the pulmonary vessels increased from the newborn to the adult animals and the maximum velocity of shortening was in

newborns much higher than in adult sheep. The responses of airways in newborn and adult sheep have not yet been investigated. We hypothesize that differences in airway smooth muscle reactivity in sheep are present at different ages.
In the present study the bronchoconstriction of newborn and adult sheep was studied in PCLS. A rationale of this study was to establish a model of ovine PCLS for the measurement of airway responses to early allergic response mediators and to allergens (after passive sensitization) in newborn and in adult animals.

Materials and Methods

Animal model

All animal procedures were approved by the Animal Ethics Committee of the University of Maastricht, The Netherlands. The newborns (n=7) were delivered spontaneously at term (147 ± 2 days GA). The lambs were euthanized directly after surgical delivery with an intravenous injection of pentobarbital. Adult sheep (n=5) were euthanized at ~18 months with pentobarbital.

Preparation of PCLS

PCLS were prepared from adult or newborn sheep lung as described in previous studies (14,17,23-25) with some modifications. Briefly, the lungs were filled via the lobular bronchus with 1.5% low-melting-point agarose solution and put onto ice until the agarose had solidified. Tissue cores (1 cm in diameter) with a penetrating airway were punched out. Those cylinders were then cut perpendicular to the airway by means of a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA) into approximately 250 µm thin PCLS. PCLS were transferred into a 10 cm cell culture dish and incubated under cell culture conditions (37°C, 5% CO_2 atmosphere) in minimal essential medium (MEM) that was frequently changed during the next 4 hours (h) and incubated overnight. The medium exchange supported the removal of tissue released mediators as well as the wash out of agarose from airways. For measurements, only slices with airways free of agarose, with beating cilia and an intact and relaxed airway smooth muscle layer were used. We only studied PCLS with comparable airway size in parallel experiments to reduce interslice variations. Electric field stimulation (EFS) on PCLS and passive sensitization studies were performed within 24 h after preparation. All other physiological measurements were conducted within 48 h after preparation.

Viability of PCLS

Viability of PCLS over three day incubation was confirmed by intracellular reduction of a tetrazolium dye to its according purple formazan (MTT-test) and constriction responses. PCLS were transferred into cavities of a standard 24−well plate (1 PCLS/well) and incubated with 900 µL MEM + 100 µL 3−(4,5−dimethyl-thiazol−2−yl)−2,5−diphenyl tetrazolium bromide solution (MTT, 7 mg/mL) for 15 min. The supernatant was discarded and the formazan was dissolved by incubation of PCLS with 200 µL formic acid/propanol (5%/95%) solution for 20 min. 100 µL of the purple supernatant were taken and transferred to 96−well plates to measure the extinction at 550 nm (Tecan GENios Microplate Reader). All reactions were carried out at room temperature and in the dark. For negative control measurements PCLS were digested with 0.2% (v/v) Triton−X100 (300 µL, 20 min, 37°C) before performing the MTT−assay.

Videomicroscopy

If not otherwise stated, PCLS were kept in cavities of standard 24-well plates and were immersed in 1 mL MEM during the experiment. The plate was then mounted on the stage of an inverted Leica DMIL microscope (Leica Microsystems, Wetzlar, Germany). The airways were imaged and digitized by videomicroscopy (SensiCam 365KL digital camera, Visitron Systems, Munich, Germany; Optimas 6.5 software, Optimas, Bothell, WA, USA). The airway area before any provocation was defined as 100%−initial airway area [%−IAA].

Airway provocations by early allergic response mediators and biogenic amines

Cumulative concentration-response curves were performed with methacholine (10^{-10} M − 10^{-4} M; 5 min/conc.; pictures every 5 s), serotonin (10^{-10} M − 10^{-4} M; 5 min/conc.; pictures every 5 s), histamine (10^{-9} M − 10^{-4} M; 5 min/conc.; pictures every 5 s), endothelin-1 (10^{-12} M − 10^{-6} M; 10 min/conc.; pictures every 10 s), leukotriene D_4 (LTD_4, 10^{-12} M − 10^{-6} M; 10 min/conc.; pictures each 5 s) and the thromboxane A_2 analogue U46619 (10^{-10} M − 10^{-5} M; 10 min/conc.; pictures every 5 s). To study a potential effect of the histamine H_2-receptor on bronchoconstriction, PCLS were pre-incubated with 10 µM cimetidine for 15 min prior to performing the histamine concentration-response curve.

Electric field stimulation (EFS)

As described before (29), EFS of PCLS was carried out in standard 12-well plates at a reaction volume of 1 mL standard MEM. The PCLS were placed in between two platinum electrodes of 12 mm distance and were mounted by a Teflon ring. The electric field was applied by a Hugo Sachs Electronics Stimulator II (Hugo Sachs Electronics, March Hugstetten, Germany). The electric stimuli were defined by a frequency of 50 Hz, pulse

duration of 1 ms, a current amplitude of 200 mA, a train width of 2.5 s and a train rhythm of 60 s. Each train lasted 3.3 min. After the first control stimulation the muscarinic antagonist atropine (10 µM) was added and incubated for 15 min prior to the second stimulation. In an additional set of EFS experiments, frequency response curves were conducted on PCLS from adult and newborn sheep. In this, the frequency was steadily increased from 0.4 Hz – 100 Hz, while the pulse duration and current amplitude were kept constant at 1 ms and 200 mA, respectively. Each frequency was applied once for 2.5 s and after a pause of one minute the next frequency was applied. The airway behavior of PCLS upon stimulation was monitored by videomicroscopy. Pictures were taken every 2.5 s. Airway area before the first stimulation was defined as 100% initial airway area (IAA).

Passive sensitization

Passive sensitization was performed by incubation with 1% serum from house dust mite (HDM)−sensitized sheep overnight. Medium was replaced by fresh serum−free MEM directly before provocation with 5000 Units of HDM from ALK−SHERAX (Wedel, Germany), which is normally used for intracutaneous testing of atopy. Airway responses were followed by videomicroscopy for 20 min. Pictures were taken every 10 s and pruned for clarity to each fifth data point.

Mast cell staining

Mast cells were stained with toluidine blue or with alcian blue and safranin. Briefly, PCLS were fixed with formaldehyde 4% (Roti® −Histofix 4%, Carl Roth GMBH & Co. KG, Karlsruhe, Germany), dehydrated and embedded in paraffin. Then, 5 µm thick sections were cut, deparaffinized, rehydrated and incubated in 1% toluidin blue for 30 min. After rinsing with water, sections were dehydrated and coverslipped. Alternatively after rehydration, sections were incubated in 0.05% Alcian Blue in 0.02 M acetate buffer, pH 5.8 with 0.2 M MgCl for 4h. After rinsing in water, sections were incubated for 5 min in 0.25% safranin 0.02 M acetate buffer, pH 5.0. After a final rinsing step, sections were dehydrated and coverslipped.

Data analysis

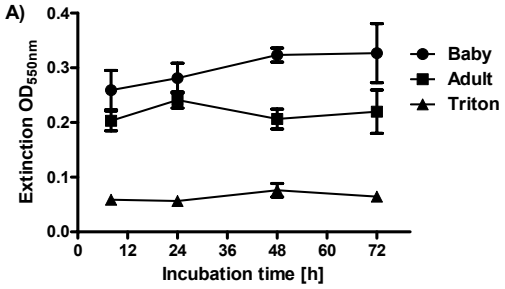
Data are shown as means ± standard error of the mean (SEM). Concentration-response curves were fitted by non-linear regression (4−parameter logistic equation). To analyze differences between curves, each parameter of the 4−parameter logistic equation was separately compared by the Extra sum-of-squares F-test. The 4-parameter logistic equation is [Y=Bottom + (Top-Bottom)/(1+10^((logEC_{50}-X)*HillSlope)], with the bottom being the maximal response (i.e. fit values aim towards the minimum in the concentration

response curves), the top being the initial situation (i.e. fit values aim towards 100%-IAA), the $logEC_{50}$ being calculated to the half maximal response and the slope initially kept variable (i.e. unconstrained from a standard value of 1). A shared concentration-response curve was plotted for adult and newborn sheep, if no difference was found in any parameter of the 4−parameter logistic equation. Minimal airway areas in EFS before and after atropine treatment were compared by Student's t−test (due to homogeneity of variance). Mixed model analysis considering changes in airway area dependent on time and treatment was performed on time courses in the passive sensitization experiments. *P*-values <0.05 were considered significant. The statistical analysis was performed by either GraphPad Prism 5 (GraphPad Software, La Jolla, CA) or SAS 9.1 (SAS Institute Inc., Cary, NC).

Results

Viability of PCLS

More than 50 slices were obtained from one lung lobe of either newborn or adult sheep. The viability of sheep PCLS was demonstrated by the MTT test and PCLS were viable for at least three days (Figure 1). In Figure 1A, high extinctions in this test indicate intact cellular reduction systems, which correlate to viability. PCLS of adult sheep had an extinction OD of 0.2 stable over 3 days compared to deterged control PCLS with an extinction OD of 0.05. PCLS of newborn sheep had also a robust extinction OD of up to 0.3 over 3 days. Functionality of airway smooth muscle contraction was shown by the application of 10^{-4} M methacholine, which evoked a strong bronchoconstriction in sheep PCLS (Figure 1B−C).



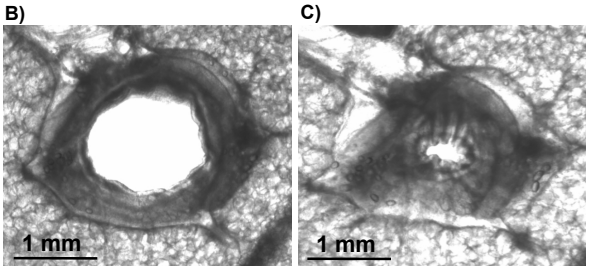


Figure 1: Viability of sheep PCLS. (A) Viability was followed by the MTT test. High extinctions indicate high viability. Data are shown as mean ± SEM, n= 3 PCLS from 3 animals per group.
Exemplary photographs (in black and white) of a PCLS before **(B)** and after **(C)** provocation with 10^{-4} M methacholine.

Airway provocations by allergic response mediators or biogenic amines in adult and newborn sheep

In sheep PCLS all of the mediators of the early allergic response in humans, except for histamine, evoked a marked bronchoconstriction (Figure 2A–F). In general, concentration-response curves are characterized by their efficacy and potency. Shared concentration-response curves in PCLS from adult and newborn sheep were found for methacholine and endothelin-1 with half-maximal responses ($logEC_{50}$ [M]) of -7.0 ± 0.1 and -7.7 ± 0.1, respectively (Figure 2A and 2D).

Age related differences in potency were found for serotonin and LTD_4. For serotonin the $logEC_{50}$ [M] values were -6.3 ± 0.4 for newborn and -6.9 ± 0.2 for adult sheep (Figure 2B), whereas for LTD_4 $logEC_{50}$ values were smaller for newborn than for adult sheep (-9.7 ± 0.4 [M] vs. -8.0 ± 0.6 [M]) (Figure 2E). Differences in efficacy were found for U46619, as airways of adult sheep responded markedly to U46619 ($logEC_{50}$ [M] = -5.9 ± 1.0; max contraction to 64.6 ± 8.4%–IAA at 10^{-5} M), whereas airways of newborn sheep were mostly unresponsive (max. contraction to 93.4 ± 6.5%–IAA at 10^{-5} M) (Figure 2F).

Airway provocation by histamine with or without cimetidine

Since in the preceding experiments no airway contraction was observed by histamine, PCLS were pretreated with or without cimetidine, which blocks the H_2–receptor. Again, airways in control PCLS, i.e. without cimetidine, did not contract in adult sheep (Figure 3). In contrast, stimulation of airways in PCLS with histamine after cimetidine pretreatment resulted in a marked concentration-dependent contraction ($logEC_{50}$ = -6.3) (Figure 3). No data were available for PCLS from newborn sheep.

Intrinsic activation of bronchoconstriction in sheep PCLS

In addition to the preceding exogenous activation of bronchoconstriction in PCLS by exogenous application of mediators, we studied whether an intrinsic activation of airway response is possible in sheep PCLS. In this regard, two approaches were chosen: EFS to study neutrally-induced bronchoconstriction and passive sensitization to evoke an early allergic response by mast cell degranulation after antigen challenge. In response to EFS, PCLS from both adult and newborn sheep, strongly contracted to 43.7 ± 13.9% IAA and 34.3 ± 6.5% IAA (Figure 4), respectively. This neurally–induced response was potently blocked by atropine resulting in an airway contraction to only 84.0 ± 7.6% IAA for PCLS from adult sheep and 78.6 ± 6.8 for PCLS from newborn sheep. Moreover, to distinguish between a difference in sensitivity of PCLS from newborn and adult sheep to EFS, frequency response curves were conducted. However, a shared curve was found (Figure 4 C), indicating equal sensitivity of PCLS from newborn and adult sheep to neural stimulation.

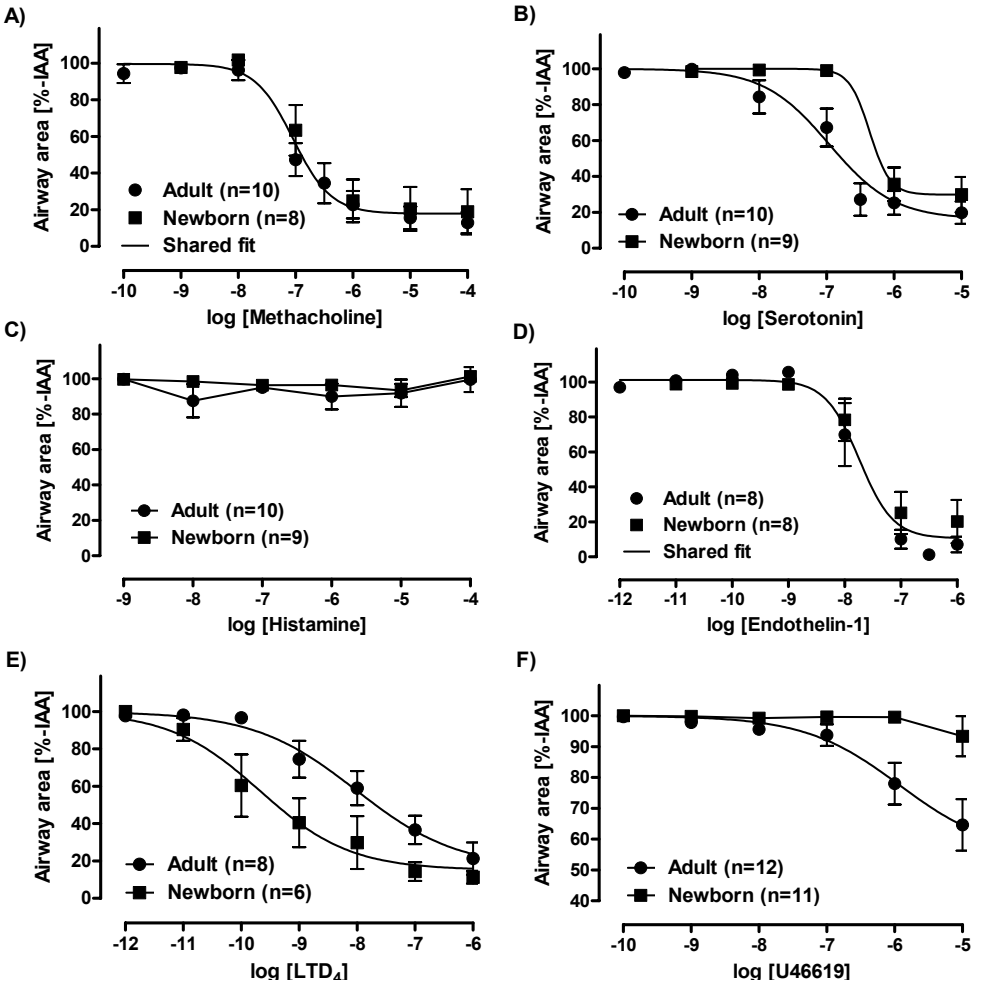


Figure 2: Concentration-response curves for common mediators of early allergic response in PCLS of adult (●) and newborn sheep (■). (**A)** methacholine, **(B)** serotonin, **(C)** histamine, **(D)** endothelin-1, **(E)** leukotriene D_4 (LTD_4), and **(F)** U46619. n = number of PCLS, whereby a total of 7 newborn and 5 adult sheep were examined. Data are shown as mean ± SEM. A shared concentration-response curve was plotted for adult and newborn sheep, if no difference was found in any parameter of the 4−parameter logistic equation.

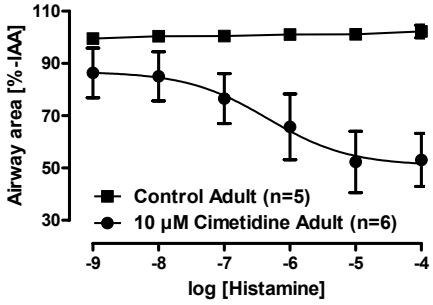


Figure 3: H_2−receptor counteracts histamine-induced bronchoconstriction in adult sheep PCLS. PCLS from adult sheep were either pre-incubated with the H_2-receptor antagonist cimetidine before histamine provocation (●) or kept untreated (control,■). Data are shown as mean ± SEM, n= number of PCLS of separate sheep. The concentration response curves were fitted by the 4−parameter logistic equation.

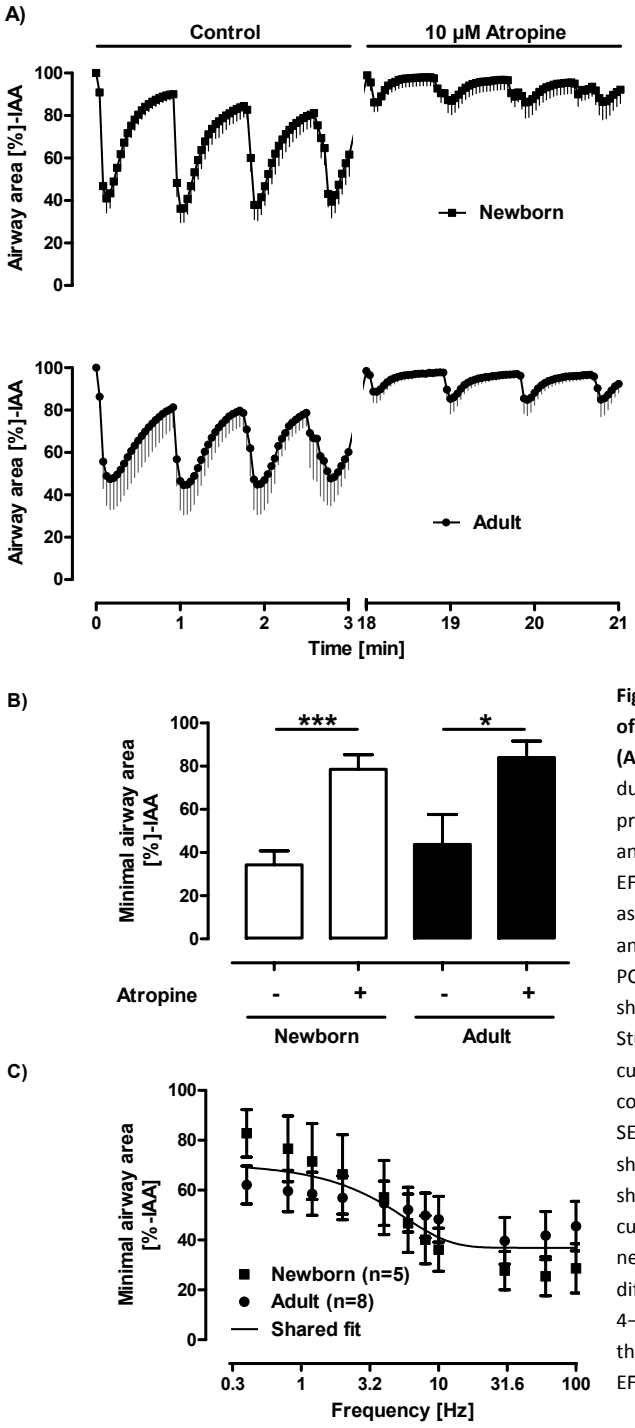


Figure 4: Electric field stimulation (EFS) of PCLS from newborn and adult sheep. **(A)** Course of airway area changes during repeated EFS in absence or presence of atropine. **(B)** Statistical analysis on minimal airway area during EFS as obtained in **(A)**, data are shown as mean ± SEM, n= 7 for newborn sheep and n= 4 for adult sheep, whereby each PCLS was taken from an independent sheep; $*P < 0.05$; $***P < 0.001$ in Student's t−test. **(C)** Frequency-response curves of EFS-induced airway contractions. Data are shown as mean ± SEM, n= 5 PCLS from three newborn sheep and n= 8 PCLS from five adult sheep. A shared frequency-response curve was plotted for adult and newborn sheep, since there was no difference in any parameter of the 4−parameter logistic equation assuming that the top is equal to 100%-IAA and EF_{50} is larger than zero Hz.

With respect to passive sensitization, provocation with 5000 U of HDM evoked a weak but significant early allergic response in PCLS from adult and newborn sheep that had been incubated with serum from HDM sensitized sheep (Figure 5A and 5B). Comparing the HDM-induced airway contractions in PCLS from adult and newborn sheep, a significant difference was found in the respective kinetics. The response to HDM was more sustained in adult than in newborn sheep. In order to study these responses, we stained for mast cells in the lung tissue since we consider them essential for the response. In accordance to the weak response, very few mast cells were found in PCLS from sheep at both ages as shown in Figure 5C and 5D. We found on average less than one mast cell per 5 µm thick section.

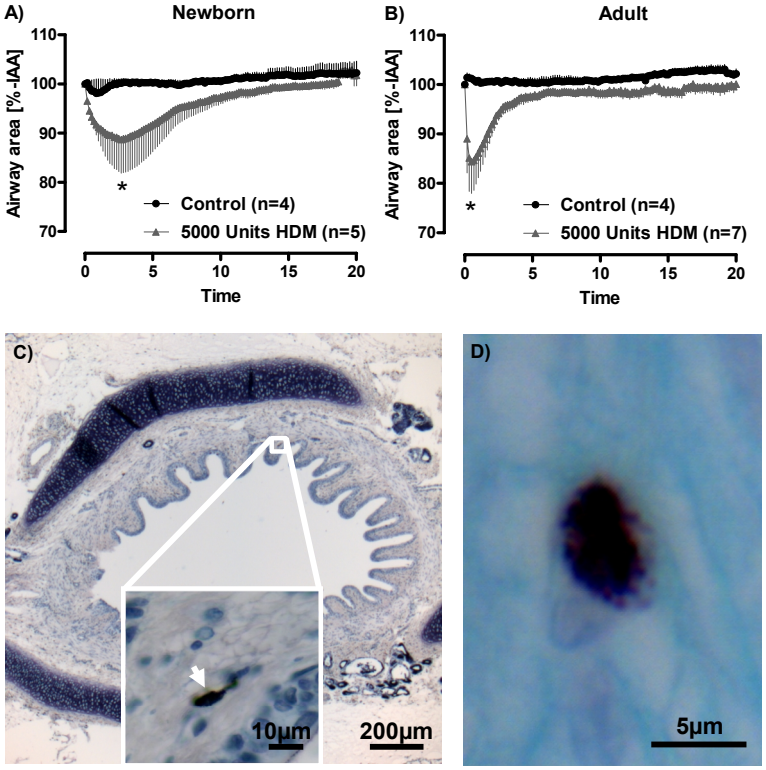


Figure 5: Early allergic response in sheep PCLS after passive sensitization. PCLS from newborn **(A)** or adult **(B)** sheep were passively sensitized with serum from actively sensitized sheep against HDM and provoked with 5000 U HDM. Data are shown as mean ± SEM, n as indicated on PCLS from different sheep. Time courses were statistically compared by mixed model analysis; *$P < 0.05$. Comparing HDM groups in **(A)** and **(B)**, courses are also significant different, which points out differences in the kinetics of allergen-induced bronchoconstriction in adult and newborn sheep. Panels **(C)** and **(D)**, less than one mast cell per 5 µm thick section was found in PCLS from sheep. PCLS were stained for mast cells with toluidine blue **(C**, **arrow)** or with alcian blue and safranin **(D)**.

Discussion

To our knowledge this is the first time, the airway reactivity of newborn and adult sheep was compared in PCLS. Many of the responses were similar in newborn and adult sheep, such as the bronchoconstriction to EFS, and to the mediators methacholine, histamine and endothelin-1. On the other hand different responses in newborn and adult sheep were found for the eicosanoids LTD_4 and thromboxane as well as the biogenic amine serotonin. The response to allergen after passive sensitization was comparable in the extent of contraction, but prolonged in adult sheep.

PCLS are viable, can readily be obtained from different species and offer a great opportunity to study airway responses under cell culture conditions (14). The microarchitecture of lung tissue, i.e. airways and vessels embedded in the parenchyma, remains intact and permit physiological measurements (23-26). Even nerves remain functional and can be specifically activated (29). In the past, PCLS from rat, mouse, guinea pig, non-human primate and human lung have been investigated (14,26). Allergic responses in sheep have been suggested to be similar to humans (18,19,21,22), but the response of airways to early allergic response mediators in newborn compared to adult sheep had not been investigated yet. Recurrent wheezing is common in young infants and toddlers (30). For some of these children asthmatic symptoms seem to remit with time, but many children develop asthmatic symptoms which persist throughout their life (12,31). Therefore, it is important to compare the allergic responses in newborn to adult lung tissue.

Given the importance of small airways for asthma and COPD (24,25,32,33), the possibility to study these airways in PCLS is of clinical relevance. Small airways are defined by their inner diameter being less than 2 mm in human (airway generations >11) and less than 780 µm in rat airways (25,34). Since sheep are comparable in weight to humans, lung/airway sizes and generations should correlate to the human structure. In this context of note, the current experiments were exclusively performed on small airways, since they were less than 2 mm in diameter. Therefore, our results reflect the situation only for the peripheral lung reducing interslice variations since airway responses can differ between large and small airways (23,29).

In the present study PCLS from sheep were viable for at least three days, not different from the viability of human PCLS (24), making it possible to study the effects of cytokines, growth factors and maybe even remodelling processes (35,36). In addition, recent work by Behrsing and colleagues (37) has demonstrated that the process of slicing itself elicits a massive cytokine response, confounding early results of cytokine measurements in response to PCLS challenge. Moreover, in contrast to our study, they also used

transcriptionally active agents like humulin, hydrocortisone and retinoic acid in their incubation medium. In the current study, we have performed frequent medium exchanges during the first 24 h after slicing to minimize the effect of endogenously released mediators. Nonetheless in general, the impact of mechanical damage conducted to create the PCLS should be noticed when cytokines and mediators are studied in PCLS.

PCLS of newborn and adult sheep responded very similar to methacholine and endothelin-1, mediators also effective in humans (17). However, the response to serotonin, the thromboxane analogue U46619 and LTD_4 depended on the age of the sheep. In newborn sheep the response to serotonin and U46619 was weaker than in adult sheep, whereas the response to LTD_4 was stronger in newborn sheep. This is an important difference compared to rodent airways that do not or at best very weakly respond to LTD4 (15). Leukotrienes contribute to airway obstruction in many human asthmatics (29). In this regard sheep PCLS, in particular newborn, may appear as a suitable animal model to study bronchoconstriction. From pharmacological and clinical point of view these results are very interesting. The strong bronchial response of newborn sheep to leukotrienes suggests that young animals will be more sensitive to the effects of montelukast, which is a leukotriene-receptor antagonist and used to prevent the wheezing and shortness of breath caused by asthma. On the other hand, in the present study it is demonstrated that adult sheep have a stronger bronchial response to serotonin. Therefore, adult sheep may be more sensitive to the effects of ketanserin, which is a highly selective antagonist for contractile serotonin 5-HT_{2A} receptors. Our studies show a lifetime dependent response pattern to different antagonist, which suggest specific disease treatment with different antagonist depending on the age of the sheep.

Compared to human PCLS, there are also differences: sheep airways reacted to serotonin rather than to histamine, which is the opposite to what is observed in human and guinea pigs PCLS, in which histamine (EC_{50} 2.7mM) is effective but serotonin is not (17,24,25). The reason for these species differences is not clear. It was suggested that it may, at least in part, be related to age differences in tissue as shown in young healthy guinea pigs versus middle-aged adults (17,24,25). However, the present study demonstrated that the bronchoconstriction in both newborn and adult sheep was the same and that age differences may not play such a crucial role if one compares responses from animal tissue with that from human tissue.

Importantly, however, the lack of responsiveness to histamine was not due to the lack of H_1-receptors. This was revealed by experiments with the H_2-receptor antagonist cimetidine (Figure 3), which has also minor affinity for H_1-, H_3- and H_4-receptors (38,39). Here, we consider effects of H_3 and H_4 activities unlikely, because H_3 is predominantly expressed in brain tissue and H_4 is attributed to immune cell responses (40,41). Moreover,

in literature there are no reports about H_3 and H_4 expression in sheep lung. Since no analysis of the different histamine receptor expression was performed in lung tissue in the present study the discussion about H_3 and H_4 remains speculative and is a clear limitation of this study. Normally, in the lungs histamine acts on H_1- and H_2-receptors and its effect is therefore balanced by the H_1-mediated contraction and the H_2-mediated relaxation of airway smooth muscles (42-45). The observation that histamine contracts tracheal and bronchial airways, but relaxes smaller bronchi and bronchioles (46), indicates that there is a shift towards predominance of H_2−receptors towards the peripheral airways. Thus, the present finding that histamine was able to contract ovine airways only after blockade of H_2-receptors, may reflect the fact that the PCLS were derived from the lung periphery. In addition, the expression of histamine H_2-receptors in the lungs may also differ depending on the type of lung sample, previous sensitization or infection, frequently leading to down-regulation of H_2-receptors (47-49). Therefore, H_2-receptor deficiency in the airways may, at least in part, explain the commonly observed airway hyperreactivity to histamine in asthmatics.

In the present study, the early allergic response in passively sensitized sheep PCLS was weak. One explanation might be that after degranulation mast cells mainly release histamine, which – as seen before – is ineffective unless H_2-receptors are blocked. Alternatively, this finding can be explained by the small number of mast cells found in the airways of PCLS from non-sensitized sheep. It was demonstrated that exposure to HDM (active sensitization) doubled the number of mast cells mostly in alveolar septa and in airway walls (22,50). Notably, these responses required at least 16 weekly HDM challenges (50), while in the present study the sheep lungs were unchallenged and PCLS were only passively sensitized with serum from actively sensitized sheep. This might explain the small numbers of mast cells in the present study. Another interesting observation in the allergen-induced bronchoconstriction after passive sensitization was the prolonged airway contraction in PCLS from adult sheep compared to newborn sheep. To our knowledge this is the first study examining the early allergic response of adult and newborn animals in parallel. Reasons, such as a sustained mediator release, diminished degradation of bronchoconstrictors or different receptor density in adult sheep, are therefore speculative. Nonetheless, this example confirms, that extreme caution is mandated selecting the appropriate animal model, when studying childhood asthma or asthma in adults as responses may differ tremendously.

We further demonstrated that ovine PCLS respond to electric field stimulation. Airway responses to electric field stimulation differ largely between species (14). PCLS from rats and marmoset airways contract by about 20%, whereas guinea pigs, sheep and humans contract at maximum by about 40−60% (14). The present study adds that the maximum

contraction (>50%) in both newborn and adult sheep did not differ. Moreover, since the sensitivity of sheep PCLS from newborn and adult sheep did not differ, one may speculate that the neural network in sheep lung is widely established by birth. The shared frequency-response curve is also in line with the exogenous application of methacholine, in which also a shared curve, i.e. same sensitivity to the agonist, was found. These findings are rounded off by the high atropine-sensitivity of EFS-induced airway contractions pointing out that lung innervation in sheep is mostly cholinergic. Hence, airway responses in sheep PCLS are a reasonable proxy for human airways, if cholinergic airway contractions are intended to study.

In our model we focused on the acute response to exogenously added inflammatory mediators and passive HDM sensitization to demonstrate that ovine PCLS are a reliable tool for *in vitro* measurement of airway responses. It was beyond the scope of this study to examine the role of cytokine and mediator secretion in response to a physiologically relevant stimulus or active sensitization. In future research it will be relevant to compare acute versus chronic responses after HDM sensitization and also to compare HDM to sensitization with other allergens. In conclusion, PCLS from sheep lungs represent a useful tool to study pharmacological airway responses for at least three days. Sheep seem well suited to study mechanisms of cholinergic airway contraction. Their airway pharmacology differs in some respects to that observed in humans. Bronchoconstriction is similar in newborn and adult sheep, except for the lipid mediators (LTD4, U46619) and serotonin. Early allergic response is weak which is probably based on a small number of mast cells.

References

1. Manning PJ, Goodman P, O'Sullivan A, et al. Rising prevalence of asthma but declining wheeze in teenagers (1995-2003): ISAAC protocol. Ir Med J 2007;100:614-615.
2. Beasley R, Crane J, Lai CK, et al. Prevalence and etiology of asthma. J Allergy Clin Immunol 2000;105:S466-472.
3. Britton J. Parasites, allergy, and asthma. Am J Respir Crit Care Med 2003;168:266-267.
4. Martinez FD, Wright AL, Taussig LM, et al. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med 1995;332:133-138.
5. Barbee RA, Dodge R, Lebowitz ML, et al. The epidemiology of asthma. Chest 1985;87:21S-25S.
6. Von Ehrenstein OS, Von Mutius E, Illi S, et al. Reduced risk of hay fever and asthma among children of farmers. Clin Exp Allergy 2000;30:187-193.
7. Riedler J, Eder W, Oberfeld G, et al. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. Clin Exp Allergy 2000;30:194-200.
8. Braun-Fahrlander C, Gassner M, Grize L, et al. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. Clin Exp Allergy 1999;29:28-34.

9. Riedler J, Braun-Fahrlander C, Eder W, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 2001;358:1129-1133.
10. von Mutius E, Braun-Fahrlander C, Schierl R, et al. Exposure to endotoxin or other bacterial components might protect against the development of atopy. Clin Exp Allergy 2000;30:1230-1234.
11. Renz H, Blumer N, Virna S, et al. The immunological basis of the hygiene hypothesis. Chem Immunol Allergy 2006;91:30-48.
12. Guilbert T, Krawiec M. Natural history of asthma. Pediatr Clin North Am 2003;50:523-538.
13. Abraham WM. Animal models of asthma. In: Busse WW, Holgate ST, editors. Asthma and rhinitis. Oxford ; Malden, MA, USA: Blackwell Science. 2000;pp.1205-1227.
14. Schleputz M, Rieg AD, Seehase S, et al. Neurally Mediated Airway Constriction in Human and Other Species: A Comparative Study Using Precision-Cut Lung Slices (PCLS). PloS one 2012;7:e47344.
15. Held HD, Martin C, Uhlig S. Characterization of airway and vascular responses in murine lungs. Br J Pharmacol 1999;126:1191-1199.
16. Dahlen SE, Hedqvist P, Hammarstrom S, et al. Leukotrienes are potent constrictors of human bronchi. Nature 1980;288:484-486.
17. Ressmeyer AR, Larsson AK, Vollmer E, et al. Characterisation of guinea pig precision-cut lung slices: comparison with human tissues. Eur Respir J 2006;28:603-611.
18. Kramer BW. Chorioamnionitis - new ideas from experimental models. Neonatology 2011l99:320-325.
19. Pringle KC. Human fetal lung development and related animal models. Clin Obstet Gynecol 1986;29:502-513.
20. Wolfs TG, Jellema RK, Turrisi G, et al. Inflammation-induced immune suppression of the fetus: a potential link between chorioamnionitis and postnatal early onset sepsis. J Matern Fetal Neonatal Med 2012;25 Suppl 1:8-11.
21. Bischof RJ, Snibson K, Shaw R, et al. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. Clin Exp Allergy 2003;33:367-375.
22. Snibson KJ, Bischof RJ, Slocombe RF, et al. Airway remodelling and inflammation in sheep lungs after chronic airway challenge with house dust mite. Clin Exp Allergy 2005;35:146-152.
23. Martin C, Uhlig S, Ullrich V. Videomicroscopy of methacholine-induced contraction of individual airways in precision-cut lung slices. Eur Respir J 1996;9:2479-2487.
24. Wohlsen A, Martin C, Vollmer E, et al. The early allergic response in small airways of human precision-cut lung slices. Eur Respir J 2003;21:1024-1032.
25. Wohlsen A, Uhlig S, Martin C. Immediate allergic response in small airways. Am J Respir Crit Care Med 2001;163:1462-1469.
26. Seehase S, Schleputz M, Switalla S, et al. Bronchoconstriction in nonhuman primates: a species comparison. J Appl Physiol 2011;111:791-798.
27. Rieg AD, Rossaint R, Uhlig S, et al. Cardiovascular agents affect the tone of pulmonary arteries and veins in precision-cut lung slices. PloS one 2011;6:e29698.
28. Belik J, Halayko A, Rao K, et al. Pulmonary vascular smooth muscle: biochemical and mechanical developmental changes. J Appl Physiol 1991;71:1129-1135.
29. Schleputz M, Uhlig S, Martin C. Electric field stimulation of precision-cut lung slices. J Appl Physiol 2011;110:545-554.
30. Robison RG, Singh AM. Chapter 11: the infant and toddler with wheezing. Allergy Asthma Proc 2012;33 Suppl 1:S36-38.
31. Been JV, Lugtenberg MJ, Smets E, et al. Preterm birth and childhood wheezing disorders: a systematic review and meta-analysis. PLoS Med 2014;11:e1001596.

32. Sturton RG, Trifilieff A, Nicholson AG, et al. Pharmacological characterization of indacaterol, a novel once daily inhaled 2 adrenoceptor agonist, on small airways in human and rat precision-cut lung slices. J Pharmacol Exp Ther 2008;324:270-275.
33. Sturton G, Persson C, Barnes PJ. Small airways: an important but neglected target in the treatment of obstructive airway diseases. Trends Pharmacol Sci 2008;29:340-345.
34. Yeh HC, Schum GM, Duggan MT. Anatomic models of the tracheobronchial and pulmonary regions of the rat. Anat Rec 1979;195:483-492.
35. Switalla S, Lauenstein L, Prenzler F, et al. Natural innate cytokine response to immunomodulators and adjuvants in human precision-cut lung slices. Toxicol Appl Pharmacol 2010.
36. Kasper M, Seidel D, Knels L, et al. Early signs of lung fibrosis after in vitro treatment of rat lung slices with CdCl2 and TGF-beta1. Histochem Cell Biol 2004;121:131-140.
37. Behrsing HP, Furniss MJ, Davis M, et al. In vitro exposure of precision-cut lung slices to 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole lysylamide dihydrochloride (NSC 710305, Phortress) increases inflammatory cytokine content and tissue damage. Toxicol Sci 2013;131:470-479.
38. Wade L, Bielory L, Rudner S. Ophthalmic antihistamines and H1-H4 receptors. Curr Opin Allergy Clin Immunol 2012;12:510-516.
39. Bielory L, Ghafoor S. Histamine receptors and the conjunctiva. Curr Opin Allergy Clin Immunol 2005;5:437-440.
40. Zampeli E, Tiligada E. The role of histamine H4 receptor in immune and inflammatory disorders. Br J Pharmacol 2009;157: 24-33.
41. Gantner F, Sakai K, Tusche MW, et al. Histamine h(4) and h(2) receptors control histamine-induced interleukin-16 release from human CD8(+) T cells. J Pharmacol Exp Ther 2002;303:300-307.
42. Bongers G, de Esch I, Leurs R. Molecular pharmacology of the four histamine receptors. Adv Exp Med Biol 2010;709:11-19.
43. Okamoto T, Iwata S, Ohnuma K, et al. Histamine H1-receptor antagonists with immunomodulating activities: potential use for modulating T helper type 1 (Th1)/Th2 cytokine imbalance and inflammatory responses in allergic diseases. Clin Exp Immunol 2009;157:27-34.
44. Chand N, Eyre P. Classification and biological distribution of histamine receptor sub-types. Agents Actions 1975;5:277-295.
45. Eyre P, Chand N. Histamine receptor mechanisms of the lung. In: Ganellin CR, Parsons ME, editors. Pharmacology of histamine receptors. Bristol ; Boston: PSG. 1982;pp.298-332.
46. Yre P. The pharmacology of sheep tracheobronchial muscle: a relaxant effect of histamine on the isolated bronchi. Br J Pharmacol 1969;36:409-417.
47. Chand N. Distribution and classification of airway histamine receptors: the physiological significance of histamine H2-receptors. Adv Pharmacol Chemother 1980;17:103-131.
48. Chand N. Is airway hyperactivity in asthma due to histamine H2-receptor deficiency? Med Hypotheses 1980;6:1105-1112.
49. Foreman JC. Histamine H2 Receptors and Lung Function. In: Arrang JM, Uvnäs B, editors. Histamine and histamine antagonists. Berlin ; New York: Springer Berlin Heidelberg. 1991;pp.285-304.
50. Van der Velden J, Barker D, Barcham G, et al. Increased mast cell density and airway responses to allergic and non-allergic stimuli in a sheep model of chronic asthma. PLoS One 2012;7:e37

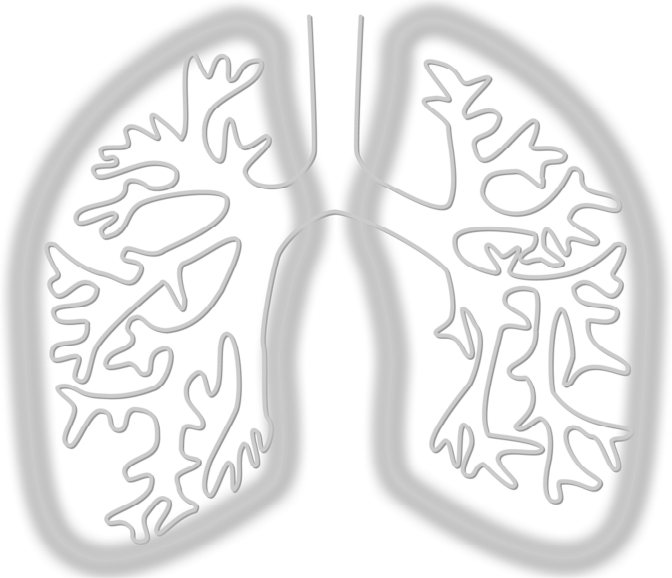
CHAPTER 5

Fetal responses to lipopolysaccharide-induced chorioamnionitis alter immune and airway responses in 7-week-old sheep

Verena A. Lambermont, Andrea J. Lee*, Jane J. Pillow, Graeme R. Polglase, Ilias Nitsos, John P. Newnham, Manfred W. Beilharz, Suhas G. Kallapur, Alan H. Jobe, Boris W. Kramer*

* both authors contributed equally

Am J Obstet Gynecol. 2011 Apr;204(4):364.e17-24



Abstract

Background: We hypothesized that fetal innate immune responses to lipopolysaccharide-induced chorioamnionitis would alter postnatal systemic immune and airway responsiveness.

Methods: Ewes received intra-amniotic injections with saline or lipopolysaccharide at 90, 100 and 110 days of gestation. Immune status and airway responsiveness were evaluated at term and at 7 weeks of age.

Results: At term, lymphocytes, monocytes and neutrophils were significantly increased (respectively 24-fold, 127-fold, and 31,000-fold) in lungs and blood monocytes became Toll-like receptor 2 responsive after lipopolysaccharide exposures. Furthermore, CD4 and CD4/CD25 lymphocytes were increased in thymus and lymph nodes. At 7 weeks, airway reactivity decreased and concentrations of CD8 cytotoxic T lymphocytes changed in the lungs and thymus relative to controls.

Conclusion: Early gestational lipopolysaccharide exposure increased leukocyte responsiveness at term. Decreased airway reactivity and changes in lymphocytes at 7 weeks postnatal demonstrate persistent effects of fetal exposure to LPS.

Introduction

Preterm birth (<37 weeks) is frequently associated with chorioamnionitis (1). Recently, chorioamnionitis was correlated with a 4-fold increased risk for developing asthma in preterm infants after correction for confounding factors (2-4). This observation is in contrast to the hygiene hypothesis (5-7), in which infections during early childhood are associated with a decreased risk of subsequent asthma and allergic disease. These conflicting observations suggest that the time of exposure to bacterial products during childhood may have different effects when compared with antenatal exposures. For example, antenatal exposure to bacterial products may increase the risk of asthma in childhood, whereas postnatal exposure to bacterial products may protect from asthma. The mechanisms to explain the different outcomes are still unclear.

Chorioamnionitis-mediated inflammation exposes the fetus to proinflammatory mediators primarily by fetal breathing and swallowing of amniotic fluid (8-10). The fetal lung responds to mediators such as lipopolysaccharide (LPS), interleukin-1 (IL-1), and live ureaplasma with a local inflammatory response. Previous experiments revealed that exposure to chorioamnionitis changes the lymphocyte population in the posterior mediastinal lymph node (PMLN) that drains the lung (11,12) and causes systemic immune modulation of blood monocytes (13-15). Furthermore, the gut responses to LPS with altered inflammatory cells in the gut wall and regional lymph nodes (8,16). These acute fetal responses were modulated further with repeated exposure to induce LPS-tolerance and cross-tolerance to other proinflammatory Toll-like receptor (TLR) agonists (13-15). However, the relationship between exposure to antenatal inflammation and postnatal disease is confounded by gestational age and neonatal lung disease in human studies.

We hypothesized that fetal innate immune responses to LPS-induced chorioamnionitis would alter postnatal systemic immune and airway responsiveness resembling asthma later in life. To test this hypothesis, we evaluated immune status at birth and at 7 weeks after LPS-mediated chorioamnionitis. We evaluated leukocytes in the bronchoalveolar lavage fluid (BALF). Transforming growth factor-beta 1 (TGF-β_1) and elastin were measured in lung tissue. CD4, CD8, CD4/CD25 and gamma-delta subpopulations of T lymphocytes were measured in the PMLN and thymus. Monocytes isolated from blood were tested *in vitro* for responses to LPS and PamCysK4 (a ligand for TLRs 1 and 2). The immunologic measurements were performed at term and at 7 weeks of age and airway reactivity to methacholine (MCh) was tested at 7 weeks after inhalation of house dust mite (HDM).

Materials and Methods

Antenatal treatment

All animal procedures were approved by the Animal Ethics Committee of the University of Western Australia, Australia. Ewes received intra-amniotic (IA) injections with saline (controls) or 10 mg *Escherichia coli* 055:B5 endotoxin (LPS; Sigma-Aldrich, St.Louis, MO) at 90, 100, and 110 days of gestation (Figure 1). At 147 days of gestation, lambs from LPS (n=5) and control (n=3) groups were delivered surgically to assess immune function of the fetus at term (term is ~147 days). The remaining ewes delivered spontaneously and airway responsiveness and immune status of lambs were evaluated at 7 weeks (LPS n=5, Control n=6). Lambs were killed with intravascular pentobarbital at term and at 7 weeks.

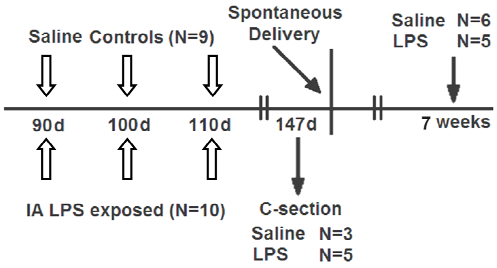


Figure 1: Timing of fetal exposure to LPS. Open arrows indicate gestational age of intra-amniotic (IA) injections with LPS or saline. Sheep model with LPS-induced chorioamnionitis. Operative delivery was performed at 147 days gestation for some animals (n=3-5 lambs), whereas the remaining ewes (n=5-6) delivered spontaneously and their lambs were studied at 7 weeks. Immune status was evaluated in postmortem tissues at 147 days and at 7 weeks. LPS, lipopolysaccharide.

Airway reactivity

At 7 weeks lambs were sedated 2 days after HDM exposure (nebulized 1 mg) with diazepam 0.25 mg/kg (Sigma NSW, Australia) and ketamine 5 mg/kg (Parnell Laboratories, NSW, Australia). They were intubated with a 6.0 mm ID tracheal tube (Portex Ltd, UK) and ventilated (Humming V, Metran, Japan) with a peak inspiratory pressure of 25 cm H_2O, an inspiratory time 0.7 second and a rate of 40 breaths per minutes. A continuous infusion of intravenous Propofol (0.3-0.6 mg/kg/h, Repose, 0.1 mg/kg/min, Norbrook Laboratories Ltd., Victoria, Australia) and Remifentanil (0.3 mg/kg/h, Ultiva 0.05 µg/kg/min; GlaxoSmithKline, Victoria, Australia) was commenced, and following confirmation of deep anaesthesia, neuromuscular blockade was achieved with vecuronium (0.1 mg/kg IV; Essex Pharma, Germany). Lambs were stabilized for 10 minutes before baseline measurements, and then received for 1 minute aerosols (1 mL) at 5 minute intervals of saline, followed by increasing concentrations (0.01%, 0.03%, 0.1%, 0.3%, 1%; w/v) of methacholine (Acetyl-β-

methylcholine chloride; Sigma). Partitioned respiratory impedance (Z_{rs}) was measured using the low-frequency forced oscillation technique (LFOT) using the FlexiVent (Module 5; Scireq, Montreal, Canada). After airway occlusion at commencement of expiration, respiratory system input impedance (Z_{rs}) measurements were obtained using a primewave (17 mutually prime frequencies from 0.5-19.75 Hz) over a 6-second apneic interval. Measurements were repeated every 30 seconds until peak response was observed (normally approximately 3 minutes).

Measurements of pressure and flow were transformed to the frequency domain and corrected for the impedance of the tracheal tube and measurement system. The partitioned airway (airway resistance - R_{aw}; airway inertance - I_{aw}) and tissue mechanical variables (tissue damping – G; tissue elastance - H) were determined by fitting the resultant impedance spectrum to the constant phase model (17). Hysteresivity (η) was calculated as G/H. Changes in airway and tissue variables were expressed relative to measurements obtained at baseline.

Bronchoalveolar lavage

After collection of the lungs, thymus and the PMLN, the left lung was lavaged 3 times with 0.9% NaCl (18). The BALFs were pooled and centrifuged at 500 rpm for 5 minutes. Differential cell counts were performed on cytospin preparations after staining with Pappenheim-staining (May-Grünwald, Giemsa) (18).

Lung tissue

Lung tissue from the right lower lobe (RLL) was snap frozen and stored at -80°C. For homogenization, a mix of lysis buffer (RIPA buffer, Sigma) and protease inhibitor (Sigma) was added to lung tissue. The lung tissue was homogenized (PRO Quick Connect Generators part no. 02-07095; PRO Scientific Inc., Oxford, CT) and centrifuged at 12 rcf (11.4 rpm) for 5 minutes at 4°C.

Enzyme-linked immunosorbent assay of TGF-β_1

Free, bound, and total TGF-β_1 (referred to R&D enzyme-linked immunosorbent assay [ELISA] kit; Minneapolis, MN as active, latent and total TGF-β_1) were measured with R&D ELISA kits (DuoSet ELISA, human TGF/β1, no. DY240; R&D Systems). Free TGF-β_1 was measured for the original sample. Total TGF-β_1 was measured after acid activation of 150 µL of the sample with 30 µL 1 M HCl for 10 minutes at room temperature and 33 µL 1 M NaOH HEPES was used to stop the activation (19). Bound TGF-β_1 was calculated as the difference between total TGF-β and free TGF-β_1.

Elastin staining

We measured elastin foci of secondary crests (concentrated vs. non-concentrated) and elastin fiber deposition in the vessel walls. Elastin fibers were stained black with a Hart's resorcin fuchsin solution (20-22), using paraffin slides that were dehydrated and stained at 60°C and counterstained with a tartrazine solution. A magnification of 200x was used to save 3 pictures randomly. Counting of elastin foci was blinded, using ImageJ 1.41o software (Rasband; National Institutes of Health, Bethesda, MD) for quantification.

Flow cytometry

Single cell suspensions of thymus and PMLN were made with a strainer, assessed for viability, and counted using a Neubauer chamber (Hawksley, England) (23). The 10^6 cells were incubated with primary monoclonal antibodies (mAbs: CD4, CD8, CD25 and TcR-1 receptor [gamma-delta T lymphocytes]; VMRD USA) for 30 minutes at 4°C. Cells were washed and incubated with fluorescence-conjugated secondary mAbs for 30 minutes at 4°C in the dark (FITC and R-PE labeled antibodies from SEROTEC, Great Britain). Cells were analyzanaled on a FACScalibur instrument (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software (Becton Dickinson). CD4/CD25 percentages are expressed relative to the population of cells expressing CD4. Other lymphocyte percentages are expressed relative to the total population of thymic lymphocytes (11).

Hydrogen peroxide assay for blood monocytes

Monocytes and macrophages were isolated with Percoll gradients from cord blood (at term) and peripheral blood (at 7 weeks) to perform functional tests (15). In brief, the isolated cells were cultured in RPMI 1640 media supplemented by 10% heat-inactivated fetal calf serum for 6 hours with LPS (a ligand for TLR4; 100 ng/mL; *E. coli* O55:B5; Sigma-Aldrich) and PamCysK4 (a synthetic ligand for TLRs 1 and 2; 5 µg/mL; EMC Microcollection, Tuebingen, Germany). PamCysK4 signals through the TLR2 pathway and is involved in maturation of the immune system. Control cells were exposed to saline (media). After washing the cells with phosphate buffered saline (PBS), the production of hydrogen peroxide by 1 X 10^6 monocytes was measured with an assay based on the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) by hydrogen peroxide under acidic conditions (Bioxytech H_2O_2-560 assay; OXIS International, Portland, OR) (13,15).

Statistical analysis

Microsoft Excel and GraphPad Prism 5 were used for statistics. Data are shown as mean ± SEM. Statistical differences between LPS and control groups were evaluated with Student *t* test and the Mann-Whitney test. Values were considered significant if $P \leq 0.05$ versus the control (saline) group.

Results

Body and organ weights at birth and at 7 weeks of age

The body weights of LPS-exposed lambs were similar to the control group at term and at 7 weeks (table 1) indicating similar growth and development. The organ weights (PMLN, thymus, spleen, total lung) were not different for the groups at birth or at 7 weeks of age.

Table 1: Physiology.

Antenatal treatment		Body weight, kg	PMLN, g/kg	Thymus, g/kg	Spleen, g/kg	Total lung, g/kg	n
At Term	Control	5.0 ± 1.0	0.13 ± 0.01	2.07 ± 1.07	1.37 ± 0.18	35.3 ± 3.3	3
	LPS	5.5 ± 1.0	0.21 ± 0.02	3.59 ± 0.43	1.74 ± 0.20	31.8 ± 2.4	5
7 weeks	Control	16.2 ± 1.3	0.23 ± 0.02	2.52 ± 0.19	4.20 ± 0.30	12.9 ± 0.4	6
	LPS	15.5 ± 1.5	0.26 ± 0.02	2.68 ± 0.48	4.62 ± 0.50	13.7 ± 0.4	5

Body weight, organ weights, and number of animals studied at birth and at 7 weeks of age. Organ weights are standardized per kilogram body weight. There were no differences to corresponding control group. LPS, lipopolysaccharide; PMLN, posterior mediastinal lymph node; n, number of animals.

Table 2: Ventilatory variables of lambs during methacholine challenge.

Treatment	PaO_2, mm Hg	$PaCO_2$, mm Hg	RR, breaths/min	PIP, cm H_2O	HR, bpm
Saline (7 weeks)	96 ± 4	53 ± 4	26.5 ± 0.5	25.8 ± 1.9	175 ± 12
LPS (7 weeks)	98 ± 1	51 ± 1	25.1 ± 0.7	26.8 ± 2.3	161 ± 7

HR, heart rate; LPS, lipopolysaccharide; $PaCO_2$, arterial carbon dioxide tension; PaO_2, arterial oxide tension; PIP, peak-inflation pressure; RR, respiratory rate.

Airway reactivity

Measurements with methacholine challenge at 7 weeks, including heart rate, peak inspiratory pressure, respiratory rate and blood gas values ($PaCO_2$, PaO_2) were not different between fetal LPS exposed and control lambs (table 2). Representative impedance spectra are shown in Figure 2.

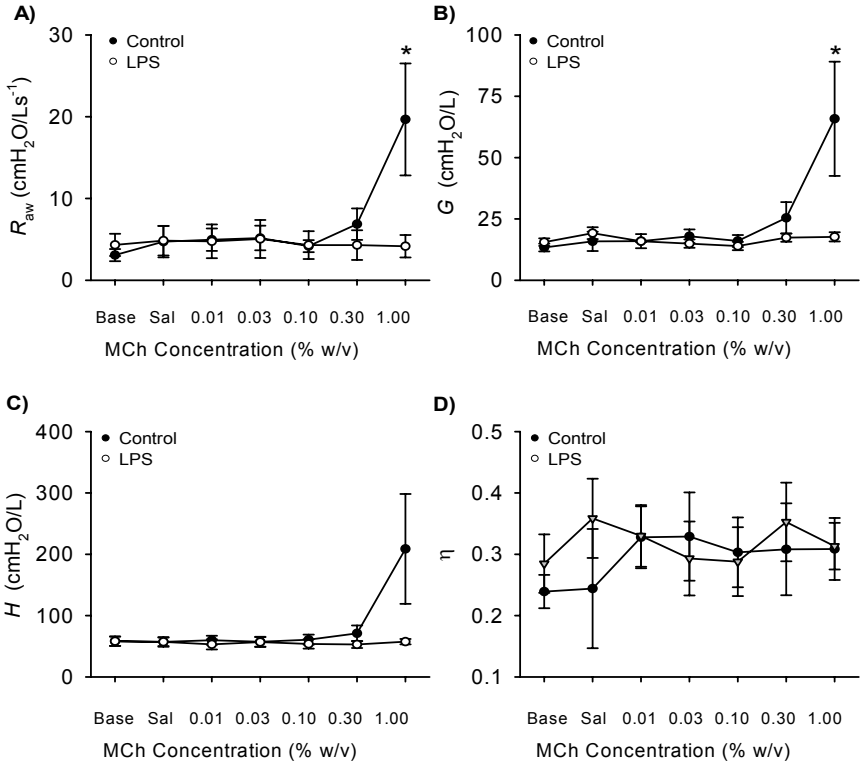


Figure 2: Dose response curves to inhaled methacholine. Responses to inhaled methacholine are expressed relative to measurements at baseline for 7-week old lambs that were exposed to intra-amniotic injections of LPS or saline (control). **(A)** Airway resistance, **(B)** tissue damping, **(C)** tissue elastance, and **(D)** hystersivity were measured to test the airway reactivity. Airway resistance (R_{aw}; P=0.008) and tissue damping (G; P=0.037) were significantly decreased with 1% w/v MCh. Tissue elastance (H; P=0.151) and hystersivity (η) showed no differences compared to the control group. LPS, lipopolysaccharide; MCh, methacholine.

There were no differences in baseline measurements between the groups. Airway resistance (R_{aw}; P=0.008; Figure 2A) and tissue damping (G; P=0.037; Figure 2B) did not increase at 1% w/v MCh in LPS-exposed lambs, whereas they increased in the saline control animals, indicating decreased airway reactivity with fetal LPS exposure. LPS-exposed lambs and control lambs did not respond to lower MCh dosing. Tissue elastance (H; Figure 2C) and hysteresivity (η; Figure 2D) were not different at any MCh concentration between LPS-exposed and control lambs.

Pulmonary inflammation

Monocytes, lymphocytes and neutrophils were greatly increased in the BALF from the LPS-exposed term fetal lambs relative to the controls 37 days after the last IA injection (*P*<0.001 versus control, Figure 3A). However, by 7 weeks there were no significant differences in the number of inflammatory cells in BALF for the LPS-exposed and control lambs (Figure 3B).

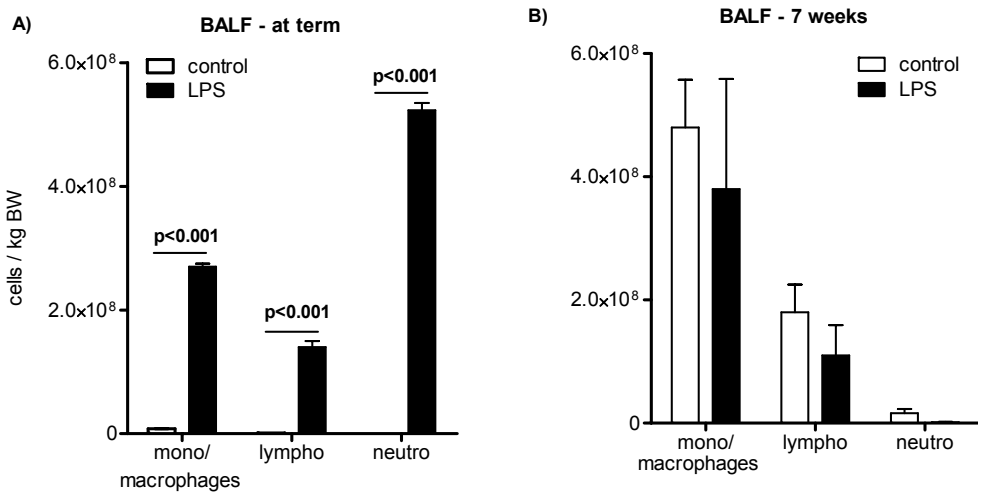


Figure 3: Leukocytes in BALF from lambs (A) at term and (B) at 7 weeks of age. Monocytes, lymphocytes and neutrophils were increased (*P*<0.001 versus control) in the LPS-exposed group at term. However, no significant differences were evident at 7 weeks, indicating resolution of the pulmonary inflammation present at term. BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide.

Hydrogen peroxide responses of blood monocytes

After stimulation *in vitro* with LPS, blood monocytes from control term lambs had no increased response, but after PamCysK4 stimulation a 6-fold increase was present (Figure 4A). At 7 weeks, blood monocytes from control lambs responded to LPS and PamCysK4 (16-fold and 14-fold increases respectively; Figure 4B). This change of monocyte responses between term and 7 weeks is explained by the maturation of the innate immune system in early postnatal life. LPS-exposed animals had a different monocyte response pattern. At term, a stimulation *in vitro* with LPS and PamCysK4 increased H_2O_2 5-fold and 9-fold, respectively (Figure 4A). This increase was still present at 7 weeks: 6-fold increase with LPS and 5-fold increase with PamCysK4 (Figure 4B). Comparing term LPS-exposed lambs and control lambs after a specific stimulation, an increased monocyte response was only present after stimulation with PamCysK4 (Figure 4A). At 7 weeks, no differences in the response were evident after PamCysK4 or LPS *in vitro* stimulation in LPS-exposed lambs when compared to control lambs (Figure 4B).

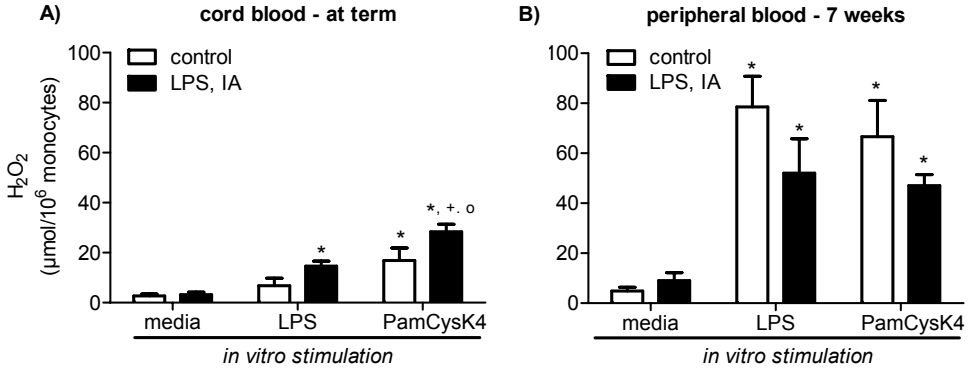


Figure 4: Hydrogen peroxide production by blood monocytes (A) at term and (B) at 7 weeks of age in cord blood. At term, hydrogen peroxide production was significantly increased after stimulation of cells with PamCysK4. At 7 weeks of age, this response was reversed, suggesting maturation of the immune system. LPS, lipopolysaccharide. (*) versus media; (+) versus control group; (o) LPS versus PamCysK4.

TGF-β_1 in lung tissue

At term large amounts of bound TGF-β_1 were detectable with only a small concentration of free TGF-β_1, in lung tissue homogenate of the control group (Figure 5A). In LPS-exposed fetuses, bound TGF-β_1 in lung homogenate was decreased relative to controls at term (P<0.002). However, there was no increase in free TGF-β_1, which is the biological active component of TGF-β_1 that can impair lung development and cause inflammation and airway remodelling. At 7 weeks of age, free TGF-β_1 concentration was increased in the lung of LPS-exposed lambs (P=0.048, Figure 5B).

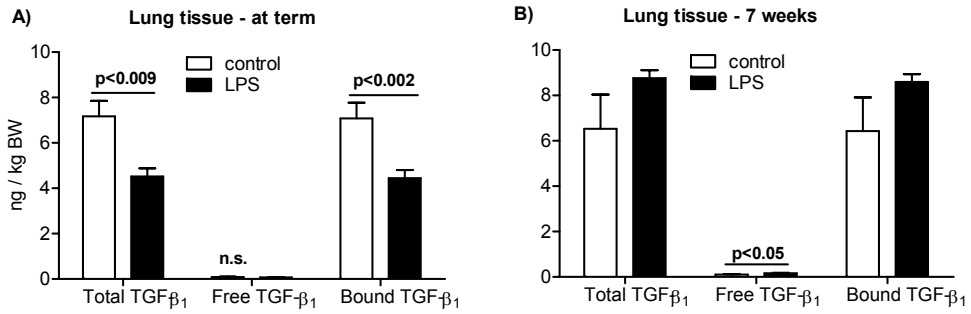


Figure 5: TGF- β_1 was measured in homogenates of lung tissue from lambs (A) at term and (B) after 7 weeks of age. At term, the concentration of total and bound TGF-β_1 was significantly decreased after LPS exposure, but free TGF-β_1 was unchanged. At 7 weeks a significant increase of free TGF-β_1 was detected in the LPS group, but no differences in bound or total TGF-β_1 were found. LPS, lipopolysaccharide; n.s., not significant; TGF-β_1, transforming growth factor-beta 1.

Elastin

At term, control and LPS-exposed lambs had a normal amount of elastin staining as concentrated elastin foci, with no differences between groups (Figure 6A) and no differences in the elastin deposition in the blood vessel walls (Figure 6B and 6C). At 7 weeks, about 80% of the elastin was nonconcentrated in both animal groups, indicating lungs alveolarization and maturation (Figure 6D).There was less elastin deposition in the blood vessel walls of the LPS-exposed lungs at 7 weeks of age than in controls (Figure 6E and 6F).

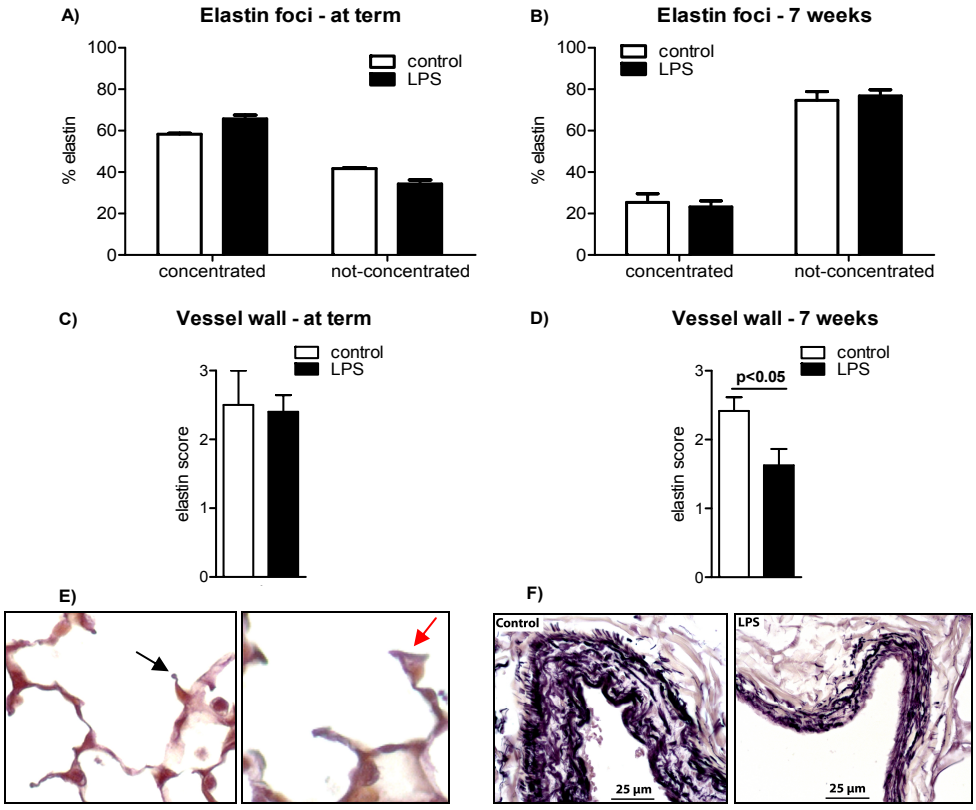


Figure 6: Elastin (stained in black) in lung tissue from lambs (A, C) at term and (B, D) at 7 weeks of age. (A, B, E) Elastin foci were distinguished between concentrated (black arrow) and non-concentrated (red arrow) foci. **(C, D, F)** Elastin scoring in the vessel wall was performed on a scale from 0-3. At 7 weeks less elastic fibers were present in the lungs after exposure to LPS. No differences in elastic foci were noticed at term and at 7 weeks of age. LPS, lipopolysaccharide.

Lymphocytes in the thymus and PMLN

Lymphocyte populations in the thymus and PMLN had an increased percentage of CD4 positive lymphocytes after LPS exposure at term (Figure 7A). CD8 positive and gamma-delta positive lymphocytes were not different. In contrast, at 7 weeks (Figure 7B), percentages of CD4 positive were not different in the LPS-exposed lambs compared to the control group. However, in the thymus, there was a lower percentage of CD8 positive lymphocytes and in the PMLN CD8 positive lymphocytes were increased. There was no effect of LPS exposure on gamma-delta positive lymphocytes in thymus and PMLN at 7 weeks of age. Fetal LPS-induced inflammation was associated with an increase in CD4/CD25 double positive lymphocytes (Figure 7C) in both thymus and PMLN at term. After 7 weeks, no differences were measured in the thymus. However, the percentage of CD4/CD25 double positive was decreased in PMLN after LPS exposure.

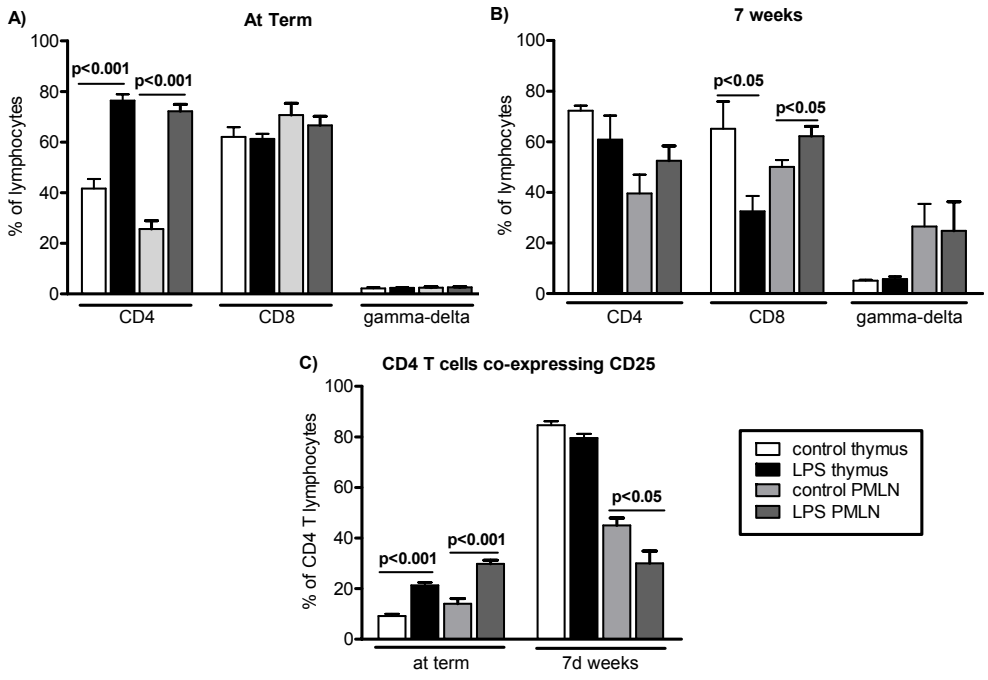


Figure 7: Percentages of specific T lymphocytes in thymus and PMLN. (A) At term, the percentages of CD4 lymphocytes were increased significantly in thymus and PMLN (34% and 46% respectively) of LPS-exposed lambs. After chorioamnionitis, CD8 lymphocytes and gamma-delta lymphocytes were not different. **(B)** The percentage of CD8 lymphocytes were decreased by 7 weeks (P=0.039) in the thymus of the LPS-exposed lambs. However, CD8 lymphocytes were increased (P<0.028) in PMLN. CD4 and gamma-delta lymphocytes showed no significant changes at 7 weeks. **(C)** CD4 co-expressing CD25 lymphocytes were increased in thymus and PMLN of 12% and 16%, respectively, at term. In the thymus no significant changes were present after 7 weeks of age. However, in the PMLN a significant decrease of CD4 lymphocytes co-expressing CD25 was noticed in LPS exposed lambs at 7 weeks (P<0.02). LPS, lipopolysaccharide; PMLN, posterior mediastinal lymph node.

Discussion

In this study, we examined the effects of fetal chorioamnionitis induced by 3 IA injections of LPS from 90 to 110 days' gestation on sheep at term and at 7 weeks of age. In previous experiments was reported profound changes in the pulmonary immune system after fetal exposure to LPS with an increase of immune cells and subsequent maturation of systemic monocytes into PU.1 expressing macrophages (24,25). In the current study, we also noticed significant increases in pulmonary immune cell populations, neutrophils in particular at term. This is remarkable as the fetal LPS exposure was 37 days before the increased neutrophils were detected. Neutrophils were not increased at 7 weeks of age. Monocytes and lymphocytes numbers that were elevated at term remained elevated at 7 weeks of age in lambs exposed as fetuses to LPS. These changes in leukocyte populations may indicate an accelerated development of the pulmonary immune system after LPS-induction.

At the systemic level, IA LPS also induced an inflammatory response (18). Previously, our group showed that the functional response of blood monocytes from preterm LPS-exposed lambs were similar to the blood monocytes of adult sheep (13,14). A functional assay in the current study demonstrated increased H_2O_2 production after PamCysK4 (a synthetic ligand for TLRs 1 and 2) stimulation of blood monocytes at term in fetal LPS-exposed animals compared with controls. This result demonstrated that blood monocytes at term were competent to respond to TLR2, because of the fetal LPS exposure. At 7 weeks, controls and LPS-exposed lambs were now similarly able to respond to PamCysK4 stimulation.

Changes in lymphocyte populations of the thymus and the PMLN of LPS-exposed preterm/fetal sheep were demonstrated in previous experiments (11). Furthermore, LPS-induced chorioamnionitis resulted in a reduced size of the thymus (23). In the current study, the percentage of cytotoxic lymphocytes decreased in the thymus after fetal LPS exposure, but increased in the PMLN of LPS-exposed lambs. This could be explained by the migration of immune effector cells to the site of inflammation. In addition, the percentage of $CD4^+CD25^+$ T lymphocytes were significantly decreased in the PMLN suggesting less suppression of effector cells by regulatory T lymphocytes (Tregs). In the literature, it was proposed that a decrease in Tregs may play a role in the dysregulation of airway inflammation in asthma (6,26,27). Normally, Tregs can suppress established airway inflammation and airway hyperresponsiveness. Schaub et al. (28) demonstrated that cord blood from offspring of atopic mothers had fewer innate-induced Tregs, indicating a potential mechanism by which intrauterine immune modulation can occur and may

influence further atopic disease development. It also points to the intrauterine environment as a critical immune modulator. Interestingly, Szépfalusi et al. (29) demonstrated that allergens could be transferred across the placenta, supporting the theory that fetal T lymphocytes are exposed during gestation to maternally derived allergens and that the uterus may not be completely sterile. However, the effect of antenatal and postnatal exposure to allergens may have reinforcing effects because exposure to allergens early in life enhanced the development of airway hyperresponsiveness and impairment of lung function at school age (30).

In our study, we tested the airway responsiveness to methacholine in lambs at 7 weeks of age. Airway resistance and tissue damping did not increase in LPS-exposed lambs at the highest MCh dosage (1.0% MCh) as it did in the controls. Interestingly, these results of decreased airway reactivity are in contrast with clinical observations of increased asthma after chorioamnionitis exposure (2). However, airway reactivity and immune dysfunction of the lungs do play a role in the development of asthma. The current study demonstrated structural changes in the vascular wall of the lungs. Less elastin was detected in blood vessels but more in lung parenchyma. This finding was surprising because elastin deposition is typically increased in blood vessels in ventilated animal models of lung injury (31). The low pulmonary blood flow *in utero* and spontaneous breathing after birth are not comparable to ventilated animal models to our model. Furthermore, decreased concentrations of bound and total TGF-β_1 were measured after chorioamnionitis at term. We speculate that the altered deposition of elastin in the vessel wall may be the result of the structural changes initiated before birth and that this is partially mediated by TGF-β_1. However these changes do not explain the decreased airway reactivity. In real life, children are exposed to many allergens, which are additional stimulators for airway reactivity and the later development of asthma. The allergen exposure was not controlled in these free living lambs to 7 weeks of age. Interestingly, Eder et al. (32) reported that exposure to LPS in early life protects against allergic sensitization, but not against asthma. This might be an explanation for the different outcome of airway reactivity in our study. Bischof et al. (33) demonstrated that age is important for asthma studies in sheep. Significant effects in the airway responsiveness were measured only after 5 months of age. Our studies at 7 weeks may have been in animals that were too young, altered responses to fetal exposure have not been tested previously. Another variable that may change the postnatal outcome is the gestational time of fetal exposure to proinflammatory stimuli.

In conclusion, our results demonstrate that early fetal exposure to LPS caused residual lung inflammation, but the pulmonary inflammation had resolved by 7 weeks. However,

minor structural and functional changes, decreased airway reactivity, and changes in lymphocytes were noted at 7 weeks. The clinical relevance of these results is the demonstration that fetal exposure to chorioamnionitis can modulate postnatal immune status and responses.

Acknowledgement

We thank Jennifer Collins for helpful comments on the manuscript.

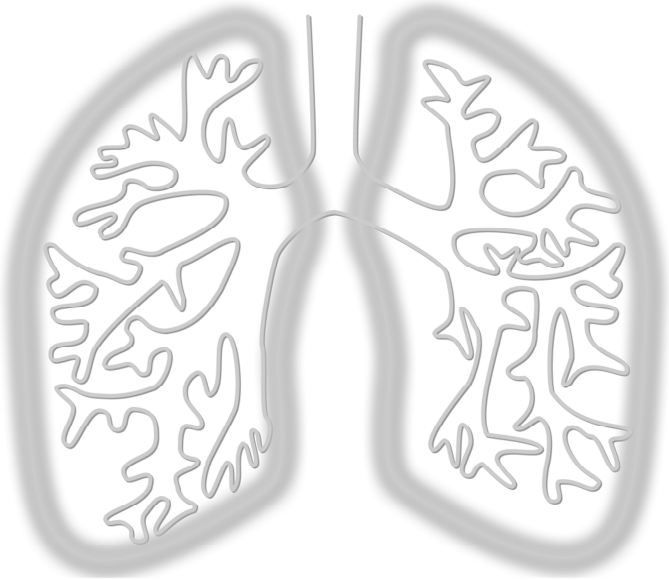
References

1. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med 2000;342:1500-1507
2. Kumar R, Yu Y, Story RE, et al. Prematurity, chorioamnionitis, and the development of recurrent wheezing: a prospective birth cohort study. J Allergy Clin Immunol 2008;121:878-884 e876
3. Getahun D, Strickland D, Zeiger RS, et al. Effect of chorioamnionitis on early childhood asthma. Arch Pediatr Adolesc Med 2010;164:187-192
4. Rusconi F, Galassi C, Forastiere F, et al. Maternal complications and procedures in pregnancy and at birth and wheezing phenotypes in children. Am J Respir Crit Care Med 2007;175:16-21
5. Strachan DP. Hay fever, hygiene, and household size. BMJ 1989;299:1259-1260
6. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002;347:911-920
7. Rook G. Review series on helminths, immune modulation and the hygiene hypothesis: The broader implications of the hygiene hypothesis. Immunology 2009;126:3
8. Wolfs TG, Buurman WA, Zoer B, et al. Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep. PLoS One 2009;4:e5837
9. Romero R, Mazor M, Wu YK, et al. Infection in the pathogenesis of preterm labor. Semin Perinatol 1988;12:262-279
10. Maloney JE, Adamson TM, Brodecky AV, et al. Diaphragmatic activity and lung liquid flow in the unanesthetized fetal sheep. J Appl Physiol 1975;39:423-428
11. Kramer BW, Kallapur SG, Moss TJ, et al. Modulation of fetal inflammatory response on exposure to lipopolysaccharide by chorioamnion, lung, or gut in sheep. Am J Obstet Gynecol 2010;202:77 e71-79
12. Moss TJ, Knox CL, Kallapur SG, et al. Experimental amniotic fluid infection in sheep: effects of Ureaplasma parvum serovars 3 and 6 on preterm or term fetal sheep. Am J Obstet Gynecol 2008;198:122 e121-128
13. Kramer BW, Ikegami M, Moss TJ, et al. Endotoxin-induced chorioamnionitis modulates innate immunity of monocytes in preterm sheep. Am J Respir Crit Care Med 2005;171:73-77
14. Kallapur SG, Jobe AH, Ball MK, et al. Pulmonary and systemic endotoxin tolerance in preterm fetal sheep exposed to chorioamnionitis. J Immunol 2007;179:8491-8499
15. Kramer BW, Kallapur SG, Moss TJ, et al. Intra-amniotic LPS modulation of TLR signaling in lung and blood monocytes of fetal sheep. Innate Immun 2009;15:101-107
16. Giannone PJ, Nankervis CA, Richter JM, et al. Prenatal lipopolysaccharide increases postnatal intestinal injury in a rat model of necrotizing enterocolitis. J Pediatr Gastroenterol Nutr 2009;48:276-282
17. Hall GL, Hantos Z, Wildhaber JH, et al. Methacholine responsiveness in infants assessed with low frequency forced oscillation and forced expiration techniques. Thorax 2001;56:42-47

18. Kramer BW, Moss TJ, Willet KE, et al. Dose and time response after intraamniotic endotoxin in preterm lambs. Am J Respir Crit Care Med 2001;164:982-988
19. Kunzmann S, Speer CP, Jobe AH, et al. Antenatal inflammation induced TGF-beta1 but suppressed CTGF in preterm lungs. Am J Physiol Lung Cell Mol Physiol 2007;292:L223-231
20. Kramer BW, Albertine KH, Moss TJ, et al. All-trans retinoic acid and intra-amniotic endotoxin-mediated effects on fetal sheep lung. Anat Rec (Hoboken) 2008;291:1271-1277
21. Bland RD, Albertine KH, et al. Impaired alveolar development and abnormal lung elastin in preterm lambs with chronic lung injury: potential benefits of retinol treatment. Biol Neonate 2003;84:101-102
22. Bland RD. Neonatal chronic lung disease in the post-surfactant era. Biol Neonate 2005;88:181-191
23. Kunzmann S, Glogger K, Been JV, et al. Thymic changes after chorioamnionitis induced by intraamniotic lipopolysaccharide in fetal sheep. Am J Obstet Gynecol 2010
24. Kramer BW, Joshi SN, Moss TJ, et al. Endotoxin-induced maturation of monocytes in preterm fetal sheep lung. Am J Physiol Lung Cell Mol Physiol 2007;293:L345-353
25. Cheah FC, Pillow JJ, Kramer BW, et al. Airway inflammatory cell responses to intra-amniotic lipopolysaccharide in a sheep model of chorioamnionitis. Am J Physiol Lung Cell Mol Physiol 2009;296:L384-393
26. Ray A, Khare A, Krishnamoorthy N, et al. Regulatory T cells in many flavors control asthma. Mucosal Immunol 2010;3:216-229
27. Robinson DS. Regulatory T cells and asthma. Clin Exp Allergy 2009;39:1314-1323
28. Schaub B, Liu J, Hoppler S, et al. Impairment of T-regulatory cells in cord blood of atopic mothers. J Allergy Clin Immunol 2008;121:1491-1499, 1499 e1491-1413
29. Szepfalusi Z, Loibichler C, Pichler J, et al. Direct evidence for transplacental allergen transfer. Pediatr Res 2000;48:404-407
30. Illi S, von Mutius E, Lau S, et al. Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. Lancet 2006;368:763-770
31. Pierce RA, Albertine KH, Starcher BC, et al. Chronic lung injury in preterm lambs: disordered pulmonary elastin deposition. Am J Physiol 1997;272:L452-460
32. Eder W, von Mutius E. Hygiene hypothesis and endotoxin: what is the evidence? Curr Opin Allergy Clin Immunol 2004;4:113-117
33. Bischof RJ, Snibson K, Shaw R, et al. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. Clin Exp Allergy 2003;33:367-375

CHAPTER 6

Summary and discussion



Prenatal inflammation is known to be a major pathological component in preterm birth and impaired lung development (1,2). In addition, preterm birth seems to be related to the development of childhood wheezing (3,4). Nevertheless, the underlying mechanisms of these inflammatory processes and the associated pathology are not completely clear. The main focus of this thesis was to give insight into the postnatal persistence of fetal immune effects following a pro-inflammatory stimulus and how the duration of exposure to antenatal inflammation may affect the fetal lungs and airways as gestation advances till near-term and the first years after birth.

Important role of inflammation during fetal development

Up to 70% of preterm deliveries prior to 30 weeks gestation are affected by chorioamnionitis through direct contact with amniotic fluid or via the placental-fetal circulation (5). Chorioamnionitis affects neonatal outcomes in an organ-dependent way (6), but is primarily attributed with alterations in lung development and may negatively affect neonatal outcomes by modulating the fetal immune system (7,8). However, results of these immune responses are limited to short term exposure to pro-inflammatory agonists. No experimental studies have examined the persistence of fetal immune modulations following a pro-inflammatory stimulus and how this may affect the fetal response as gestation advances till near-term.

Therefore, in **chapter 2**, we hypothesized that lipopolysaccharide (LPS)-induced chorioamnionitis would cause persistent fetal pulmonary responses as the lungs develop further *in utero*. To characterize the effects of LPS on the fetal lung, we assessed lung homeostasis after single or repetitive doses of LPS. Consistent with our hypothesis, intra-amniotic LPS exposure resulted in changes in the fetal lung inflammatory responses in the course of gestation with involvement of an alveolar macrophage response shown by an increased PU.1 expression (a hematopoietic transcription factor). In addition, delayed increases in Toll-like receptor (TLR)2 and TLR6 mRNA levels 15 days after the initial exposure to the TLR4 agonist LPS might have clinical implications for the chronic and often polymicrobial organisms associated with chorioamnionitis (9). However, our finding of reduced mast cell populations soon after LPS exposure is in contrast with our hypothesis that LPS exposure would increase mast cells. Mast cells in the lower airways normally contribute to the development of asthma and allergic diseases (10). In addition, there is a possible relation between chorioamnionitis, preterm birth, and the development of asthma later in life (3,4). Therefore, further investigation of mast cell populations and function are needed to provide insights into the mechanisms and development of asthma and allergic diseases after preterm birth.

Besides immunologic changes, structural changes measured by elastin foci, resulted in more diffusely localized elastin foci in the more immature fetal lambs, a more normal distribution with localization of elastin foci at the tips of alveolar septa was re-established near-term, indicating recovery with advancing gestation. The mechanism by which the fetal lung repairs the abnormal elastin response is unknown. Importantly, overall differences in the results were demonstrated after a single or repetitive dose of LPS, which suggests that the pulmonary immune response may be dependent on the intensity and/or persistence of the LPS exposure. With chorioamnionitis in human pregnancies, fetuses will be continuously exposed to bacteria until delivery, which will challenge the immune system permanently. Therefore, it is important to mimic this continuous exposure of LPS during further research.

In **chapter 3** we were interested in the gender differences during prenatal lung development. In the perinatology, gender differences for short- and long-term outcomes of the infants are often reported (11-15). It was suggested that slower lung maturation among male fetuses is a major contributing factor to gender differences in neonatal mortality (15). In addition, an increased vulnerability of perinatal mortality among male neonates might also be the result of gender differences in the development and function of the immune system (16). In our study, we did not detect any potentially underlying differences in pulmonary and systemic markers of inflammation after antenatal exposure to LPS. However, we observed larger improvements in lung compliance after LPS-induced intrauterine inflammation among female preterm fetuses compared with males. A decrease in lung compliance was detectable 2 days after intrauterine LPS exposure only in male fetuses. Conversely, lung compliance was enhanced 7 days after LPS, when lung compliance was again better in female than in male fetuses. We propose that these differences may explain at least part of the female advantage in survival as well as in outcome among survivors after chorioamnionitis and preterm birth. To examine the mechanism behind this female advantage we analyzed the mean alveolar size and alveolar wall thickness without any differences noticed. This suggests that the female advantage in lung gas volume is not based on lung histology, but might contribute to the theory that the surfactant deficiency in male lungs might play a key role in the female advantage after preterm birth (17-20). Further investigations are required to test this proposed mechanism between sexes.

Effect of LPS-induced inflammation on lung development after birth

Exposure to antenatal inflammation is a double-edged sword to the fetal lung: on the one hand, it enhances lung maturation; on the other hand, it stops the further development of the alveoli resulting in a reduced surface area for gas exchange (1,2,21). These very same infants will need supplemental oxygen, which is the clinical definition of bronchopulmonary dysplasia (BPD) (22-24). Long-term studies showed persisting lung function abnormalities in children with BPD resulting in airway obstruction, airway hyperreactivity and hyperinflation (25,26). Chorioamnionitis is correlated with a 4.4-fold increased risk for developing physician-diagnosed asthma in preterm babies after correction for confounding factors (3). This last observation is in contrast to the hygiene hypothesis, in which infections during early childhood are associated with a decreased risk of subsequent asthma and allergic diseases (27-29). The relationship between antenatal inflammatory exposures and postnatal abnormalities seems to be confounded by the fetal response to inflammation. Hardly anything is known about how this fetal response to inflammation may affect the lung development during the first years after birth.

In **chapter 4**, we firstly were interested in the differences of airway smooth muscle reactivity in sheep at different ages without antenatal inflammation. A model of ovine precision-cut lung slices (PCLS) was established for the measurement of airway responses to early allergic response mediators and to allergens (after passive sensitization) in newborn and adult animals. Many of the responses were similar in newborn and adult sheep, such as the bronchoconstriction to electric field stimulation (EFS), to allergen (after passive sensitization) and to the mediators methacholine, histamine and endothelin-1. However, the response to leukotriene D_4 (LTD_4), the thromboxane analogue U46619 and serotonin depended on the age of the sheep. The response to serotonin and U46619 was weaker in newborn sheep than in adult sheep, whereas the response to LTD_4 was stronger in newborn sheep. The notable differences between newborn and adult sheep demonstrate the importance of age in such studies, since leukotrienes contribute to airway obstruction in many human asthmatics (30).

Therefore, in **chapter 5** we focused on the effects of intra-amniotic LPS-induced chorioamnionitis at 7 weeks old sheep, which is comparable to 4-5 years of age in humans. We hypothesized that fetal innate immune responses to antenatal inflammation would alter postnatal systemic immune and airway responsiveness. Our results demonstrated that early fetal exposure to LPS caused residual lung inflammation, but the pulmonary inflammation had resolved by 7 weeks. However, minor structural and functional changes, decreased airway reactivity and changes in lymphocytes demonstrate persistent effects of fetal exposure to LPS at 7 weeks postnatal. Interestingly, the results of decreased airway

reactivity are in contrast with clinical observations of increased asthma after chorioamnionitis exposure (3). However, airway reactivity and immune dysfunction of the lungs do play a role in the development of asthma. Bischof et al. demonstrated that significant effects in the airway responsiveness were measured after 5 months of age (31). This suggests that examination of the airway reactivity at 7 weeks may have been in animals that were too young. The clinical relevance of our results is the demonstration that fetal exposure to antenatal inflammation can modulate postnatal immune status and responses.

Implications and future perspectives

To come up with new preventive and therapeutic strategies for asthma, it is highly important to understand the mechanisms that drive normal lung development and how they are driven off course by the events that surround preterm birth (Figure 1).

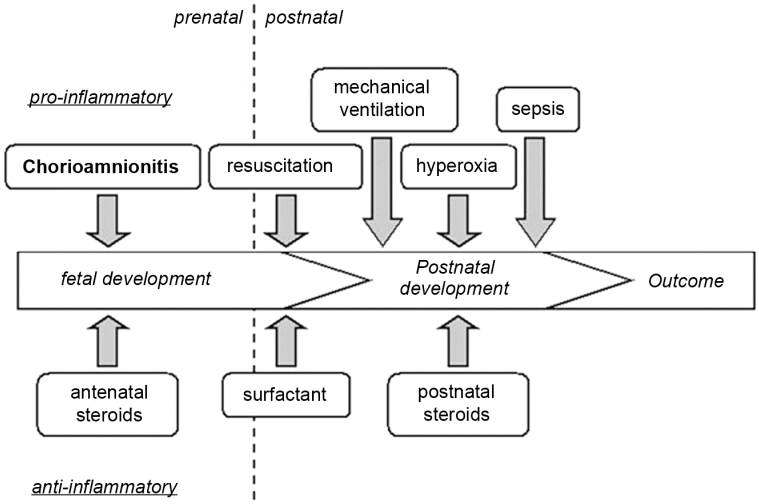


Figure 1: Overview of the most common antenatal and postnatal events to which preterm infants are exposed. Both antenatal and postnatal exposures can influence the neonatal immune response and subsequent neonatal outcome (6).

In recent years, the fetal immune response toward the perinatal events has been identified as a key determinant for neonatal outcomes (32,33). An accurate and timely immune response towards pathogens is essential to protect against tissue damage and disease (34). After controlling the infection it is essential that resolution of the inflammation is initiated by control mechanisms to prevent further tissue damage and to enable wound healing (35,36). However, it seems that the preterm infants have a limited ability to resolve the pro-inflammatory processes, especially when exposed to antenatal

events such as chorioamnionitis. It has been demonstrated that monocytes from preterm sheep function differently in respect to initiation and resolution of inflammation compared to adult cells and that their function can be further modulated by various stimuli such as endotoxin (lipopolysaccharide [LPS]), glucocorticoids and surfactant, which are all common exposures for a preterm infant (37-39) (Figure 1). This sustained inflammation in the neonate seems to be closely associated with an increased risk for adverse outcomes such as pulmonary complications, but also brain damage and intestinal complications (6,40). The follow-up studies of former preterm showed clearly that their lung function and airway reactivity remains impaired (41,42).

Asthma is a chronic lung disease characterized by episodes of airflow obstruction. The prevalence and incidence of asthma is increasing worldwide in the last few decades (43,44), especially among children and adolescents (45,46). Asthma has become the most common chronic disease among children in many countries and is one of the major causes of hospitalization among those younger than 15 years of age (47). It is the leading cause of missed school days due to the chronic condition. Differences in major activity limitations for children with and without asthma are substantial. Children with asthma are almost five times more likely to have activity limitations than children without asthma. In addition, to the considerable distress for the children and inconvenience for the families, childhood asthma is a major source of medical expense for society. Medical treatment with anti-inflammatory agents (especially inhaled steroids) and bronchodilators is necessary to prevent and control asthmatic attacks. The total annual health care expenditure on a single asthmatic child is 2.8 times higher than the costs of a non-asthmatic child (47).

Although, many treatments already exist that suppress the symptoms of the disease, these treatments do not cope with the cause of the disease. Clearly, childhood asthma has become a major public health problem that needs considerable effort to reverse the increasing trend in its incidence. Fetal life exposures are thought to contribute to these increases (3,48,49). Although asthma is probably a heterogeneous disease or syndrome, four factors and/or events consistently emerge for their ability to significantly influence asthma development in the first decade of life: 1) prematurity, which is associated with changes in pulmonary function, including those that have found effects extending into adolescence; 2) immune response aberrations, which appear to be defined best by the concept of cytokine dysregulation; 3) gene-environment interactions that need to occur at a critical time-period in the development of the immune system or the lung; and 4) lower respiratory tract infections, in particular respiratory syncytial virus (RSV). However, it remains to be established how any one or all of these factors, either independently or interactively, influence the development of childhood asthma. Therefore, children with asthma after prematurity need long-term follow-up studies.

Previous animal studies have provided some insight into the long-term consequences after preterm birth. It may well be modulated to a significant degree by perinatal factors. However, many new questions have arisen, requiring new animal studies, clinical studies and alternative approaches to become answered. Future studies should evaluate the effects of cytokine dysregulation such as T helper 1/T helper 2 (Th1/Th2) imbalance. Balanced Th1/Th2 immunity is a physiologic state among healthy individuals, and excessive responses to either Th1 or Th2 direction are harmful (50). Th1-type immune responses are involved in delayed type immune responses against microbe, whereas Th2-type immune responses are associated with immunoglobulin E (IgE) production and allergic inflammation. The postnatal downregulation of the Th2 immunity is a complex process with involvement of various cells and mediators (44,51). Among many cytokines and chemokines, Th1 cell secreted interferon-gamma (IFN-γ) has been suggested to have an important role in downregulating the postnatal Th2 immunity. In addition, chorioamnionitis is associated with a strong pro-inflammatory response with increased levels of TNF-α, IL-6, and IL-8 (52,53). These inflammatory cytokines may be a response to infection and also a trigger for premature delivery. Specifically, human decidual cells increase the production of delivery promoting factors such as prostaglandin (PG)E2 and PGF2 in response to inflammatory cytokines including IL-1a, IL-1b, and TNF-α (54). COX-2 plays a predominant role in PG formation during inflammation and LPS is well described as a potent stimulus to COX-2 induction (55, 56). Some of the cytokines which are a response to infection may be involved in chronic respiratory disease development. For example, TNF-α variants have been found to be associated with recurrent wheezing after RSV bronchiolitis (57) and IL-8 levels were increased in children who developed wheezing illnesses in response to rhinovirus with an additional risk of persistent wheezing till follow up at age 3 years (58,59). Thus, the same cytokines which are generated as part of the inflammatory response to chorioamnionitis may play a role in asthma and responses to respiratory viral illness (60). However, only a fraction of children develop recurrent wheezing following RSV infections, despite the fact that nearly all children have been infected at least once by 2 years of age. Thus, although RSV infections may have the potential of targeting the inflammatory response to the lower airway, they may only be able to do so during a vulnerable time-period of immune system or lung development. This developmental component may further reflect important gene–environment interactions that regulate both short- and long-term airway physiological alterations that manifest themselves clinically as childhood asthma. The obtained knowledge from these future studies can be used to test the clinical association of chorioamnionitis and asthma with the ultimate aim of prevention.

Conclusion

In summary, our findings provide new insights into how the lung development is influenced by antenatal inflammation as gestation advances and during the first period after birth. Although our studies were performed in fetal and newborn lambs in which the developing lung is highly similar to that in humans, they were also limited by this observational approach. To confirm the association between chorioamnionitis-induced preterm birth and asthmatic symptoms, a more optimized animal model needs to be developed. Nonetheless this thesis has identified a potential link between activation of the prenatal immune status and impaired lung development, resulting in altered airway reactivity postnatal, and should provide a basis for future research towards the understanding and treating of asthma in early childhood after preterm birth.

References

1. Kramer BW. Antenatal inflammation and lung injury: prenatal origin of neonatal disease. J Perinatol 2008;28 Suppl 1:S21-27.
2. Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Hum Pathol 1998;29:710-717.
3. Kumar R, Yu Y, Story RE, et al. Prematurity, chorioamnionitis, and the development of recurrent wheezing: a prospective birth cohort study. J Allergy Clin Immunol 2008;121:878-884 e876.
4. Getahun D, Strickland D, Zeiger RS, et al. Effect of chorioamnionitis on early childhood asthma. Arch Pediatr Adolesc Med 164:187-192.
5. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med 2000;342:1500-1507.
6. Gantert M, Been JV, Gavilanes AW, et al. Chorioamnionitis: a multiorgan disease of the fetus? J Perinatol 2010;30 Suppl:S21-30.
7. Moss TJ, Nitsos I, Kramer BW, et al. Intra-amniotic endotoxin induces lung maturation by direct effects on the developing respiratory tract in preterm sheep. Am J Obstet Gynecol 2002;187:1059-1065.
8. Wolfs TG, Jellema RK, Turrisi G, et al. Inflammation-induced immune suppression of the fetus: a potential link between chorioamnionitis and postnatal early onset sepsis. J Matern Fetal Neonatal Med 2012;25 Suppl 1:8-11.
9. Czikk MJ, McCarthy FP, Murphy KE. Chorioamnionitis: from pathogenesis to treatment. Clin Microbiol Infect 17:1304-1311.
10. Brown JM, Wilson TM, Metcalfe DD. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. Clin Exp Allergy 2008;38:4-18.
11. Sheiner E, Levy A, et al. Gender does matter in perinatal medicine. Fetal Diagn Ther 2004;19:366-369.
12. Eogan MA, Geary MP, O'Connell MP, et al. Effect of fetal sex on labour and delivery: retrospective review. BMJ 2003;326:137.
13. Feinstein U, Sheiner E, Levy A, et al. Risk factors for arrest of descent during the second stage of labor. Int J Gynaecol Obstet 2002;77:7-14.
14. Perelman RH, Palta M, Kirby R, et al. Discordance between male and female deaths due to the respiratory distress syndrome. Pediatrics 1986;78:238-244.

15. Khoury MJ, Marks JS, McCarthy BJ, et al. Factors affecting the sex differential in neonatal mortality: the role of respiratory distress syndrome. Am J Obstet Gynecol 1985;151:777-782.
16. Shames RS. Gender differences in the development and function of the immune system. J Adolesc Health 2002;30:59-70.
17. Carey MA, Card JW, Voltz JW, et al. It's all about sex: gender, lung development and lung disease. Trends Endocrinol Metab 2007;18:308-313.
18. Carey MA, Card JW, Voltz JW, et al. The impact of sex and sex hormones on lung physiology and disease: lessons from animal studies. Am J Physiol Lung Cell Mol Physiol 2007;293:L272-278.
19. Torday JS, Nielsen HC. The sex difference in fetal lung surfactant production. Exp Lung Res 1987;12:1-19.
20. Torday JS, Nielsen HC, Fencl Mde M, et al. Sex differences in fetal lung maturation. Am Rev Respir Dis 1981;123:205-208.
21. Kramer BW, Jobe AH. The clever fetus: responding to inflammation to minimize lung injury. Biol Neonate 2005;88:202-207.
22. Jobe AJ. The new BPD: an arrest of lung development. Pediatr Res 1999;46:641-643.
23. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med 2001;163:1723-1729.
24. Jobe AH. Antenatal associations with lung maturation and infection. J Perinatol 2005;25 Suppl 2:S31-35.
25. Koumbourlis AC, Motoyama EK, Mutich RL, et al. Longitudinal follow-up of lung function from childhood to adolescence in prematurely born patients with neonatal chronic lung disease. Pediatr Pulmonol 1996;21:28-34.
26. Northway WH, Jr., Moss RB, Carlisle KB, et al. Late pulmonary sequelae of bronchopulmonary dysplasia. N Engl J Med 1990;323:1793-1799.
27. Rook GA. Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis. Immunology 2009;126:3-11.
28. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002;347:911-920.
29. Renz H, Blumer N, Virna S, et al. The immunological basis of the hygiene hypothesis. Chem Immunol Allergy 2006;91:30-48.
30. Okunishi K, Peters-Golden M. Leukotrienes and airway inflammation. Biochim Biophys Acta 2011;1810:1096-1102.
31. Bischof RJ, Snibson K, Shaw R, et al. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. Clin Exp Allergy 2003;33:367-375.
32. Azizia M, Lloyd J, Allen M, et al. Immune status in very preterm neonates. Pediatrics 2012;129:e967-974.
33. Been JV, Rours IG, Kornelisse RF, et al. Histologic chorioamnionitis, fetal involvement, and antenatal steroids: effects on neonatal outcome in preterm infants. Am J Obstet Gynecol 2009;201:587 e581-588.
34. Medzhitov R, Schneider DS, et al. Disease tolerance as a defense strategy. Science 2012;335:936-941.
35. Leviton A, Fichorova R, Yamamoto Y, et al. Inflammation-related proteins in the blood of extremely low gestational age newborns. The contribution of inflammation to the appearance of developmental regulation. Cytokine 2011;53:66-73.
36. Leviton A, Hecht JL, Allred EN, et al. Persistence after birth of systemic inflammation associated with umbilical cord inflammation. J Reprod Immunol 2011;90:235-243.
37. Kramer BW, Ikegami M, Moss TJ, et al. Antenatal betamethasone changes cord blood monocyte responses to endotoxin in preterm lambs. Pediatr Res 2004;55:764-768.
38. Kramer BW, Jobe AH, Ikegami M. Monocyte function in preterm, term, and adult sheep. Pediatr Res 2003;54:52-57.
39. Willems CH, Urlichs F, Seidenspinner S, et al. Poractant alfa (Curosurf(R)) increases phagocytosis of apoptotic neutrophils by alveolar macrophages in vivo. Respir Res 2012;13:17.

40. Wolfs TG, Buurman WA, Zoer B, et al. Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep. PLoS One 2009;4:e5837.
41. Greenough A. Long term respiratory outcomes of very premature birth (<32 weeks). Semin Fetal Neonatal Med 2012;17:73-76.
42. Fawke J, Lum S, Kirkby J, et al. Lung function and respiratory symptoms at 11 years in children born extremely preterm: the EPICure study. Am J Respir Crit Care Med 2010;182:237-245.
43. Haahtela T, Lindholm H, et al. Prevalence of asthma in Finnish young men. BMJ 1990;301:266-268.
44. Remes ST, Korppi M. On roots of childhood asthma: the role of respiratory infections. Ann Med 2005;37:26-32.
45. Yunginger JW, Reed CE, O'Connell EJ, et al. A community-based study of the epidemiology of asthma. Incidence rates, 1964-1983. Am Rev Respir Dis 1992;146:888-894.
46. Peat J, Bjorksten B. Primary and secondary prevention of allergic asthma. Eur Respir J 1998;Suppl 27:28s-34s.
47. Lozano P, Sullivan SD, Smith DH, et al. The economic burden of asthma in US children: estimates from the National Medical Expenditure Survey. J Allergy Clin Immunol 1999;104:957-963.
48. Kennedy JD, Edward LJ, Bates DJ, et al. Effects of birthweight and oxygen supplementation on lung function in late childhood in children of very low birth weight. Pediatr Pulmonol 2000;30:32-40.
49. Halvorsen T, Skadberg BT, Eide GE, et al. Pulmonary outcome in adolescents of extreme preterm birth: a regional cohort study. Acta Paediatr 2004;93:1294-1300.
50. Johnston SL. Viruses and asthma. Allergy 1998;53:922-932.
51. Martinez FD, Holt PG. Role of microbial burden in aetiology of allergy and asthma. Lancet 1999;354 Suppl 2:SII12-15.
52. Murtha AP, Sinclair T, Hauser ER, et al. Maternal serum cytokines in preterm premature rupture of membranes. Obstet Gynecol 2007;109:121-127.
53. Jacobsson B, Mattsby-Baltzer I, Hagberg H. Interleukin-6 and interleukin-8 in cervical and amniotic fluid: relationship to microbial invasion of the chorioamniotic membranes. BJOG 2005;112:719-724.
54. Mitchell MD, Edwin S, Romero RJ. Prostaglandin biosynthesis by human decidual cells: effects of inflammatory mediators. Prostaglandins Leukot Essent Fatty Acids 1990;41:35-38.
55. McAdam BF, Mardini IA, Habib A, et al. Effect of regulated expression of human cyclooxygenase isoforms on eicosanoid and isoeicosanoid production in inflammation. J Clin Invest 2000;105:1473-1482.
56. Rouzer CA, Marnett LJ. Cyclooxygenases: structural and functional insights. J Lipid Res 2009;50 Suppl:S29-34.
57. Goetghebuer T, Isles K, Moore C, et al. Genetic predisposition to wheeze following respiratory syncytial virus bronchiolitis. Clin Exp Allergy 2004;34:801-803.
58. Gern JE, Martin MS, Anklam KA, et al. Relationships among specific viral pathogens, virus-induced interleukin-8, and respiratory symptoms in infancy. Pediatr Allergy Immunol 2002;13:386-393.
59. Lemanske RF, Jr., Jackson DJ, Gangnon RE, et al. Rhinovirus illnesses during infancy predict subsequent childhood wheezing. J Allergy Clin Immunol 2005;116:571-577.
60. Lemanske RF, Jr. The childhood origins of asthma (COAST) study. Pediatr Allergy Immunol 2002;13 Suppl 15:38-43.

Nederlandse samenvatting (Dutch summary)

Vroeggeboorte na antenatale inflammatie als onderliggende oorzaak van bronchiale hyperreactiviteit

Vroeggeboorte

Over vroeggeboorte, ook wel prematuriteit genoemd, wordt gesproken bij een zwangerschapsduur van minder dan 37 weken (normale duur is 37-42 weken). Het komt voor bij 5-13% van alle zwangerschappen in de Westerse wereld. In 2008 werden in Nederland 7,7% van alle kinderen te vroeg geboren. Vroeggeboorte is de belangrijkste oorzaak van ziekte en sterfte bij zuigelingen. Hoe korter de zwangerschapsduur, hoe groter de kans is op ziekte en sterfte. Dus elke week dat een zwangerschap langer duurt, stijgt de kans op overleven. Hoewel maar 7,7% van alle kinderen geboren wordt voor de 37ste zwangerschapsweek, maken zij 75% uit van alle zuigelingensterfte in Nederland in 2008. Vroeggeboorte is niet alleen risicovol rondom de bevalling maar kan ook één jaar na de bevalling nog ongunstige effecten hebben voor het kind en mogelijk zelfs nog later in de ontwikkeling.

Vroeggeboorte kan verschillende oorzaken hebben en wordt grofweg ingedeeld in twee hoofdcategorieën. De eerste categorie is de vroegtijdige zwangerschaps-beëindiging op basis van medische indicatie of gezondheidsrisico voor moeder of kind. Bij deze geïndiceerde vroeggeboorte wordt ingegrepen door middel van een keizersnede. Bij de tweede categorie, de spontane vroeggeboorte, is de meest waarschijnlijke oorzaak een bacteriële infectie van de vruchtvliezen (chorion en amnion) en het vruchtwater (amnionvocht). Deze bacteriële infectie wordt chorioamnionitis genoemd en is de oorzaak van 70% van alle vroeggeboorten. Het is dus de meest voorkomende oorzaak. Meestal vertoont de moeder geen klinische symptomen van deze infectie en kan dit pas na de bevalling vastgesteld worden door de placenta (dit is de moederkoek) in een laboratorium te laten onderzoeken. Het is niet duidelijk hoe deze bacteriële infecties ontstaan tijdens de zwangerschap, maar waarschijnlijk banen de bacteriën zich een weg vanuit de vagina naar de baarmoeder en de vruchtvliezen om uiteindelijk in het vruchtwater terecht te komen. Doordat de ongeboren baby het vruchtwater inslikt en inademt zal de baby geïnfecteerd raken. Er zijn verschillende soorten bacteriën bekend die in de vruchtvliezen en het vruchtwater voorkomen van zuigelingen met chorioamnionitis. Het immuun systeem van de zuigelingen reageert op een stof in het buitenmembraan van deze bacteriën. Deze stof is lipopolysaccharide (afgekort: LPS) en wordt in het wetenschappelijk onderzoek vaak gebruikt om een infectie na te bootsen.

In de kindergeneeskunde wordt daarnaast vaak gezien dat het verschil in geslacht van invloed is op de zuigelingensterfte bij vroeggeboorte. De zuigelingensterfte is bij te vroeg geboren jongetjes hogere dan bij meisjes. Er wordt gedacht dat de langzamere longrijping onder ongeboren jongetjes een belangrijke factor is die bijdraagt tot een verschil in zuigelingensterfte tussen jongens en meisjes, maar ook een verschil in de ontwikkeling van het immuun systeem kan hieraan bijdragen.

Bronchiale hyperreactiviteit en astma ontwikkeling

Blootstelling aan chorioamnionitis heeft twee kanten: enerzijds zorgt het voor een versnelde longrijping, anderzijds stopt het de verdere ontwikkeling van de longblaasjes. Het gevolg is dat er minder en erg grote longblaasjes ontstaan. De longen zijn nog onderontwikkeld met een te kleine longoppervlakte waardoor te vroeg geboren baby's (prematuren) niet genoeg zuurstof binnen krijgen en met extra zuurstof beademd moeten worden. Deze behandeling is noodzakelijk voor de overleving van de prematuren maar gaat ook gepaard met schade aan de onderontwikkelde longen. Hierdoor ontwikkelen deze kinderen chronische longschade (bronchopulmonale dysplasie; afgekort als BPD). Langdurige beperking van de longfunctie bij kinderen met BPD leidt uiteindelijk tot luchtwegvernauwing en overmatige reactie van de luchtwegen op prikkels, dit wordt 'bronchiale hyperreactiviteit' genoemd. Het is bij prematuren bekend dat chorioamnionitis gerelateerd is aan een vier keer hoger risico op het ontwikkelen van astma op latere leeftijd.
Als men echter kijkt naar infecties die ontstaan in de vroege kinderjaren, dus na de geboorte, dan blijkt dat astma minder voorkomt bij boerenkinderen (meer contact met bacteriën en schimmels) en antroposofische kinderen (meer kinderziektes, want ze zijn niet gevaccineerd uit levensbeschouwingsmotief). Bij deze kinderen is er sprake van een hogere infectiegraad op jonge leeftijd en dit lijkt een beschermende factor te zijn voor het ontwikkelen van astma. Hieruit blijkt dus het belang van de periode van blootstelling aan een infectie of deze tijdens de zwangerschap of na de geboorte plaatsvindt.
Teruggaand naar de relatie tussen een bacteriële infectie tijdens de zwangerschap en een veranderde longontwikkeling op latere leeftijd, lijkt dit veroorzaakt te worden door een verstoorde reactie van de ongeboren baby op deze infectie. Het is echter nauwelijks bekend hoe deze infectie de longontwikkeling ongunstig beïnvloedt tijdens de zwangerschap en de eerste jaren na de geboorte. Kennis van deze processen is dan ook noodzakelijk voor een beter inzicht in de ontwikkeling van astma na vroeggeboorte en de ontwikkeling van preventie- en behandelopties voor astma.

Dit proefschrift

In dit proefschrift is onderzocht hoe de normale longontwikkeling en longfunctie beïnvloed wordt door een ontsteking van de vruchtvliezen en het vruchtwater gedurende de zwangerschap tot aan de kindertijd. Het zijn namelijk vooral de lange termijn effecten die nauwelijks onderzocht zijn. Voor dit onderzoek is gebruik gemaakt van een schaapmodel, omdat de laatste stadia van schaapfoetussen sterke gelijkenis hebben met de mens. Verder geldt voor schapen dat een leeftijd van 7 weken ongeveer overeenkomt met een leeftijd van 4-5 jaar bij de mensen.
In **hoofdstuk 2** wordt beschreven hoe de longontwikkeling en het immuunsysteem van schaapfoetussen reageert op de blootstelling aan een ontsteking in het verloop van de zwangerschap. Hiervoor werden de longen onderzocht op 3 verschillende momenten in de zwangerschap. Uit dit onderzoek blijkt dat de immuunrespons in de longen afhankelijk is van de intensiteit en continuïteit van de blootstelling aan LPS. In menselijke zwangerschappen wordt de ongeboren baby voortdurend blootgesteld aan bacteriën tot aan de bevalling en zal het immuunsysteem dus voortdurend geprikkeld worden. Het is dan ook belangrijk om deze continue blootstelling van LPS na te bootsen in toekomstig onderzoek. In **hoofdstuk 3** is onderzocht of de longontwikkeling verschilt tussen de nog ongeboren jongens en meisjes na blootstelling aan een ontsteking in het vruchtwater. Uit dit onderzoek blijkt dat het longvolume, en daarmee ook de longrijping, groter is bij meisjes dan bij jongens na blootstelling aan chorioamnionitis. Deze versnelde longrijping bij ongeboren meisjes is een voordeel in de overleving na vroeggeboorte. Het mechanisme achter dit voordeel lijkt niet gebaseerd te zijn op de longstructuur volgens dit onderzoek, maar kan wel bijdragen tot de theorie dat 'surfactant'-tekort een belangrijke rol speelt. Surfactant is een stof dat zorgt voor een verlaagde oppervlakte spanning, vergelijkbaar met zeep in water. Eerdere studies hebben aangetoond dat deze stof in de longen bij ongeboren meisjes vroeger in de zwangerschap stijgt dan bij de ongeboren jongens, mogelijk speelt dit een rol in het verschil van longrijping tussen de beide geslachten. Deze theorie zal in de toekomst verder onderzocht gaan worden.
Om het effect van chorioamnionitis op een astmatische aandoening na de geboorte verder te kunnen onderzoeken in schapen, is een onderzoeksmodel ontwikkeld waarbij de samentrekkingen van de luchtwegen gevisualiseerd en onderzocht kan worden. In **hoofdstuk 4** is dit onderzoeksmodel verder uitgewerkt bij schaapfoetussen die niet zijn blootgesteld aan een ontsteking in de baarmoeder. Bij deze schaapfoetussen werden na de geboorte de luchtwegen blootgesteld aan allergische prikkelingen, om zo de normale reactiviteit van de luchtwegen te kunnen onderzoeken. Hierbij werd aangetoond dat er een duidelijk verschil is in samentrekkingen van de luchtwegen tussen pasgeboren

schapen en 18 maanden oude schapen. Het is belangrijk dat dit verschil is aangetoond omdat dit het belang van leeftijd weergeeft voor het verschil in resultaten bij dergelijke studies. In **hoofdstuk 5** is verder gekeken naar de effecten van blootstelling aan LPS bij 7 weken oude schapen. De resultaten toonden aan dat de vroege blootstelling aan LPS zorgt voor activatie van het immuun systeem in de longen, maar dat deze activatie met 7 weken bijna helemaal is verdwenen. Bij 7 weken worden wel kleine structurele en functionele veranderingen gezien, verminderde reactiviteit van de luchtwegen waargenomen en aanwezigheid van bepaalde immuun cellen (lymfocyten) aangetoond. Interessant is dat de resultaten van verminderde luchtweg reactiviteit in tegenstelling is tot de klinische waarnemingen van een toename van astma na blootstelling aan chorioamnionitis. Dit komt mogelijk doordat het onderzoek van de luchtwegreactiviteit bij 7 weken nog te vroeg is, stijging in luchtwegreactiviteit treedt waarschijnlijk op latere leeftijd pas op. Dit zal in de toekomst verder onderzocht gaan worden. De klinische relevantie van de resultaten in dit hoofdstuk is het bewijs dat blootstelling van de ongeboren baby aan een ontsteking in het vruchtwater van invloed is op veranderingen in het immuun systeem vlak na de geboorte en op latere leeftijd structurele en functionele veranderingen laat zien.

Conclusie

Een bacteriële infectie van de vruchtvliezen en het vruchtwater staat bekend als een belangrijke oorzaak voor vroeggeboorte en een gestoorde longontwikkeling van de zuigeling. Daarnaast lijkt vroeggeboorte gerelateerd te zijn aan de ontwikkeling van een piepende ademhaling tijdens de kindertijd. Er is een dringende behoefte aan kennis naar de onderliggende mechanismen van deze ontstekingsprocessen en de gestoorde longontwikkeling op latere leeftijd. Het onderzoek dat gepresenteerd wordt in dit proefschrift heeft hieraan een bijdrage geleverd door aan te tonen dat blootstelling aan een ontsteking in het vruchtwater resulteert in een mogelijk verband tussen activatie van het immuun systeem en een gestoorde longontwikkeling tijdens de zwangerschap, met als gevolg een veranderde luchtwegreactiviteit na de geboorte. Deze resultaten bieden nieuwe inzichten in hoe de ontwikkeling van de longen wordt beïnvloed door een infectie tijdens de zwangerschap en de eerste jaren na de geboorte. Het onderzoek in dit proefschrift geeft een basis voor toekomstig onderzoek naar meer inzicht en de behandeling van astma in de vroege jeugd van te vroeg geboren kinderen.

Valorization

The main focus of this dissertation was to give insight into the postnatal persistence of fetal immune effects following a pro-inflammatory stimulus and how the duration of exposure to antenatal inflammation may affect the fetal lungs and airways as gestation advances till near-term and the first years after birth.

Relevance and target groups

Chorioamnionitis affects neonatal outcomes in an organ-dependent way, but is primarily attributed with alterations in lung development and may negatively affect neonatal outcomes by modulating the fetal immune system. However, results of these immune responses are limited to short term exposure to pro-inflammatory agonists. In the last years translational research with various animal models has been helpful to answer some basic questions about the effect of various fetal exposures on different organs. However, the various animal models differ in their developmental biology compared to humans. Development of the ovine lung is highly similar to that of humans. Sheep undergo the alveolar phase of lung development in utero and these animals are large enough to manipulate them and to induce an intrauterine inflammation for example by lipopolysaccharide (LPS). Sheep have, in contrast to mice, a bronchial circulation, bronchial glands and mast cells that respond to stimuli similarly to the human lung with both early and late phase responses. In addition, sheep can be sensitized to house dust mite antigen which is a common human antigen in asthma. Recently, airway remodelling (increased airway collagen and smooth muscle with hyperplasia of goblet cells) following repeated airway exposures of sheep to house dust mite (HDM) was reported. Sheep respond to HDM sensitization and airway challenges with a complete allergic/asthma response similar to that which occurs in humans. Therefore, preterm sheep are considered as an excellent translational model to study the effects of antenatal events on the developing fetus till adolescence in relation to the development of asthma.

Asthma is the most common chronic disease affecting children, resulting in constriction of the airway muscles and swelling of bronchioles; breathing passages are narrowed and breathing becomes very difficult. The outcome is a wheezing phenotype or childhood asthma which limits children's daily activities and affects their social activities. Specific risk factors during pregnancy can influence the risk of wheezing in early childhood. Maternal smoking, low maternal age, early bottle feeding, low birth weight and prematurity are associated with lower respiratory tract illness in the first 2 years of life. Prematurity and low birth weights are considered significant risk factors for reduced lung function at school age and the development of childhood asthma. The link with preterm birth is of significant public health relevance because of the increasing incidence of both entities. Wheezing is common in young infants and toddlers with 27% of all children having at least one

wheezing episode by the age of 9 years. For some of these children these asthmatic symptoms seem to remit with time, but many children develop asthmatic symptoms which persist throughout their life and are associated with more severe symptoms ending in the loss of lung function. About 15% of the wheezing infants develop persistent wheezing and clinical asthma later in life. Therefore, surviving preterm children are the target population for the clinical application of the identified mechanisms.

Activities, innovations and implementations

In summary, our findings have identified a potential link between activation of the prenatal immune status and impaired lung development, resulting in altered airway reactivity postnatal, and should provide a basis for future research towards the understanding and treating of early childhood asthma after preterm birth. The obtained knowledge from these future studies can be used to test the clinical association of chorioamnionitis and asthma with the ultimate aim of prevention. In addition, our findings demonstrated that infections may have the potential of targeting the inflammatory response to the lower airway, but may only be able to do so during a vulnerable time-period during development of the immune system or lung. This developmental component may further reflect important gene-environment interactions that regulate both short- and long-term airway physiological alterations that manifest themselves clinically as childhood asthma. Good and large clinical and biological databases during pregnancy and after preterm birth are needed to substantiate this association in patients.

Gender differences were observed in this dissertation. For example, female preterm fetuses had larger improvements in lung compliance after an intrauterine inflammation compared with males, which may explain at least part of the female advantage in survival after chorioamnionitis and preterm birth. Our findings suggest that the female advantage in lung gas volume is not based on lung histology, but might contribute to the theory that the surfactant deficiency in male lungs might play a key role in the female advantage after preterm birth. These results demonstrate the importance to differentiate between sexes in future experiments and outcomes. Databases and prediction models will benefit from this finding in the analysis and concept of new studies where the gender and lung development must be taken into account.

Furthermore, the knowledge of airway smooth muscle reactivity obtained with sheep precision-cut lung slices (PCLS) in this dissertation have been demonstrated to be useful for the measurement of airway responses to different early allergic response mediators and to allergens in this dissertation. It provided new inside in mechanisms of cholinergic airway contraction. Medication affecting airway constriction can be tested in this model to pave the way for clinical studies. This is a new step towards the understanding and

treating of early childhood wheezing after preterm birth which has to be tested in clinical trials in ongoing research collaboration between pharmacology and pediatrics.

Dankwoord / Acknowledgements

Het is gelukt, mijn proefschrift is klaar! 2014 is een jaar waarin ik niet alleen mijn promotietraject afrond maar waarin ik ook mijn geneeskundeopleiding afrond. Voor mij dus een bijzonder jaar! Uiteraard zou dit nooit gelukt zijn zonder een fantastische groep van collega's, vrienden en familie om me heen. Graag wil ik op deze wijze een woord van dank richten aan iedereen die op enigerlei wijze heeft bijgedragen aan de totstandkoming van dit proefschrift.

Mijn eerste dank gaat uit naar mijn promotoren, **prof. dr. Boris Kramer** en **prof. dr. Luc Zimmermann**, en copromotor **Privatdozent dr. Christian Martin**. Jullie hebben mij de gelegenheid gegeven om dit promotietraject te doorlopen, bedankt voor deze waardevolle ervaring.
Boris, voor jou een speciale dank! Het was een groot avontuur om dit promotietraject te combineren met mijn geneeskundeopleiding, niet iedereen was ervan overtuigd dat dit zou gaan lukken. Jij hebt altijd vertrouwen in me gehad en dat heb ik al die jaren enorm gewaardeerd. Je bent een fantastische mentor en jouw inspirerende en positief kritische begeleiding heeft mij gebracht tot waar ik nu ben. Dank daarvoor!

Ik dank de leden van de beoordelingscommissie, **prof. dr. Edward Dompeling**, **prof. dr. Sidarto Bambang Oetomo**, **prof. dr. Irwin Reiss**, **prof. dr. Emiel Wouters**, **prof. dr. Servaas Morré** voor hun bereidheid om dit proefschrift op wetenschappelijke inhoud te beoordelen.

Om de voorbije jaren door te komen, helpt de steun van een groep geweldige collega's die klaarstaan om bij te springen in tijden van nood en om even te ontspannen tijdens een kopje koffie/thee/water. Dank aan **Nico Kloosterboer** en **Lilian Kessels** die mij vele malen hebben geholpen in het lab. Hoofd van het lab **Tim Wolfs,** voor jou een woord van dank. Jouw komst heeft een frisse en kritisch blik over de afdeling gebracht, dit heeft mij gestimuleerd om verder te groeien en evolueren als onderzoeker. Verder wil ik graag **Eduardo Villamor**, **Danilo Gavilanes** en **Freek van Iwaarden** bedanken voor hun wetenschappelijk input tijdens de research meetings over de jaren. Ook dank aan de andere medewerkers, promovendi en oud-promovendi bij de afdeling Kindergeneeskunde, **Bea Zoer, Coen Willems, Jennifer Collins, Elke Kuypers, Reint Jellema, Daan Ophelders, Monique Willems, Maria Nikiforou, Sizzle Vanterpool, Leon Janssen, Saskia van der Sterren, Rob Moonen, Jasper Been, Kim van de Kant, Ester Klaassen,** en **Sasha Hutchinson** voor de gezellige tijd tijdens mijn onderzoekstijd bij Kindergeneeskunde. **Tamara Cauberg-Smits, Peggy van den Heuvel** en **Natascha Vrijhoeven-Pieters**, bedankt voor de onmisbare secretariële ondersteuning.

Tijdens mijn PhD periode heb ik niet alleen veel tijd doorgebracht in het lab, maar ook veel uren in het CPV (Centrale Proefdier Voorzieningen). Graag wil ik dan ook de medewerkers van het CPV, **Saskia Seeldrayers**, **Barry Plooyer**, **Joyce Suyk**, **Sanne Bout**, **Petra Dijkstra**, **Frans Slangen** en **Noortje Peeters** bedanken voor hun hulp en toewijdingen bij het opzetten en uitvoeren van de schaapexperimenten. Daarnaast dank aan de studenten **Luc Ploum**, **Sophie Kienhorst**, **Pim Boden**, **Manuel Noben**, **Anne-Sophie Warda** en collega's **Marjolein Caron** en **Yixuan Zhang** voor jullie hulp bij nachtdiensten van de bevallingen van de schapen. **Marco Schlepütz** vielen Dank für deine Hilfe bei der Verarbeitung des Schafenmaterials und für die lustige Gespräche. Das Wort 'Kliko' und den Satz 'kapot gemaakt' werde ich immer mit einem Lächeln erinnern. Außerdem Dank an deine Kollegen **Stefan Uhlig**, **Constanze Dassow** und **Peter König** an den Universitäten von Aachen und Lübeck für die angenehme Zusammenarbeit. Darüber hinaus möchte ich **Steffen Kunzmann** an die Universität von Würzburg danken für die nette Zusammenarbeit.
Zoals uit bovenstaand al naar voren komt werkt een goede wetenschapper niet geïsoleerd op het lab maar streeft naar het functioneren in een internationaal netwerk en het overleggen met collega's van alle windstreken met verschillende expertises. Behalve de samenwerking met collega's in Duitsland, heb ik ook de kans gehad om samen te werken met onze collega's in Perth, Australië.
I would like to thank the research-team in Perth at the University of Western Australia for performing the annual sheep experiments and their additional scientific input: **Jane Pillow**, **John Newnham**, **Graeme Polglase**, **Ilias Nitsos**, **Suhas Kallapur**, **Matthew Kemp**, **Manfred Beilharz**, and **Andrea Lee**. Furthermore, I would like to address a special 'thank you' to **Alan Jobe** for his invaluable scientific remarks and suggestions during my PhD-project. You have taught me a great deal about writing scientific papers, I always looked forward to receive new useful feedback and comments on my manuscript from you. Thank you for that, you are a true inspiration!

Voordat ik aan mijn promotietraject begon, heb ik tijdens mijn afstudeerstage van moleculaire levenswetenschappen (MLW) een aantal geneeskunde studenten ontmoet die op dat moment hun WESP (wetenschapsparticipatie van geneeskunde) deden bij kindergeneeskunde. Voor mij waren zij een inspiratiebron om de geneeskundeopleiding te gaan doen. **Nienke Wieringa**, **Thijs Flinsenberg**, **Heike Heineman**, **Patricia Sanders** en **Marijn Schuurman**, ik heb met veel plezier geluisterd naar jullie boeiende verhalen over het vak geneeskunde, dank daarvoor. Tijdens de geneeskundeopleiding heb ik een ontzettend lieve vriendin leren kennen. **Charelle Willems**, op jou kan ik rekenen, je staat altijd voor me klaar. Bedankt voor je steun en gezelligheid. Ik vind het erg fijn dat je mijn paranimf wilt zijn.

De weg die ik bewandeld heb is geen doorsnee route geweest en voor advies kon ik dan ook altijd terecht bij studieadviseur **Guido Tans**. Voor jou ook een woord van dank voor je adviezen om de combinatie studeren en promoveren tot een goed einde te brengen.
Dan wil ik via deze weg graag nog iemand bedanken die ik vaak op de meest onverwachte momenten tegenkom en altijd een hoop plezier en gezelligheid met zich meebrengt. **Eddy de Haan**, waar ik je ook tegenkom, op het lab, in het ziekenhuis of ergens in de gangen van de universiteit, je bent altijd in voor een gezellig praatje. Dank voor de gezellige en grappige verhalen, dat ga ik zeker missen.

Aan het einde van een lange werkdag is er niets zo fijn als thuis te komen en samen te zijn met de mensen van wie je houdt en die er altijd voor je zijn. Lieve **pappa** en **mamma**, bedankt voor jullie onvoorwaardelijke steun en vertrouwen. Ik weet dat jullie me soms wel eens achter het behang konden plakken, maar jullie hebben me het doorzettingsvermogen gegeven om te komen waar ik nu ben. Bedankt voor de veilige thuishaven waar ik steeds op terug kon vallen; zonder jullie had ik dit nooit gekund! Ook mijn grote broer wil ik graag bedanken. **Frank**, bedankt voor je steun en betrokkenheid, jouw geduld en support hebben me steeds weer vooruit geholpen. Ik ben er trots op dat je vandaag mijn paranimf bent!

Lieve **Mike**, als laatste wil ik jou bedanken dat je op mijn pad bent gekomen. Bij jou heb ik de ontspanning gevonden in deze hectische jaren van onderzoek en studeren. Bedankt voor je liefde en geduld, ik ben ‘happy’ met je!

Curriculum Vitae

Verena Anna Carolus Lambermont was born on October 16th 1984 in Heerlen, the Netherlands. In 2003 she graduated from high school at Sophianum in Gulpen and subsequently started her study in Molecular Life Sciences at Maastricht University. After completion of her Bachelor's degree in 2006, she stayed in Maastricht to pursue a Master's degree in Clinical Molecular Sciences. During this period she did an internship, entitled "Antenatal exposure to inflammation affects immune response to postnatal sensitization to house dust mite in sheep", under supervision of prof. dr. Boris Kramer at the Department of Pediatrics of the Maastricht University Medical Center for which she obtained her Master's degree in 2008.
At this point her interest in the clinical care for patients was triggered and she started Medical School at Maastricht University. In 2011 she obtained her Bachelor's degree and continued in Maastricht to pursue a Master's degree in Medicine in 2014. Next to her medical school she did her PhD research. The end of her senior internship in 2008 was the beginning of her PhD project. During her PhD at the research group of prof. dr. Boris Kramer, she focused on aspects of lung development and bronchial hyperreactivity after antenatal exposure to inflammation, resulting in the current thesis.

Verena Lambermont werd geboren op 16 oktober 1984 in Heerlen, Nederland. In 2003 studeerde ze af van het Sophianum te Gulpen waarna ze aan haar studie Moleculaire Levenswetenschappen aan de Universiteit van Maastricht begon. Na het behalen van haar Bachelor diploma in 2006, startte ze een Master opleiding in Klinische Moleculaire Wetenschappen aan de Universiteit van Maastricht. Tijdens haar opleiding volgde ze een stage, getiteld "antenatale blootstelling aan een inflammatie beïnvloedt de immuun-respons op postnatale sensitisatie voor huisstofmijt in schapen", onder begeleiding van prof. dr. Boris Kramer bij de afdeling kindergeneeskunde van het Maastricht Universitair Medisch Centrum waarmee ze haar Master diploma behaalde in 2008.
Op dat moment is haar interesse voor de klinische zorg van patiënten ontstaan en begint ze aan de opleiding Geneeskunde aan de universiteit van Maastricht. In 2011 behaalt ze haar Bachelor diploma en vervolgt ze de opleiding in Maastricht om uiteindelijk een Master diploma in de Geneeskunde te behalen in 2014. Haar Geneeskunde opleiding heeft ze gecombineerde met een PhD traject. Tijdens haar PhD bij de onderzoeksgroep van prof. dr. Boris Kramer, heeft ze zich vooral gericht op aspecten van longontwikkeling en bronchiale hyperreactiviteit na antenatale blootstelling aan een ontsteking, dat resulteerde in dit proefschrift.

List of Publications

List of Publications

1. **Lambermont VA***, Schlepütz M*, Dassow C, König P, Uhlig S, Kramer BW, Martin C. Comparison of airway responses in sheep of different age in precision-cut lung slices (PCLS), PLoS One 2014, *In press*. *Both authors contributed equally.
2. **Lambermont VA**, Kuypers E, Collins JJ, Pillow JJ, Newnham JP, Polglase GR, Nitsos I, Kemp MW, Jobe AH, Kallapur SG, Kramer BW. *Effects of intra-amniotic lipopolysaccharide exposure on the fetal lamb lung as gestation advances*. Pediatr Res. 2014 Apr;75(4):500-6.
3. Engel M, Nowacki RM, Reiss LK, Uhlig S, Willems CH, Kloosterboer N, van Iwaarden JF, Sewing AC, Seehase M, **Lambermont VA**, Collins JJ, Zimmermann LJ, Vos GD, Kramer BW. *Comparison of Recruitment Manoeuvres in Ventilated Sheep with Acute Respiratory Distress Syndrome*. Lung. 2013 Feb;191(1):77-86.
4. Schlepütz M, Rieg AD, Seehase S, Spillner J, Perez-Bouza A, Braunschweig T, Schroeder T, Bernau M, **Lambermont V**, Schlumbohm C, Sewald K, Autschbach R, Braun A, Kramer BW, Uhlig S, Martin C. *Neurally mediated airway constriction in human and other species: a comparative study using precision-cut lung slices (PCLS).* PLoS One. 2012;7(10):e47344.
5. **Lambermont VA,** Been JV, Kunzmann S, Vanterpool SF, Newnham JP, Kallapur SG, Jobe AH, Kramer BW. *Sex differences in lung gas volumes after lipopolysaccharide-induced chorioamnionitis in fetal sheep*. Gend Med. Aug 2012;9(4):278-286.
6. Gantert M, Jellema RK, Heineman H, Gantert J, Collins JJ, Seehase M, **Lambermont VA**, Keck A, Garnier Y, Zimmermann LJ, Kadyrov M, Gavilanes AW, Kramer BW. *Lipopolysaccharide-induced chorioamnionitis is confined to one amniotic compartment in twin pregnant sheep*. Neonatology. May 2012;102(2):81-8.
7. **Lambermont VA***, Lee AJ*, Pillow JJ, Polglase GR, Nitsos I, Newnham JP, Beilharz MW, Kallapur SG, Jobe AH, Kramer BW. *Fetal responses to lipopolysaccharide-induced chorioamnionitis alter immune and airway responses in 7-week-old sheep*. Am J Obstet Gynecol. Apr 2011 Apr;204(4):364.e17-24. *Both authors contributed equally.

Award nomination

Nominated at ESPR 2011 for the 'Young Investigators Award', Newcastle, UK.