Annamari Salminen

SURFACTANT PROTEINS AND CYTOKINES IN INFLAMMATION-INDUCED PRETERM BIRTH

EXPERIMENTAL MOUSE MODEL AND STUDY OF HUMAN TISSUES
ANNAAMARI SALMINEN

SURFACTANT PROTEINS AND CYTOKINES IN INFLAMMATION-INDUCED PRETERM BIRTH
Experimental mouse model and study of human tissues

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in Auditorium F202 of the Department of Pharmacology and Toxicology (Aapistie 5), on 21 October 2011, at 12 noon

UNIVERSITY OF OULU, OULU 2011
Salminen, Annamari, Surfactant proteins and cytokines in inflammation-induced preterm birth. Experimental mouse model and study of human tissues
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Oulu, Finland

Abstract
Prematurity is the main cause of morbidity and mortality in infants. In 25–40% of the cases preterm birth is associated with intrauterine inflammation. Surfactant proteins (SPs) A, C, and D have roles in innate immunity. In the female reproductive tract and amniotic fluid (AF), these proteins may modulate the inflammatory responses leading to preterm birth.

The aim of the present study was to establish a mouse model of lipopolysaccharide (LPS)-induced preterm birth of live-born pups and to study the activation of the innate immune system. By using mice overexpressing either rat SP-A (rSP-A) or rSP-D the roles of SP-A and SP-D in inflammatory responses were investigated. In addition, the expression of SP-C in gestational tissues was analyzed. The association of SP-C single nucleotide polymorphism (Thr138Asn) with spontaneous preterm birth and preterm premature rupture of membranes (PPROM) was investigated in a homogenous northern Finnish population of mothers and infants.

Wild-type (WT) mice were injected with a single dose of intraperitoneal LPS at 16 or 17 days of gestation (term 19–20 days) leading to preterm delivery of live-born fetuses. After LPS, cytokine levels increased rapidly in maternal serum and in the uterus. This maternal inflammatory response was followed by the modest inflammatory activation in fetal and feto-maternal compartments. In fetal lung the expression of SP-A and SP-D was downregulated.

The overexpression of SP-A or SP-D was evident in gestational tissues of rSP-A or rSP-D mice, respectively. In addition, excess of these proteins was detected in AF. Overexpression of either rSP-A or rSP-D modulated the LPS-induced inflammatory response related to preterm birth. Most notably, the expression of IL-4 and IL-10 in uteri and IL-10 in fetal membranes was lower in overexpressing animals. SP-C was detected in mouse and human placentas, fetal membranes, and in uteri of pregnant mice. The fetal SP-C polymorphism strongly associated with the duration of PPROM.

The present study provides new information about the molecular events in inflammation-induced preterm birth, particularly about the roles of cytokines and SPs in this process. Understanding of the mechanisms involved in preterm parturition may provide means for prevention and management of preterm births in the future.

Keywords: cytokines, endotoxins, innate immunity, PPROM, preterm birth, surfactant proteins

Tutkimuksen tavoitteena oli luoda hiirimalli, jossa ennenaikainen synnytys saadaan aikaan lipopolysakkarin (LPS:n) injektiolla vatsaonlaisen. Hiirimallin avulla tutkittiin puolustusjärjestelmän aktivaatiota sekä äidin että sikiön kudoksissa. Rotan SP-A:ta (rSP-A:ta) tai rSP-D:stä yli-ilmentävien hiirten avulla selvitettiin, muuttavatko nämä proteiinit ennenaikaiseen synnytymään johtavia tulehdusreaktioita. Lisäksi määritettiin SP-C:n ilmentymisen hiiren ja ihmisen kohdussa, sikiökaloilla ja istukassa. SP-C-geenin yhden emäksen polymorfin (Thr138Asn) liittymistä ennenaikaiseen synnytykseen tai sikiökalojen puhkeamiseen tutkittiin homogeenisessä pohjoismelodialaisessa tutkimuspopulaatiossa.


Tutkimus antaa lisätietoa tulehduksen aiheuttaman ennenaikaisen synnytyksen mekanismista sekä sytokiinien ja SP-A:n- ja SP-D:n osuuksista synnyttystapahtumassa. Mekanismin ymmärtäminen on erittäin tärkeää, jotta ennenaikaiset synnytykset voitaisiin tulevaisuudessa ehkäisämmät tehokkaammin.

Asiakirjat: endotoksiinit, ennenaikainen synnytys, PPROM, surfaktantiproteiinit, synnynnäinen immuniteetti, sytokiinit
Acknowledgements

This study was carried out at the Department of Pediatrics and Biocenter Oulu, University of Oulu, during the years 2003–2011.

I wish to express my deepest gratitude to my supervisor Professor Mikko Hallman, for providing me the opportunity to work in this interesting research project as a member of his group. His enormous patience, never-ending ideas, and encouragement during all these years have been invaluable. I am also grateful to my other supervisors Reetta Vuolteenaho and Reija Paananen for guidance and help with scientific, practical, and sometimes even personal matters. Especially I would like to thank Reetta and Reija for believing in me and my project from the beginning. There wouldn’t be this thesis without you two.

I am grateful to Professor Ganesh Acharya and Professor Boris Kramer for accepting the invitation to review my thesis with such a short notice. Their valuable comments truly helped me to improve the manuscript. Sandra Hänninen is acknowledged for revising the language of this thesis.

I wish to thank all my other co-authors: Helena Autio-Harmainen, Stephan Glasser, Pentti Jouppila, Aino Luukkanen, and Anu Tuohimaa are all acknowledged. Ritva Saastamoinen (ex Haataja) and Minna Karjalainen are acknowledged especially for answering my questions about genetics over and over again, Marja Ojaniemi for help with the manuscripts, and Juhani Metsola for assisting me with the mouse studies.

I warmly thank all the current and former members of the Hallman group for their support, useful advice, and friendship. I am also grateful for all the joyful moments we have spent together. We really should have written down all the things that have been said and done! I owe my special thanks to Maarit Haarala and Mirkka Ovaska for their excellent technical assistance, and Tanja Kanniala for extracting mRNA from hundreds of tissue samples. Marjatta Palohimo at the office is acknowledged for her always helpful attitude and kind advice throughout these years.

I wish to express my gratitude to the personnel of the Laboratory Animal Center, including Sanna Haapalainen, Jukka Ketola, Seija Seijänperä, Tuula Stranius, and especially Sirpa Tausta for taking excellent care of our mice and making so many things so much easier. I would also like to thank Riitta Vuento for preparing tissue sections and performing the immunohistochemical stainings and Antti Viklund for keeping my computer in good condition.
I thank all my friends. Piaia and Riina are acknowledged for almost lifelong friendship and “Tokotytöt” of OSN for all those hilarious moments that one can never forget. I would also like to mention here my two golden girls “Emma” and “Ringa”, without whom my life would not fulfilled. Especially Ringa deserves thanks for keeping me sane although trying to drive me insane sometimes.

Above all, I wish to express my warmest gratitude to my family, my mother Anna-Kaija, sister Veera, and especially my dear fiancé Juha for his patience, understanding, and support.

This work was supported financially by the Alma and K.A. Snellman Foundation, Oulu, Finland, Biocenter Oulu, Foundation for Pediatric Research, the Oulu University Scholarship Foundation, the Paulo Foundation, and the Sigrid Juselius Foundation.

Oulu, August 2011

Annamari Salminen
### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ATII</td>
<td>Alveolar type II cell</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric Bead Array</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CRD</td>
<td>Carbohydrate recognition domain</td>
</tr>
<tr>
<td>dpc</td>
<td>Days <em>post coitum</em></td>
</tr>
<tr>
<td>hSP</td>
<td>Human surfactant protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipopolysaccharide binding protein</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeat</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemo-attractant protein</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inhibitory protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PPROM</td>
<td>Preterm premature rupture of membranes</td>
</tr>
<tr>
<td>proSP-C</td>
<td>Surfactant protein C proprotein</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptor</td>
</tr>
<tr>
<td>RDS</td>
<td>Respiratory distress syndrome</td>
</tr>
<tr>
<td>RPA</td>
<td>RNase Protection Assay</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>rSP</td>
<td>Rat surfactant protein</td>
</tr>
<tr>
<td>SIRPα</td>
<td>Signal inhibitory regulatory protein α</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SP</td>
<td>Surfactant protein</td>
</tr>
<tr>
<td>SP^{−}</td>
<td>Surfactant protein deficient</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. Additionally, some unpublished data are presented.


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1 Introduction

From fertilization to parturition, normal human pregnancy is tightly regulated to maintain uterine quiescence to allow the growth and development of the fetus. Prior to birth, changes occur in myometrial contractility and cervical extracellular matrix that eventually result in the delivery of the fetus. The factors involved in the cascade leading to birth are still incompletely understood, but it is likely that parturition is the end result of a complex interaction of various factors, including inflammatory mediators, steroid hormones, and paracrine molecules (Mendelson 2009, Peltier 2003).

Preterm birth is the main cause of perinatal mortality and morbidity in developed countries. There is increasing evidence suggesting that the signals leading to preterm parturition are similar to the signals associated with term birth – just taking place too early. Bacterial infections and consequent inflammatory processes have been implicated as principal mechanisms responsible for preterm birth. In addition, many other factors (e.g. genetic factors, multiple pregnancy, and previous history of preterm birth) predisposing to preterm delivery have been recognized (Goldenberg et al. 2008, Slattery & Morrison 2002). However, the prediction of preterm birth is still very problematic because many of the mothers exhibit none of the known risk factors. In addition, various clinical applications focused on the prevention of preterm delivery have not succeeded in reducing the risk of prematurity (Iams et al. 2008).

Surfactant proteins (SPs) A and D are hydrophilic collagenous glycoproteins that have roles in surfactant homeostasis and innate immunity. SP-A and SP-D can directly interact with a variety of microorganisms and enhance their uptake and clearance. In addition to binding to pathogens, these proteins are known to interact with receptors on host cells and modulate cellular responses to various stimuli. In the female reproductive tract and in amniotic fluid, SP-A and SP-D can be postulated to have roles in modulating the inflammatory responses related to initiation of labor (Kingma & Whitsett 2006, Kishore et al. 2006, Wright 2005). Most notably, SP-A secreted by the fetal lung has been suggested to be the labor-inducing signal (Condon et al. 2004). SP-C is a highly hydrophobic protein that has been considered to be exclusively lung-specific and to have roles merely in the functions of pulmonary surfactant. Recently it has been proposed that SP-C possesses an immunomodulatory role as well (Mulugeta & Beers 2006). In addition, expression of SP-C in many nonpulmonary tissues has become evident (Brauer et al. 2007a, Brauer et al. 2009, Eikmans et al. 2005, Mo et al. 2007, Sati
et al. 2010). In humans, an SP-C polymorphism has been associated with very preterm birth (Lahti et al. 2004).

In the present study, a mouse model of inflammation-induced preterm birth of live-born pups was developed. This model was used to study the LPS-induced activation of the inflammatory cascade in maternal and fetal compartments related to preterm birth. By using mice overexpressing rat SP-A (rSP-A) or rSP-D (Elhalwagi et al. 1999, Fisher et al. 2000), the roles of SP-A and SP-D in modulating the LPS-induced inflammation were characterized. In addition, the expression of SP-C in gestational tissues was analyzed and the possible involvement of this protein in the process of prematurity was evaluated.

This study provides additional knowledge about the roles of cytokines and surfactant proteins in inflammation-induced preterm parturition and may provide a novel means for the prevention and management of premature births in the future.
2 Review of the literature

2.1 Mammalian immune system

The mammalian immune system can be considered to be a multilayer system consisting of three major defense mechanisms: external barriers and innate and adaptive immune responses. The external barriers can be classified either as physical barriers (such as skin and mucous membranes) that prevent the entry and colonization of many microbes, and chemical barriers that include stomach acids and destructive enzymes found in secretions. Innate immunity serves as a first line host defense against pathogens and it is conserved within many species, from flies to mammals (Medzhitov 2007, Moser & Leo 2010). The adaptive immune system provides a second line of defense, usually at the later stage of infection (Medzhitov 2007, Shanker 2010). Innate and adaptive immunity are not completely separate systems, but rather they work together in a complex way to provide wide-ranging protection against environmental pathogens.

Innate immunity is the host defense system that is present even before the infection. Innate immunity responses are mediated by phagocytes including macrophages, dendritic cells, neutrophils, and monocytes. These cells can recognize microbial components, known as pathogen-associated molecular patterns (PAMPs), through a limited set of germ-line encoded pattern recognition receptors (PRRs) (Medzhitov 2007). In general, the recognition of PAMP by PRR triggers a sequence of events that eventually leads to different inflammatory responses, including the production and secretion of cytokines and chemokines, the activation of macrophages and monocytes, and the activation of the adaptive immunity system. The innate immunity responses were originally thought to be completely nonspecific, but it is now evident that different PRRs react with different PAMPs and activate specific signaling pathways leading to distinct responses (Kawai & Akira 2010). Innate immunity is particularly important in newborn infants, whose adaptive immune system has not yet been fully developed.

In contrast to innate immunity responses that are characterized by the rapid protection against microorganisms and lack of memory and learning process, adaptive immunity is involved in the elimination of pathogens in the late phase of infection and the generation of immunological memory (Medzhitov 2007). The specificity and diversity of the adaptive immune system arise from the clonal
selection of T- and B-lymphocytes that express antigen-specific receptors generated early in the lymphocyte development (Li et al. 2004b)

2.1.1 Pattern recognition receptors

Pattern recognition receptors are germ-line encoded receptors that recognize different PAMPs and initiate the innate immunity responses. Based on their functions, PRRs can be divided into three groups: secreted, endocytic, and signaling receptors (Medzhitov 2007). Membrane-bound toll-like receptors (TLRs) and soluble collectins are among the best characterized PRRs (Kawai & Akira 2010, Medzhitov 2007).

Toll-like receptors

Toll protein of Drosophila melanogaster is the founding member of the TLR family. Toll was first identified as an essential factor for Drosophila embryogenesis (Anderson et al. 1985). Later it was demonstrated to have a critical role in mediating the antifungal immune responses of flies (Lemaitre et al. 1996) suggesting that mammalian TLRs may also participate in innate immunity. At present, ten members of the TLR family have been identified in humans and thirteen in mice. Many of the identified TLRs have been found in other mammalian species as well (Kawai & Akira 2010).

Mammalian TLRs are expressed at the highest levels in tissues that are constantly exposed to environmental factors such as lung and the gastrointestinal tract. In addition, TLRs are present in various cells of the immune system, including macrophages, dendritic cells, B cells, and specific types of T cells (Zarember & Godowski 2002). At the cellular level, the expression of TLRs has been specifically detected either in the plasma membrane or in intracellular vesicles. TLRs recognize a wide variety of structural components such as lipopeptides, LPS, single- and double-stranded RNA, and flagellins that are unique to bacteria, viruses and fungi (for a recent review, see Kawai & Akira 2010). The ligand recognition by TLR leads to receptor dimerization and activation of a complex signaling cascade that evokes the host inflammatory response. The broad specificity of TLRs to distinguish between various bacterial and viral compounds is achieved by structural variations in the ligand recognition domain, heterodimerization of the receptors, and activation of distinct signaling pathways (reviewed in Kawai & Akira 2010 and O’Neill & Bowie 2007). TLR4
was the first identified mammalian homolog of *Drosophila* Toll and is the best characterized TLR to date. TLR4 will be discussed further in section 2.4.

**Collectins**

Collectins are collagenous glycoproteins that belong to the large calcium-dependent lectin superfamily of proteins characterized by one or more C-type lectin domain. Nine different collectins have been characterized so far: SP-A, SP-D, mannose binding lectin (MBL), collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1), conglutinin, collectin-43 (CL-43), collectin-46 (CL-46) (for a review, see van de Wetering *et al.* 2004), and collectin kidney 1 (CL-K1) (Keshi *et al.* 2006). Of these, SP-A, SP-D, MBL, CL-K1, conglutinin, CL-43, and CL-46 are the secreted type of collectins, while CL-L1 and CL-P1 localize to the cytosol and cell membrane. Members of the collectin family have been found in many vertebrate species (van de Wetering *et al.* 2004).

Collectins represent an important group of pattern receptor molecules, having the ability to bind various invading microorganisms, including Gram-positive and Gram-negative bacteria, viruses, and fungi. Binding of collectins to pathogens facilitates microbial clearance through different mechanisms, including aggregation, complement activation, opsonization, and activation of phagocytosis. In addition, collectins can modulate the inflammatory responses of host cells to various stimuli. Biological responses are greatly dependent on the collectin, but also on the nature of the pathogen, the duration of exposure, cell type and the state of activation of the cell (reviewed in van de Wetering *et al.* 2004, Wright 2005). SP-A and SP-D are among the best characterized collectins regarding their roles in innate immunity, and they will be discussed in more detail in section 2.5.

**2.1.2 Cytokines in inflammation**

Cytokines are a diverse group of small and soluble proteins that are produced by almost all nucleated cells. These modulators have specific effects on virtually every systemic reaction of an organism, including immune responses, inflammatory processes, wound healing, embryogenesis, organ development, mitosis, cell survival/death, and cell transformation. Some cytokines even behave like classical hormones. Cytokines act in a complex network in which a biological effect of one cytokine is often modified or augmented by another. In addition, a
specific cytokine can have multiple biological activities and different cytokines can have the same activity (Haddad 2002).

The expression of the cytokines that stimulate the innate immunity responses is initiated after the recognition of invading microorganisms, most notably via TLRs (Haddad 2002, Kawai & Akira 2010). Proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-8 favor inflammation, whereas the anti-inflammatory cytokines such as IL-4, and IL-10 control the action of proinflammatory cytokines promoting healing (Opal & DePalo 2000). The net effect of the cytokine response is the balance between both types. However, it should be noted that a clear-cut classification of cytokines as pro- or anti-inflammatory may be misleading, since many of the cytokines (e.g. IL-6) can express both properties depending on various environmental factors, stimuli, and activation status of the cells (Haddad 2002, Opal & DePalo 2000). TNF-α and IL-1 are produced rapidly and transiently in response to microbial challenge. These “early-response” cytokines are especially important in initiating the cytokine cascade and controlling the first line immunomodulatory effects, resulting in a synchronized host defense against invading pathogens (Mizgerd et al. 2001).

2.2 Preterm birth

Spontaneous preterm birth, defined as a birth at less than 37 weeks of gestation (term 40 weeks) following the onset of preterm labor or preterm premature rupture of membranes (PPROM), is the foremost cause of perinatal morbidity and mortality in developed countries (Goldenberg et al. 2008). During the years 1987–2009, preterm birth complicated 5.1–5.5% of all pregnancies in Finland (http://www.stakes.fi). In the USA the preterm delivery rate is about 12–13% and it has been steadily increasing during the past decades, as in many other countries as well (Goldenberg et al. 2008). The pathogenesis of preterm labor is still poorly understood, but various factors that may contribute to spontaneous preterm birth have been recognized. These include previous history of preterm birth, multiple pregnancy and associated complications, PPROM, intrauterine growth restriction, genetic factors, infections, ethnic origin, socioeconomic status, smoking, substance misuse, poor nutrition, young maternal age, low maternal weight, and maternal medical disorders. However, rather than being caused by a single factor, preterm birth is more likely the final common pathway of multiple pregnancy complications (Goldenberg et al. 2008, Slattery & Morrison 2002). Recently the
role of the fetus in the initiation of labor has been emphasized (Challis et al. 2005).

Despite the advancing knowledge of risk factors and mechanisms related to preterm parturition, identification of new biochemical markers, and the introduction of new pharmaceuticals and methods of treatment, an intervention to decrease the risk of prematurity remains to be discovered (Goldenberg et al. 2005, Iams et al. 2008, Slattery & Morrison 2002). Improvements in neonatology have increased the survival rates of preterm infants, and the survival of almost all infants born after 32 completed weeks of gestation can now be expected (Slattery & Morrison 2002). However, premature babies are still at risk for various chronic disabilities including respiratory, neurologic, and metabolic disorders. Because the morbidity is inversely related to gestational age, any treatment that prolongs the length of pregnancy would have a profound effect on the welfare of these infants (Saigal & Doyle 2008, Slattery & Morrison 2002).

### 2.2.1 Intrauterine infection and preterm birth

Intrauterine bacterial infections and consequent inflammatory processes have been implicated in 25–40% of preterm births. However, this may be an underestimation because infections are difficult to detect in many cases (Goldenberg et al. 2008). The frequently identified bacteria in the amniotic cavity of women in spontaneous preterm labor include *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Peptostreptococcus* spp, and *Bacteroides* spp. In addition many other microorganisms (e.g. *Escherichia coli*) are occasionally associated with increased risk of preterm birth. Many of these bacteria are actually present on the normal vaginal flora (Goldenberg et al. 2000, Goldenberg et al. 2008, Slattery & Morrison 2002).

Microbes can enter the amniotic cavity via several routes: ascending from the vagina and the cervix, through the placenta by hematogenous dissemination, through the fallopian tubes from the peritoneal cavity, and by accidental introduction during invasive procedures. Of these, the ascending route is the most common. The infection within the uterus can be detected between the maternal tissues and fetal membranes, within the fetal membranes, within the placenta, within the amniotic fluid, within the umbilical cord, or within the fetus itself (Goldenberg et al. 2000, Goldenberg et al. 2008). In addition, several non-genital tract infections, including pyelonephritis and asymptomatic bacteriuria, pneumonia,
and periodontal disease, have been suggested to increase the risk of preterm birth (Agueda et al. 2008, Goodnight & Soper 2005, Smaill 2007).

Mechanism of inflammation-induced preterm birth

Throughout the pregnancy the uterus remains in a relatively quiescent state and the cervical canal is closed with a mucus plug. A transition from uterine quiescence to the activated state precedes labor. Bacteria are thought to initiate the complex signaling cascade related to preterm labor through the release of endotoxins and exotoxins that are recognized by PRRs. Activation of receptor proteins leads to the production of proinflammatory cytokines and chemokines by leukocytes and other cells in the uterus, placenta, and fetal tissues. The cytokines and chemokines together with bacterial toxins stimulate the synthesis and release of prostaglandins and initiate the chemotaxis, infiltration, and activation of inflammatory cells (Goldenberg et al. 2000, Romero et al. 2006). Prostaglandins have multiple roles in parturition. Upregulation of the gap junction proteins in the myometrium permits the formation of the gap junctions allowing the free passage of ions and small molecules between the smooth muscle cells and enables coordinated, powerful contractions to begin (Challis 2000). Furthermore, prostaglandins activate matrix metalloproteinases (MMPs), which degrade the extracellular matrix in the cervix and fetal membranes. Cervical ripening is essential for successful birth and occurs independently of uterine contractions (Olson 2003, Vadillo-Ortega & Estrada-Gutierrez 2005).

The initiation of the signaling cascade presented above may take place in fetal and/or maternal compartments, but it might as well occur in extrauterine tissues (Goldenberg et al. 2000, Peltier 2003). The role of the fetus in providing the signals for preterm parturition has been supported by the finding that a systemic fetal proinflammatory cytokine response markedly decreases the time to delivery (Romero et al. 1998). Despite the initial site of the inflammation, the end result is most likely an interplay of various organs. It should be noted that the mechanism presented here is a very simplified illustration and other mediators may have an important role as well. However, pregnancies in the third trimester and deliveries without signs of infection are also characterized with increased levels of proinflammatory cytokines in the amnion, chorio-decidua, and myometrium (Romero et al. 2006). This indicates that a common component in the initiation of labor is inflammation at the maternal-fetal interface mediated by proinflammatory cytokines, regardless of the presence of infection.
2.2.2 Preterm premature rupture of membranes

During pregnancy, the fetus is surrounded and cushioned by amniotic fluid that is enclosed within a sac formed by the fetal membranes. This barrier protects the fetus from invading microorganisms that could potentially cause infection. At term pregnancy, the rupture of membranes is a normal process involved in parturition and occurs before or after the onset of labor. PPROM, defined as spontaneous rupture of fetal membranes prior to 37 completed weeks of gestation at least 1 hour before the regular uterine contractions, is the presenting symptom in 25–40% of the cases of all preterm births (Goldenberg et al. 2008, Parry & Strauss 1998). The underlying cause of PPROM is often unknown, but the risk factors are generally similar to those described for spontaneous preterm birth (DeFranco et al. 2007). In many cases PPROM is associated with infections that are thought to cause weakening of the fetal membranes and their breakage (Menon & Fortunato 2007). Labor almost always follows PPROM relatively soon, although the delay between PPROM and the onset of labor can vary up to several weeks, especially in earlier gestational weeks. Prolonged PPROM decreases the incidence of neonatal morbidity related to prematurity, but predisposes to the development of intrauterine infection and consequent complications (Caughey et al. 2008, Parry & Strauss 1998).

2.2.3 Genetic influences of preterm birth

The likelihood of recurrence of PPROM or preterm labor within the same mother or family and the racial disparities in preterm birth rates suggest the influence of genetic factors on preterm birth (Menon & Fortunato 2007, Plunkett & Muglia 2008). The higher risk for preterm delivery among black women compared with white women is evident even after correction for confounding social and economic factors (Goldenberg et al. 1996). Familial and twin studies all support the possibility of an underlying genetic basis for preterm parturition (Menon & Fortunato 2007, Plunkett & Muglia 2008). Because the important role for inflammation and infection in the initiation of the signaling cascade leading to delivery is well established, most of the genetic studies have focused on the genes involved in immunity as a possible etiologic mechanism for preterm birth. For example, common polymorphisms in the genes of several inflammatory cytokines or their receptors and in TLR4 gene have been associated with an increased risk for preterm labor or PPROM. Several polymorphisms associated with adverse
pregnancy outcomes have also been identified in the genes that are involved in the remodeling of connective tissue (e.g. MMPs) or the control of uterine contractility and placental function (Plunkett & Muglia 2008). In addition, a polymorphism in the SP-C gene has been associated with very preterm birth in humans (Lahti et al. 2004). Recently a new susceptibility gene IGF1R increasing the risk for preterm birth in infants has been identified (Haataja et al. 2011), further supporting the role of the fetus in determining the fate of pregnancy. A more detailed description of the genetic variation associated with prematurity is presented in the extensive review by Plunkett & Muglia (2008).

Although there is increasing evidence about the genetic influence of the polymorphisms on the risk of preterm birth, more studies are still needed about their complex interplay with other factors including environmental stimuli and other candidate genes. The future expectations are that the population of women at highest risk of preterm birth could be identified and treated with patient-specific therapies aimed at delaying or preventing prematurity.

2.2.4 Animal models of preterm birth

Much of our information about the inflammatory pathways promoting preterm parturition is derived from different animal models. Several approaches have been developed to mimic the inflammation-induced preterm birth in animals such as mice, rats, rabbits, sheep, and nonhuman primates. These procedures include the administration of live or killed bacteria, bacterial products (e.g. LPS), inflammatory cytokines, or other inflammatory agents into the peritoneal cavity, cervix, amniotic fluid, or uterus (e.g. Davies et al. 2000, Gravett et al. 1994, Kaga et al. 1996, Rounioja et al. 2003, Schlafer et al. 1994, reviewed in Elovitz & Mrinalini 2004). Intraperitoneal administration represents a systemic model of inflammation-induced preterm birth that in humans is associated with conditions that promote bacteremia or sepsis, whereas the other routes may better reflect the ascending infection. However, administration of inflammatory agents into the amniotic fluid or uterus requires invasive procedures which may limit the usability of these methods.

The induction of an inflammatory response related to preterm birth by bacteria or bacterial products in animals results in elevated levels of several proinflammatory cytokines, e.g. IL-1β, IL-6, IL-8 and TNF-α, detected either as increased messenger RNA (mRNA) synthesis in gestational tissues or as higher concentrations of these proteins in maternal serum and in amniotic fluid (e.g.
Gravett et al. 1994, Hirsch et al. 1995, Kajikawa et al. 1998, Kallapur et al. 2001, Kramer et al. 2001, Reznikov et al. 1999). Similar increases have been observed in human pregnancies with preterm labor (Goldenberg et al. 2005, Romero et al. 2006). Therefore, the roles of specific cytokines in the initiation of preterm labor have been extensively studied. In different animal models, preterm labor can be induced by IL-1 or TNF-α (Baggia et al. 1996, Bry & Hallman 1993, Romero et al. 1991, Sadowsky et al. 2006, Yoshimura & Hirsch 2005), but not by IL-6 or IL-8 (Sadowsky et al. 2006, Yoshimura & Hirsch 2003). However, rather than working alone, cytokines are known to interact with each other in a complex manner and to have redundant roles. For example, mice deficient in either the IL-1 receptor or IL-1β are not different from wild-type mice in their susceptibility to inflammation-induced preterm delivery (Hirsch et al. 2002, Reznikov et al. 1999, Wang et al. 2006), but the combined signaling via IL-1 and TNF-α receptors is needed (Hirsch et al. 2006). Similarly it has been demonstrated that neither the treatment with IL-1 receptor antagonist or soluble TNF receptor Fc fusion protein, nor the overexpression of IL-1 receptor antagonist prevents the endotoxin-induced preterm birth in mice (Fidel et al. 1997, Yoshimura & Hirsch 2005). In contrast to previous studies, Holmgren et al. (2008) demonstrated that the systemic pretreatment of mice solely with anti-TNF-α prior to LPS injection decreases the preterm delivery rate.

Anti-inflammatory cytokine IL-10 is a feasible candidate for the maintenance of pregnancy because it has been shown to control the synthesis of several proinflammatory cytokines, including TNF-α, IL-6, and IL-1α both in vitro and in vivo (de Waal Malefyt et al. 1991, Fiorentino et al. 1991, Fortunato et al. 1996, Fortunato et al. 1997, Robertson et al. 2006). In addition, high concentrations of IL-10 have been associated with preterm labor in humans, especially in the cases of evident infection (Gotsch et al. 2008, Greig et al. 1995). In rats IL-10 has been shown to prevent preterm birth induced by LPS (Terrone et al. 2001). Regardless of these facts, studies with IL-10-deficient mice have revealed that this cytokine is not essential for successful pregnancy (White et al. 2004), but these mice are more susceptible to LPS-induced inflammation leading to preterm fetal loss or intrauterine growth restriction (Robertson et al. 2006).

Although the inflammatory events are conserved between many species, reproduction is species-specific and a particular animal model may not be suitable for every avenue of study (Elovitz & Mrinalini 2004, Hirsch & Wang 2005, Kemp et al. 2010, Mitchell & Taggart 2009). Therefore, the results from animal studies
are not always applicable to human parturition as such and must be interpreted with caution.

2.3 Lipopolysaccharide

Lipopolysaccharide (LPS), one of the best-characterized PAMPs, is the main building block of the outer membrane of Gram-negative bacteria. For the most part, LPS is incorporated into the bacterial cell wall surrounded by a thick capsule that restrains its toxicity. LPS can be released into the bloodstream during bacterial multiplication, dying, or lysis (Rietschel et al. 1994, Van Amersfoort et al. 2003) or due to some antibiotics (Crosby et al. 1994). Free LPS is clearly harmful to the host and can induce systemic inflammation and sepsis followed by multiorgan failure in high concentrations (Danner et al. 1991, Opal 2007). However, the potentially lethal consequences of LPS are due to the host inflammatory response to endotoxin, rather than the intrinsic properties of LPS itself.

The LPS molecule is composed of three different parts: lipid A, core polysaccharides and O-antigen repeats (Figure 1). Hydrophobic lipid A, responsible for the toxic effects of LPS (Galanos et al. 1985, Kotani et al. 1985), forms the outer monolayer of the bacterial outer membrane and is required for the growth of most Gram-negative bacteria. Core polysaccharides and O-antigen repeats of five common sugars are located on the bacterial cell surface and are not essential for bacterial growth. Instead, they help to build up resistance against antibiotics, the complement system, and other stressful situations caused by the environment. LPS molecules containing lipid A and a core of varying length form different rough LPS serotypes. LPS that contains highly immunogenic O-antigen is called smooth LPS (Raetz & Whitfield 2002, Van Amersfoort et al. 2003). The detailed structure of LPS varies from one bacterium to another and these variations may contribute to the different pathogenic properties of bacteria (Wilkinson 1996).
Recognition of LPS occurs through a series of interactions with LPS binding protein (LBP), CD14, MD-2, and TLR4, the members of the LPS-receptor complex. In addition to TLR4, other receptors for LPS have been identified as well. These include macrophage scavenger receptors, $\beta_2$-integrins, and selectins (for a review, see Van Amersfoort et al. 2003). The interaction between LPS and the LBP/CD14/MD-2/TLR4 receptor complex will be discussed in more detail in section 2.4.3.

### 2.4 TLR4

Toll-like receptor 4 was recognized as a receptor for LPS after the characterization of mice of the C3H/HeJ and C57BL10/ScCr strains that are defective in responding to LPS. C3H/HeJ mice carry a point mutation in the TLR4 gene, whereas C57BL10/ScCr mice exhibit a deletion of the gene (Poltorak et al. 1998, Qureshi et al. 1999). The role of TLR4 in LPS recognition was further confirmed using TLR4 deficient mice that are hyporesponsive to LPS and lipid A (Hoshino et al. 1999). In addition to LPS, TLR4 recognizes a wide variety of PAMPs derived from microbial and endogenous origins (reviewed in Takeda et al. 2003).

#### 2.4.1 Structure of TLR4

TLR4 is structurally similar with other TLR family receptors. TLRs are type I transmembrane glycoproteins that are composed of extracellular, transmembrane,
and intracellular signaling domains (Carpenter & O'Neill 2009, Jin & Lee 2008). The extracellular domain contains leucine-rich repeat (LRR) motifs that are responsible for PAMP recognition (Matsushima et al. 2007). The intracellular Toll/IL-1 receptor (TIR) domain that shares homology with interleukin-1 receptor family members is required for signal transduction (Carpenter & O'Neill 2009, Jin & Lee 2008).

2.4.2 Expression of TLR4

TLR4 is expressed mainly by immunomodulatory cells, such as monocytes, macrophages, and dendritic cells (Muzio et al. 2000). However, the presence of either TLR4 mRNA or protein has been reported in various other cells as well, including placental trophoblasts (Holmlund et al. 2002, Kumazaki et al. 2004), uterine endometrial cells (Herath et al. 2006), and cervical smooth muscle cells (Watari et al. 2000).

In general, the cell surface expression of TLR4 is rather low (Janssens & Beyaert 2003, Visintin et al. 2001) and may be the factor limiting the response to LPS. On the other hand, it has been suggested that in the absence of ligand TLR4 circulates between the plasma membrane and Golgi and that localization and trafficking of TLR4 would partly regulate the LPS responsiveness of the cell (Husebye et al. 2006, Latz et al. 2002). The regulation of TLR4 expression in response to various inflammatory stimuli is crucial for the proper function of the innate immune system. Despite numerous studies in this field, the exact mechanism is still incompletely understood and both upregulation and downregulation of TLR4 have been reported (reviewed in Janssens & Beyaert 2003). For example, intraperitoneally administered LPS increases the expression of TLR4 in mouse lung, whereas the levels in liver, placenta, and fetal lung remain unchanged (Harju et al. 2005, Ojaniemi et al. 2006, Rounioja et al. 2005). In contrast, LPS decreases TLR4 expression in murine macrophages (Matsuguchi et al. 2000, Medvedev et al. 2000, Nomura et al. 2000).
2.4.3 TLR4 signaling

LPS recognition

Although TLR4 is essential for LPS signaling, TLR4 alone is not sufficient to mediate LPS responsiveness to host cells (Kirschning et al. 1998, Shimazu et al. 1999), and the direct binding between TLR4 and LPS has not even been reported. Instead, the recognition of LPS in mammalian cells occurs through a series of interactions between LBP, CD14, TLR4, and MD-2 (Figure 2). The signaling cascade is initiated by the binding of LPS to CD14 on monocytes or monocytically-derived cells (Wright et al. 1990). LPS can bind directly to CD14 (Haziot et al. 1993), but in the presence of low concentrations of LPS the binding is facilitated by LBP found in serum (Gegner et al. 1995, Hailman et al. 1994, Tobias et al. 1986). CD14 that lacks the cytosolic domain has a role in the presentation of LPS to the TLR4/MD-2 complex and in the modulation of LPS recognition (Akashi et al. 2000). MD-2 binds directly to LPS and TLR4 allowing LPS recognition (Hyakushima et al. 2004, Shimazu et al. 1999, Viriyakosol et al. 2001).

Signal transduction

Ligand binding to the extracellular domain of TLR4 induces receptor homodimerization. Thereafter, the intracellular domain of the activated receptor complex recruits adaptor proteins including myeloid differentiation primary response gene 88 (MyD88), MyD88 adapter-like (MAL aka TIRAP; TIR domain-containing adaptor protein), TIR domain-containing adaptor including Interferon β (TRIF), and TRIF-related adaptor molecule (TRAM) (reviewed in O’Neill & Bowie 2007). Two distinct intracellular TLR4 signaling pathways can be activated in response to ligand recognition: MyD88-dependent and MyD88-independent pathways (Figure 2). The MyD88 pathway leads to a complex signaling cascade of several protein kinases resulting in the translocation of nuclear factor κB (NF-κB) to the nucleus and the induction of proinflammatory cytokine genes, whereas the MyD88-independent pathway is involved in the production of type I interferons. However, the activation of either pathway alone is not sufficient for the induction of the inflammatory response by TLR4 (for a recent review, see Kawai & Akira 2010).
The TLR4 pathway has been shown to be the most important for the elimination of LPS. However, full resistance to Gram-negative bacteria includes the crosstalk of different innate immunity receptors. For example, it has been demonstrated that LPS upregulates the expression of TLR2 in a process that is dependent on TLR4-signaling, since the upregulation of TLR2 expression is impaired in the tissues of TLR4-deficient mice (Fan et al. 2003, Ojaniemi et al. 2006). In addition, lipid A structures that are unable to signal through TLR4 can transmit the inflammatory response through TLR2 (Girard et al. 2003).

2.5 Surfactant proteins

Surfactant proteins A, B, C, and D are components of pulmonary surfactant, a surface-active lipoprotein complex synthesized and secreted by alveolar type II (ATII) cells. About 10% of the surfactant is proteins, and 90% is composed of lipids (Perez-Gil 2008, Serrano & Perez-Gil 2006). The main function of the surfactant is to reduce the surface tension of alveoli at the air-water interface. Reduced tension lowers the work of breathing and prevents alveolar collapse at...
the end of expiration (Serrano & Perez-Gil 2006). Lack of surfactant due to immaturity in prematurely born infants causes disturbance of alveolar gas exchange leading to respiratory distress syndrome (RDS), the major cause of neonatal mortality and morbidity (Farrell & Avery 1975). In addition, pulmonary surfactant has another important role in facilitating the clearance of pathogens and preventing the development of infections (Chroneos et al. 2010, Haagsman et al. 2008). The hydrophobic surfactant proteins B and C are mainly involved in the surface tension-lowering properties (Weaver & Conkright 2001), whereas the hydrophilic SP-A and SP-D are the primary modulators of the host defense functions (Chroneos et al. 2010, Haagsman et al. 2008). However, optimal surfactant function requires the presence of all SPs, and there is accumulating evidence indicating that SP-B and SP-C are involved in immunomodulation as well (Chroneos et al. 2010). SP-A, SP-C, and SP-D were the focus of this study and they will be discussed in more detail in the following sections.

2.5.1 Structure of SP-A and SP-D proteins

Surfactant proteins A and D are hydrophilic, collagenous proteins that are synthesized as primary translation products having sizes of approximately 26–36 kDa and 43 kDa, respectively. In the human genome there are two SP-A genes, SP-A1 and SP-A2 (Floros et al. 1986, Katyal et al. 1992), and one SP-A pseudogene (Korfhagen et al. 1991). Other species, with the exception of primates, are known to have only one SP-A gene (Gao et al. 1996). The two human SP-A proteins share about 96% identity.

SP-A and SP-D monomers share a common structural homology with other collectins. They contain a short amino-terminal region, followed by a collagen-like domain consisting of Gly-X-Y repeats, where X and Y can be any amino acid, but are usually proline and hydroxyproline. The collagen-like domain is thought to have several distinct functions, including involvement in receptor-mediated effects. The collagen-like domain is connected to a calcium-dependent carbohydrate recognition domain (CRD) via a short α-helical coiled-coil neck domain that is essential for the trimerization of the monomers. The broad specificity of collectins to recognize a large repertoire of glycoconjugates on a variety of cell surfaces resides mainly in their CRD (reviewed in Kishore et al. 2006, van de Wetering et al. 2004, and Wright 2005).

The basic structural unit of collectins is composed of three polypeptide chains that are held together via interactions of the collagenous tails and stabilized by
disulfide bonds in the cysteine-rich N-terminal region (Kishore et al. 2006, van de Wetering et al. 2004, Wright 2005). Trimeric subunits further combine to form multimers of varying degrees. SP-A exists mainly as octadecamers of six trimeric subunits, forming a bouquet-like structure (Spissinger et al. 1991), but smaller oligomeric forms have been detected as well (Hickling et al. 1998). It is generally thought that natural human SP-A in lung consists of hetero-oligomers of a ratio 2:1 of SP-A1 to SP-A2 (Voss et al. 1991). SP-D trimers predominantly form dodecamers of four trimeric subunits adapting a cruciform shape (Crouch et al. 1994b). Furthermore, SP-D dodecamers can contribute to the formation of higher order multimeric arrangements (Crouch et al. 1994a, Crouch et al. 1994b). The degree of oligomerization can significantly affect the function of collectins, demonstrated as differences in carbohydrate, LPS, and phospholipid binding specificity and in pathogen neutralization and opsonization efficiency (e.g. Kishore et al. 1996, Sanchez-Barbero et al. 2005, Sorensen et al. 2009, White et al. 2008, Yamada et al. 2006). The structure of SP-A and SP-D is illustrated in Figure 3.

Fig. 3. Structural organization of SP-A and SP-D (modified from Kingma & Whitsett 2006).
2.5.2 Receptors for SP-A and SP-D

SP-A and SP-D can modulate cellular functions by binding to a variety of cell-surface receptors present either on immune or ATII cells (see section 2.5.6). By binding to the LBP/CD14/MD-2/TLR4-receptor complex, SP-A and SP-D can modulate the LPS-elicited cellular responses. In addition, collectins can have an effect on inflammatory cytokine production via a mechanism that is not dependent on the LPS signaling pathway by binding to signal inhibitory regulatory protein α (SIRPα) or CD91/calreticulin. Additional receptors recognizing SP-A or SP-D, including surfactant protein receptor 210 kDa, CD93, glycoprotein 340, and P63 have been recognized as well (reviewed in Kishore et al. 2006).

2.5.3 Tissue distribution of SP-A and SP-D

SP-A and SP-D in lung

In lung the major sites of SP-A and SP-D synthesis are ATII cells and non-ciliated respiratory epithelial cells called Clara cells, but mRNA and protein of both SPs have been detected in bronchial epithelium and submucosal glands as well (Endo & Oka 1991, Khoor et al. 1993, Mori et al. 2002, Phelps & Floros 1988, Voorhout et al. 1992, Williams & Benson 1981, Wong et al. 1996). In human fetal lung low levels of SP-D mRNA and protein have been detected already at 16 weeks of gestation (Dulkerian et al. 1996), before the appearance of SP-A that is undetectable at 8 to 20 weeks of gestation (Ballard et al. 1986, Mori et al. 2002). However, Mori et al. (2002) demonstrated that production of SP-D does not begin until about 21 weeks of pregnancy. They excluded all cases associated with PPROM or infection, claiming that only the natural course of the SP-D expression was detected in their study. It is not clear from the previous report whether there were any fetal complications such as infection (Dulkerian et al. 1996). In mice the expression of SP-D becomes evident at day 17 of gestation (term 19–20 days). Depending on the study the expression of SP-A has been reported to be initiated either at 15 or 17 days post coitum (dpc) (Alcorn et al. 1999, Korfhagen et al. 1992, Wong et al. 1996). Gestational periods can vary slightly between mouse strains and may contribute to differences in these studies. The level of SP-A and SP-D synthesis in the lung increases prior to birth both in human and mouse

**SP-A and SP-D in nonpulmonary tissues**

In addition to the lung, both SP-A and SP-D have been detected in several nonpulmonary cells and tissues, although the synthesis of SP-D appears to be somewhat more widespread (Tables 1 and 2). Many of these nonpulmonary sites, including urinary and reproductive systems, skin, gastrointestinal tract, and salivary glands, are important first line barriers against invading pathogens. Therefore, the functions of collectins in these tissues may be similar to those described in the lung, although additional roles are possible as well. It should be noted that in many tissues the presence of protein but not mRNA has been reported (Table 2). This raises the question of whether SPs are actually produced in these tissues or if their presence is due to a paracrine process. For example, at least part of the SP-A protein in fetal membranes has been suggested to originate from amniotic fluid (Lee et al. 2010).
Table 1. SP-A and SP-D in nonpulmonary tissues.

<table>
<thead>
<tr>
<th>Source</th>
<th>SP-A</th>
<th>SP-D</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal gland</td>
<td>X</td>
<td>Madsen et al. 2000, Stahlman et al. 2002</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>X</td>
<td>Madsen et al. 2000</td>
<td></td>
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<tr>
<td>Eustachian tube</td>
<td>X</td>
<td>X</td>
<td>Kankavi 2003, Paananen et al. 1999, Paananen et al. 2001</td>
</tr>
<tr>
<td>E.g. cornea, lacrimal gland</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>E.g. esophagus, colon, intestine, stomach</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gall bladder</td>
<td>X</td>
<td>Herias et al. 2007, Madsen et al. 2000</td>
<td></td>
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<tr>
<td>Lymph node</td>
<td>X</td>
<td>Herias et al. 2007</td>
<td></td>
</tr>
<tr>
<td>E.g. epididymis, prostate, testis</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammary gland</td>
<td>X</td>
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<tr>
<td>Nasal cavity</td>
<td>X</td>
<td>X</td>
<td>Kim et al. 2007</td>
</tr>
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<td>X</td>
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<tr>
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<tr>
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<td>X</td>
<td>Herias et al. 2007, Madsen et al. 2000, Mo et al. 2007</td>
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<td>Madsen et al. 2000, Stahlman et al. 2002</td>
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<td>X</td>
<td>Herias et al. 2007, Madsen et al. 2003, Stahlman et al. 2002</td>
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<td>X</td>
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<td>Akiyama et al. 2002, Stahlman et al. 2002</td>
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1 The presence of SP-A1 and SP-A2 may differ between the human tissues.
<table>
<thead>
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<th>Source</th>
<th>SP-A</th>
<th>SP-D</th>
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<td></td>
<td>mRNA</td>
<td>Protein</td>
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<tr>
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<td>Fetal membranes</td>
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1 The presence of SP-A1 and SP-A2 may differ between the human tissues.
2.5.4 SP-A and SP-D in amniotic fluid

Amniotic fluid is in constant circulation between fetal airways and the amniotic cavity due to spontaneous breathing movements by the fetus (Hallman et al. 1997). This allows the secretion of different compounds, including SPs from fetal lungs to the amniotic fluid. Both SP-A and SP-D can be detected in human amniotic fluid in the second trimester of pregnancy, although the reported time points vary notably between the studies, ranging from 14 to 26 weeks of gestation (Chaiworapongsa et al. 2008, Han et al. 2007, Leth-Larsen et al. 2004, Miyamura et al. 1994, Pryhuber et al. 1991). Because the expression of SP-A in fetal lung is not evident until 20 weeks of gestation, it can be speculated whether the SP-A detected in amniotic fluid early in the second trimester could originate from nonpulmonary tissues such as fetal membranes (Han et al. 2007, Sun et al. 2006).

In mouse amniotic fluid SP-A is first detected at 15 dpc as nonreducible trimers and supratrimERIC oligomers, whereas high molecular mass SP-A oligomers are evident from 17 dpc onwards (Lee et al. 2010). However, in a study utilizing a different mouse line, SP-A was not detected until 17 dpc (Condon et al. 2004).


2.5.5 Roles of SP-A and SP-D in surfactant homeostasis

In lung lavage most SP-A is associated with surfactant lipids. Additionally, SP-A binds to ATII cells (Kuroki et al. 1988, Kuroki & Akino 1991, Wright et al. 1989). According to initial in vitro studies, SP-A has been proposed to modulate the uptake and secretion of phospholipids by ATII cells, enhance the uptake of phospholipids by alveolar macrophages, regulate surface film formation, and contribute to the formation and maintenance of tubular myelin, a highly ordered array of phospholipids and proteins found in alveoli (reviewed in Haagsman & Diemel 2001 and Hawgood & Poullain 2001).

In contrast to SP-A, SP-D does not predominantly associate with the major surfactant phospholipids in lung lavages (Crouch et al. 1991, Persson et al. 1989) suggesting that SP-D is actually not a real surfactant protein and therefore would
not have a role in surfactant homeostasis. However, in some conditions SP-D has been shown to associate with phosphatidylinositol and glucosylceramide, the lipids present in surfactant (e.g. Kuroki et al. 1991, Kuroki et al. 1992, Persson et al. 1992, Sano et al. 1998).

The relevance of these in vitro observations has been questioned by the characterization of mice deficient in either SP-A or SP-D as discussed in section 2.5.8.

2.5.6 Immunomodulatory functions of SP-A and SP-D

Interaction of SP-A and SP-D with microorganisms

SP-A and SP-D are pattern receptor molecules that can interact with PAMPs expressed on the surface of various invading microorganisms, including *Chlamydia*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, respiratory syncytial virus (RSV), and Influenza A virus (for recent reviews see Haagsman et al. 2008, Kishore et al. 2006, Kuroki et al. 2007, and Wright 2005). After pathogen recognition, SP-A and SP-D can participate in the innate host defense via several mechanisms (see section 2.1.1) that in many cases are collectin specific (Haagsman et al. 2008, van de Wetering et al. 2004, Wright 2005).

Interaction of SP-A and SP-D with LPS

Both SP-A and SP-D can modulate the LPS-elicited inflammatory response by interacting with LPS. SP-A binds directly to the lipid A moiety of rough LPS (Garcia-Verdugo et al. 2005, Sano et al. 1999, Song & Phelps 2000, Van Iwaarden et al. 1994), whereas SP-D poorly recognizes lipid A and binds rather to the core domain (Kuan et al. 1992, Wang et al. 2008a, Wang et al. 2008b). The interactions between the collectins and LPS occur both in the presence and in the absence of calcium, indicating that binding can occur via CRD but also independently from the lectin property. Participation of the neck-region of SP-D has been proposed (Garcia-Verdugo et al. 2005, Kuan et al. 1992, Sano et al. 1999, Sano et al. 2000, Van Iwaarden et al. 1994, Wang et al. 2008b, Yamazoe et al. 2008). It has been demonstrated in many studies that both SP-A and SP-D lack the ability to bind to smooth serotypes of LPS and to cause their aggregation (Kuan et al. 1992, Sano et al. 1999, Van Iwaarden et al. 1994, Yamazoe et al. 2008).
This could be explained by the O-antigen region and complete core polysaccharides that may create a steric constraint and mask the SP-A and SP-D binding sites in smooth LPS molecule. However, binding of SP-D to specific serotypes of smooth LPS of *Klebsiella* has been demonstrated as well (Sahly *et al.* 2002).

**Interaction of SP-A and SP-D with components of the LPS receptor complex**

In addition to interactions with LPS, both SP-A and SP-D can modulate the LPS-induced cellular responses by binding directly to CD14 (Sano *et al.* 1999, Sano *et al.* 2000), MD-2 (Nie *et al.* 2008, Yamada *et al.* 2006, Yamazoe *et al.* 2008), and TLR4 (Ohya *et al.* 2006, Yamada *et al.* 2006, Yamazoe *et al.* 2008), the components of the LPS receptor complex. The mechanisms of receptor recognition may vary between SP-A and SP-D. According to Sano *et al.* (1999) binding of SP-A to CD14 inhibits the binding of CD14 to smooth LPS but increases the binding to rough forms. Similarly, SP-A prevents the interaction of the TLR4/MD-2 complex with smooth but not with rough LPS (Yamada *et al.* 2006). The hypothesis is that smooth LPS, which is not a ligand for SP-A, fails to interact with CD14 or TLR4/MD-2 that are already bound to SP-A, whereas rough LPS may effectively associate with the components of the receptor complex through SP-A. However, in another study SP-A decreased the binding of CD14 to rough LPS as well and reduced the ability of LBP to transfer rough LPS to CD14, indicating that SP-A may alter the function of the LBP/CD14 receptor complex (Garcia-Verdugo *et al.* 2005). SP-D attenuates the binding of both serotypes of LPS to CD14 and to the TLR4/MD-2 complex, demonstrating that SP-D also influences the inflammatory response by altering the LPS receptor function (Sano *et al.* 2000, Yamazoe *et al.* 2008).

**Interaction of SP-A and SP-D with CD91/calreticulin or with signal inhibitory regulatory protein α**

SP-A and SP-D can also have an effect on inflammatory cytokine production via mechanism that is not dependent on the LPS signaling pathway by binding to SIRPa or CD91/calreticulin (Gardai *et al.* 2003). Calreticulin is a multifunctional protein that lacks the transmembrane domain but can interact with CD91, which may act as a signaling partner (Basu *et al.* 2001, Coppolino & Dedhar 1998).
binding to calreticulin via collagen-like domains, collectins can participate in the clearance of apoptotic cells (Vandivier et al. 2002). SIRPα is a transmembrane glycoprotein that is involved in transmitting signals for phagocytosis. By binding to SIRPα, SP-A and SP-D can suppress phagocytosis by alveolar macrophages (Janssen et al. 2008). The ability of SP-A and SP-D to function as both anti- and proinflammatory factors utilizing calreticulin/CD91 and SIRPα receptors was proposed by Gardai et al. (2003). According to their model the CRD domain of SP-A or SP-D binds to SIRPα in the absence of pathogens blocking the production of proinflammatory mediators. Binding of the microbial ligand by CRD reverses the collectin function, as the collagenous tails bind to calreticulin/CD91 stimulating the production of proinflammatory cytokines via activation of NF-κB (Figure 4).

The quaternary structure of SP-D differs from that of SP-A as demonstrated in section 2.5.1. The tail domains of SP-D are buried and head domains exposed, which may prevent the action of SP-D as a proinflammatory mediator via the collagenous tails as presented above. However, S-nitrosylation of SP-D results in disruption of the multimers revealing the trimeric subunits that are then available for interaction with calreticulin/CD91. S-nitrosylated SP-D can act as a proinflammatory mediator enhancing macrophage migration and chemokine production (Guo et al. 2008).

![Fig. 4. Dual effects of SP-A and SP-D (modified from Gardai et al. 2003).](image_url)
Effect of SP-A and SP-D on inflammatory responses

The modulatory role of SP-A and SP-D on innate immunity responses has been extensively studied in vitro, especially for SP-A. The results have been conflicting since both anti- and proinflammatory effects have been reported.

SP-A has been shown to directly increase the production and release of TNF-α, IL-1, IL-6, and IL-8 by various cells including alveolar macrophages and the monocytic cell line THP-1 (e.g. Huang et al. 2004, Kremlev et al. 1997, Rubovitch et al. 2007). However, in many studies SP-A has no effect on the baseline production of TNF-α by these cells (Salez et al. 2001, Sano et al. 1999, Stamme et al. 2002).

Although smooth LPS is not a ligand for SP-A, SP-A can modulate cellular responses elicited by it. For example, SP-A decreases smooth LPS-induced expression and secretion of TNF-α, IL-10, and macrophage inhibitory protein-2 (MIP-2, analog of IL-8 in mice) by macrophages, mononuclear phagocytes, or macrophage-like U937 cells (Alcorn & Wright 2004, Chabot et al. 2003, Garcia-Verdugo et al. 2005, Gardai et al. 2003, McIntosh et al. 1996, Salez et al. 2001, Sano et al. 1999), but fails to attenuate or even increases the proinflammatory responses induced by rough LPS (Sano et al. 1999, Song & Phelps 2000). These results correlate with the finding that SP-A reduces the binding of smooth but not rough LPS to the TLR4/MD-2/CD14 receptor complex. However, there is increasing evidence that the effects of SP-A on rough LPS-stimulated production of proinflammatory cytokines can be inhibitory as well. This may be due to the ability of SP-A to reduce the LBP-mediated transfer of rough LPS to CD14 (Garcia-Verdugo et al. 2005, Stamme et al. 2002) or the inhibition may be independent from the LPS signaling pathway (Alcorn & Wright 2004). SP-D has been shown to suppress inflammatory responses elicited by both LPS serotypes (Gardai et al. 2003, Yamazoe et al. 2008).

2.5.7 SP-A and SP-D as mediators of intrauterine inflammation and parturition

It has been demonstrated in several studies that SP-A and SP-D are present in gestational tissues and in amniotic fluid (see section 2.5.4 and Table 2). Considering the well characterized role of SP-A and SP-D in innate immunity, it seems conceivable that these proteins have a role in controlling the intrauterine inflammatory responses related to parturition as well.
The first evidence about the direct role for SP-A as the regulator of parturition came from Condon et al. (2004), who demonstrated that injection of SP-A into mouse amniotic fluid initiates labor. They showed that SP-A elevates the expression of IL-1β and NF-κB by amniotic fluid macrophages and increases the migration of macrophages of fetal origin to myometrium. Thereby they hypothesized that SP-A secreted from fetal lungs in increasing amounts with advancing gestation acts as a hormone that initiates the proinflammatory signaling cascade in the myometrium leading to labor. However, in a study by Lee et al. (2010), amniotic fluid SP-A was demonstrated to bind to human amnion in vitro and to downregulate the expression of several genes, including IL-1β, suggesting that SP-A may mediate the anti-inflammatory responses during pregnancy. In addition to fetal membranes, uterine cells contain binding sites for SP-A as well. In myometrium SP-A has been proposed to contribute to the initiation, regulation, and maintenance of uterine contractions associated with labor (Breuiller-Fouche et al. 2010, Garcia-Verdugo et al. 2007, Garcia-Verdugo et al. 2008). These studies further support the role of SP-A as a labor inducing factor, although the mechanism differs from that initially described in mice.

Interestingly the levels of SP-D in the uterus seem to be hormonally controlled. In humans the presence of SP-D in endometrium increases towards the secretory phase of the menstrual cycle (Leth-Larsen et al. 2004), whereas in mice the SP-D mRNA levels are highest at estrus and lowest at diestrus of the estrus cycle (Oberley et al. 2007a). Treatment of mice with progesterone arrests the animals in diestrus and makes them more susceptible to infection, which correlates with the low levels of SP-D (Oberley et al. 2007a). In humans, several studies have addressed the potential role of sex hormones in the susceptibility to reproductive tract infections caused by various pathogens (Sonnex 1998).

2.5.8 Mouse models of SP-A or SP-D deficiency and excess

Additional information about the role of SP-A and SP-D in innate immunity of the lung and in surfactant homeostasis has been obtained from studies utilizing SP-A or SP-D deficient and overexpressing mice.

SP-A deficient mice

In pathogen-free conditions, the deletion of SP-A gene expression does not affect the survival of mice deficient in SP-A (SP-A−/−). The most notable change in the
The surfactant structure of SP-A−/− mice is the complete lack of tubular myelin. However, the lung structure, volume, and compliance are normal. In addition there are no differences in the levels of other SPs, phospholipid composition, secretion and clearance, or in the distribution and morphology of ATII cells (Ikegami et al. 1997, Korfhagen et al. 1996). Under physiological conditions the surfactant from SP-A−/− mice adsorbs rapidly forming stable films that have nearly identical properties compared with wild-type surfactant (Ikegami et al. 1998, Korfhagen et al. 1996). Overexpression of rSP-A in a SP-A−/− background corrects the structural and functional defects of the surfactant (Ikegami et al. 2001). However, both human SP-A1 and SP-A2 gene products are necessary for the formation of tubular myelin as demonstrated with humanized SP-A1 and SP-A2 transgenic mice (Wang et al. 2010).

In the presence of pathogens or inflammatory stimuli, SP-A−/− mice exhibit defects in innate host defense, detected as decreased killing and phagocytosis of various pathogens, enhanced inflammatory response, and the production of increased levels of proinflammatory cytokines in their bronchoalveolar lavage fluid (BALF) compared with wild-type mice (for reviews, see Kishore et al. 2006 and Wright 2005). For example, SP-A−/− mice are more susceptible to inflammation induced by *Haemophilus influenzae* (LeVine et al. 2000), influenza A virus (LeVine et al. 2002), LPS (Borron et al. 2000), RSV (LeVine et al. 1999), and *Ureaplasma* (Famuyide et al. 2009). However, the specific nature and magnitude of the response varies considerably depending on the experimental settings.

**SP-A overexpressing mice**

In SP-A overexpressing mice, rSP-A is expressed under the control of the human SP-C (hSP-C) promoter (Elhalwagi et al. 1999). The activity of the hSP-C promoter increases with advancing gestation and it has been proposed to drive the expression exclusively to the lung (Glasser et al. 1990, Wert et al. 1993). The level of SP-A in alveoli of overexpressing mice is increased 7- to 8-fold compared with control levels, but the increase has no effect on the lung structure, surfactant function, surfactant pool sizes, or the distribution of saturated phosphatidylcholine between the air space and lung tissue. However, elevated levels of SP-A enhance the resistance of surfactant to protein inhibitors, indicating that SP-A may have a role in maintaining the integrity of pulmonary surfactant in the case of lung injury. The structure of alveolar macrophages in SP-A
overexpressing mice is abnormal (Elhalwagi et al. 1999), but the effect of SP-A overexpression on innate immunity functions has not been evaluated.

**SP-D deficient mice**

In contrast to SP-A⁻ mice, mice deficient in SP-D (SP-D⁻) have a much more complex phenotype. Although SP-D⁻ mice grow and survive normally when they are housed in a barrier facility, they develop several perturbations in surfactant homeostasis that were not expected based on *in vitro* studies (see section 2.5.5). To some extent these changes seem to be dependent on the genetic background of the mice (Atochina et al. 2004, Botas et al. 1998, Korfhagen et al. 1998).

Surfactant phospholipids accumulate progressively in the lung tissue and alveolar space of SP-D⁻ mice, but the lipid composition is not different from wild-type mice (Botas et al. 1998, Korfhagen et al. 1998). The total protein content and SP-A and SP-B levels in the BALF of SP-D⁻ mice are either increased or decreased, depending on the genetic background (Atochina et al. 2004, Botas et al. 1998, Korfhagen et al. 1998). The deficiency of SP-D additionally alters the ATII cell morphology and increases the size and number of alveolar macrophages (Atochina et al. 2004, Botas et al. 1998, Korfhagen et al. 1998, Wert et al. 2000), which produce higher levels of hydrogen peroxide contributing to increased MMP activity and emphysematous phenotype (Wert et al. 2000). Despite the pulmonary changes evidenced in SP-D⁻ mice, there are no significant differences between SP-D deficient and wild-type surfactant *in vitro* (Botas et al. 1998). The pulmonary abnormalities of SP-D⁻ mice can be corrected with the overexpression of rSP-D (Fisher et al. 2000), but not with overexpression of rSP-A (Zhang et al. 2006), suggesting a prominent role for SP-D in surfactant metabolism.

The altered response to pathogens is evident in SP-D⁻ mice as well, but in some cases the response is pathogen-specific. For example, the clearance of *H. influenzae* and Group B Streptococci is not decreased in SP-D⁻ mice, although deficient uptake of bacteria by alveolar macrophages and increased inflammation and inflammatory cell recruitment are evident. Higher levels of superoxide and hydrogen peroxide produced by alveolar macrophages from SP-D⁻ mice likely contribute to the effective bacterial killing (LeVine et al. 2000). In contrast, SP-D⁻ mice have an increased inflammatory response after Influenza A, LPS, and RSV challenge compared with wild-type mice (LeVine et al. 2001, LeVine et al. 2004)
In addition to lung, delayed clearance of pathogens has been characterized in SP-D deficient ocular surface (Mun et al. 2009).

**SP-D overexpressing mice**

In rSP-D overexpressing mice rat protein is expressed under the hSP-C promoter as well. SP-D content in BALF of these overexpressing mice is increased 30- to 50-fold compared with wild-type littermates, but the elevated levels have no effect on lung morphology, phospholipid content, surfactant metabolism, or the expression of SPs. In addition, the number and the structure of alveolar macrophages are normal (Fisher et al. 2000). Surprisingly rSP-D overexpressing mice immunosuppressed with depletion of CD4 lymphocytes are more susceptible to *Pneumocystis* infection (Vuk-Pavlovic et al. 2006).

**2.5.9 Clinical implications of SP-A and SP-D**

Although immunomodulatory and pulmonary roles of SP-A and SP-D have been established, not many human diseases resulting from the altered function or decreased levels of either SP-A or SP-D have been identified. However, a variety of clinical conditions, including cystic fibrosis (Griese et al. 2004), RDS (Dahl et al. 2006) and acute RDS (Greene et al. 1999), are associated with altered levels of SP-A or SP-D in the blood or BALF of these patients. In premature infants the concentration of SP-D in umbilical cord blood and capillary blood has been shown to be twice as high as in mature infants. Among these preterm infants the concentration of SP-D depends on several perinatal conditions, such as intrauterine growth retardation and PPROM (Dahl et al. 2006).

Recently, two rare mutations in the SP-A2 gene have been detected in individuals with familial pulmonary fibrosis and lung cancer. These mutations probably disrupt the protein structure resulting in perturbations in protein trafficking through the endoplasmic reticulum and secretion (Wang et al. 2009). Common polymorphisms in the SP-A and SP-D genes have been associated with a number of different pulmonary diseases (for a review, see Pastva et al. 2007), including RDS (Haataja et al. 2001, Hilgendorff et al. 2009, Rämet et al. 2000), RSV (Lahti et al. 2002, Löfgren et al. 2002), and tuberculosis (Floros et al. 2000, Madan et al. 2002). In addition, a common variation in the SP-A gene has been associated with otitis media (Pettigrew et al. 2006, Rämet et al. 2001). The Met11Thr polymorphism in SP-D contributes to the assembly of SP-D oligomers,
resulting in differences in the microbial binding properties (Leth-Larsen et al. 2005). Moreover, the same single nucleotide polymorphism (SNP) influences the concentration of SP-D in serum (Heidinger et al. 2005, Leth-Larsen et al. 2005) confirming that genetic factors may modulate the susceptibility to different immune-related diseases.

2.5.10 Structure of SP-C protein and synthesis in lung

Biophysically active human SP-C is one of the most hydrophobic proteins known. This 3.7 kDa protein is encoded by exon 2 of the SP-C gene and has a membrane-spanning domain and cytosolic N-terminal domain that are highly conserved among species (Weaver & Conkright 2001). Synthesis of SP-C has been well characterized in lung, where it is produced in ATII cells as a 21 kDa integral transmembrane precursor protein of 191 or 197 amino acids, depending on alternative splicing at the beginning of exon 5 (Glasser et al. 1988, Warr et al. 1987). Mature SP-C is generated via four proteolytic cleavages as SP-C proprotein (proSP-C) is trafficked through the regulated secretory pathway as illustrated in Figure 5. For the proper processing of proSP-C, the simultaneous intracellular production of SP-B is needed. SP-B deficiency leads to additional deficiency of mature SP-C and accumulation of aberrantly processed SP-C containing the N-terminal propeptide in the lung tissue and alveolar spaces (Clark et al. 1995, Nogee et al. 2000, Vorbroker et al. 1995).
Fig. 5. A) Exon-intron structure of the human SP-C gene. B) Processing of proSP-C in lung. ER = endoplasmic reticulum, MVB = multivesicular body, LB = lamellar body (modified from ten Brinke et al. 2002).

### 2.5.11 Functions of SP-C

**Pulmonary functions**

SP-C has important roles in the lung, where it reduces the surface tension by promoting the formation, stabilization, and maintenance of a biologically active surface film and enhancing the surfactant film function (e.g. Oosterlaken-Dijksterhuis et al. 1991, Qanbar et al. 1996, Yu & Possmayer 1990, for reviews, see Mulugeta & Beers 2006 and ten Brinke et al. 2002). In addition, SP-C increases the reuptake of phospholipids into isolated ATII cells (Horowitz et al. 1996), suggesting a role for SP-C in surfactant catabolism. Many of the functions
of SP-C overlap with the functions of SP-B (Serrano & Perez-Gil 2006, Weaver & Conkright 2001). Based on these functional roles, the exogenous surfactant preparations containing SP-C and SP-B can be effectively used for the treatment of RDS in preterm infants to improve lung function (Seger & Soll 2009).

Mutations in the SP-C gene that include missense, frameshift, or splice mutations and result in the production of an abnormal proSP-C have been linked to many respiratory disorders including pulmonary fibrosis, interstitial lung disease, and RDS (e.g. Nogee et al. 2001, Poterjoy et al. 2010, Thomas et al. 2002, for a review, see Beers & Mulugeta 2005). The presence of a mutation usually only in one allele suggests a dominant-negative effect on phenotype. Many of the mutations found in the SP-C gene are located in the BRICHOS-domain of the C-terminus of proSP-C (Beers & Mulugeta 2005, Guillot et al. 2009). BRICHOS is a highly conserved region of approximately 100 residues that can be found in a number of proteins associated with degenerative and proliferative diseases in several organs. BRICHOS has been proposed to act as a chaperone, preventing the misfolding and aggregation of the proprotein (Sanchez-Pulido et al. 2002). The observations that mutations in the BRICHOS domain of proSP-C cause misfolding and accumulation of the protein in endoplasmic reticulum are consistent with this hypothesis (Mulugeta et al. 2005, Mulugeta et al. 2007). In addition, a common genetic variation in the SP-C gene has been associated with RDS, severe RSV infection, and asthma (Lahti et al. 2004, Puthothu et al. 2006).

The role of SP-C in pulmonary function has been further demonstrated with different SP-C deficient (SP-C−/−) mouse models that will be discussed in detail in section 2.5.13.

Role in LPS recognition

The role of SP-C in innate host defense was first raised by Augusto et al. (2001, 2002 and 2003), who demonstrated that SP-C binds to the lipid A moiety of LPS and to CD14. Binding to LPS has been proposed to occur through the hydrophilic site of SP-C (Augusto et al. 2002). The interaction between SP-C and LPS was further verified by Li et al. (2004a), who reported that mature SP-C, but not an incompletely processed 6 kDa form, binds to LPS. The N-terminal region of SP-C presumably interacts with CD14 as well, competing with the binding to LPS. However, in the absence of serum and thus in the absence of LBP, the binding of
CD14 to LPS is enhanced by SP-C, indicating that SP-C may act as LPS-presenting molecule (Augusto et al. 2003).

2.5.12 Tissue distribution of SP-C

Expression of SP-C in lung

The expression of SP-C in lung is initiated at an earlier stage of fetal development compared with hydrophilic SPs (see section 2.5.3) and it is restricted to ATII cells (Glasser et al. 1991, Horowitz et al. 1991, Phelps & Floros 1991). In human fetal lung SP-C mRNA and proprotein are detectable from 13–15 weeks of gestation onward (Khoor et al. 1994, Liley et al. 1989). In mice SP-C expression is first evident at 11 dpc (Wert et al. 1993). The levels of SP-C increase towards the term pregnancy (Liley et al. 1989, Wert et al. 1993).

The presence of SP-C in nonpulmonary tissues

There is increasing evidence that SP-C is not completely a lung-specific protein, but it is additionally present in many nonpulmonary tissues. The expression of SP-C has been detected in eye (Brauer et al. 2007a), salivary glands (Brauer et al. 2009), skin (Mo et al. 2007), and kidney (Eikmans et al. 2005) and SP-C proprotein in eye and tear fluid (Brauer et al. 2007a), salivary glands and saliva (Brauer et al. 2009), skin (Mo et al. 2007), and placenta (Sati et al. 2010). Interestingly Glasser et al. reported already in 2003 that all SP-C−/− mice develop conjunctivitis beyond 6 months of age and there is deterioration of coat condition in most SP-C−/− mice after 2 months of age, but the influence of SP-C in these conditions was not discussed. The oral cavity, skin, and eye are constantly exposed to a multitude of exogenous factors like microbes, and the regulation of the uterine inflammatory response is of great importance. Considering the proposed role of SP-C in the innate immune defense, the presence of SP-C in these tissues does not seem surprising. Additional roles in the eye and mouth may be related to the formation of a stable and functional tear film and enhancement of the transport of tear fluid and saliva through the nasolacrimal ducts and excretory duct system, respectively (Brauer et al. 2007a, Brauer et al. 2009). However, the exact functions of SP-C in nonpulmonary tissues remain to be solved.
2.5.13 Mouse models of SP-C deficiency and excess

SP-C deficient mice

Although absence of SP-C at birth is not lethal, the deletion of SP-C leads to pulmonary disorders of varying degrees based on the genetic background, age, and environment of SP-C<sup>−/−</sup> mice (Glasser et al. 2001, Glasser et al. 2003). The initial study by Glasser et al. (2001) demonstrated that the deletion of SP-C has no adverse effect on pulmonary function or lung morphogenesis. However, subtle abnormalities in lung mechanics and the instability of the surfactant at low lung volumes were observed, supporting the importance of SP-C at the end of expiration. The lung structure of SP-C<sup>−/−</sup> mice in another background is normal at birth, but a progressive pulmonary disease characterized by thickening of the alveolar walls, monocytic cell infiltration, emphysema, epithelial cell dysplasia and accumulation of intracellular lipids can be detected as early as 2 months after birth. These pathological findings resemble those seen in idiopathic interstitial pneumonitis (Glasser et al. 2003). Although it was reported in the initial study that SP-C<sup>−/−</sup> mice are healthy and survive and grow normally (Glasser et al. 2001), it was later informed that they develop pneumonitis after 1 year of age (Glasser et al. 2003). The more severe pulmonary inflammation in SP-C<sup>−/−</sup> mice compared with wild-type mice after microbial challenge supports the role for SP-C in regulating the innate immunity responses (Glasser et al. 2008, Glasser et al. 2009).

SP-C overexpressing mice

Two different mouse lines overexpressing either mouse mature SP-C peptide or a hSP-C mutation associated with interstitial lung disease have been developed. Elevated levels of SP-C in the ATII cells of these mice result in accumulation of the protein in the secretory pathway. The toxic effects of aggregation are detected as neonatal lethality and delayed or disrupted lung organogenesis, evidenced with little branching morphogenesis or sacculcation, large cystic structures, and decreased levels of SP-A, SP-B and endogenous SP-C. The severity of lung immaturity correlates with the increased SP-C levels (Bridges et al. 2003, Conkright et al. 2002).
3 Outlines of the present study

Despite years of research and improvements in modern therapies, prematurity remains the biggest unsolved problem in perinatal and neonatal medicine in Western countries. The pathogenesis of prematurity is poorly understood but intrauterine bacterial infections and consequent inflammatory processes have been implicated as one mechanism responsible for preterm delivery. However, the mechanism needs to be further studied. The specific aims of the present study were:

1. To create a mouse model of LPS-induced preterm birth of live-born pups to study the activation of the maternal and fetal innate immune systems related to preterm birth.
2. To study the influence of the overexpression of either rSP-A or rSP-D under the hSP-C promoter on the inflammatory response related to preterm birth in mice.
3. To investigate the possible involvement of SP-C in the process of preterm parturition and to identify the gestational tissues that express SP-C.
4 Materials and methods

4.1 Experimental mouse model of prematurity

4.1.1 Animals (I-III)

All studies were performed under protocols approved by the Animal Research Committee of the University of Oulu or the Finnish Animal Ethics Committee. C57BL/6 and FVB wild-type mice and mice overexpressing rSP-A (Elhalwagi et al. 1999) or rSP-D (Fisher et al. 2000) under the control of the hSP-C promoter were used in these studies. Prior to experiments rSP-A and rSP-D mice were backcrossed onto the C57BL/6 background over 6 generations. Mice were maintained in a conventional mouse facility with a light/dark cycle of 12 hours and free access to food and water. Female mice aged 3–5 months were mated with males of the same strain or a transgenic line. Gestational age was determined by the appearance of a vaginal plug, designated as day 0 of pregnancy (± 12 hours). The dams were randomized to receive either LPS or phosphate-buffered saline (PBS). After the injections mice were housed in a temperature-controlled room (+22 °C).

4.1.2 Establishment of the mouse model of preterm birth (I, II)

In preliminary experiments the LPS dose needed to induce preterm birth of live-born offspring in C57BL/6 strain was established. Purified E. coli LPS (serotype 0111:B4, Sigma-Aldrich, St Louis, MO, USA) dissolved in PBS at a final concentration of 0.25 mg/ml was used. Dams were intraperitoneally injected at 16 or 17 dpc with different doses of LPS. Doses of 10–50 μg/mouse (~ 0.4–2 mg/kg) used in these preliminary experiments were chosen based on previous publications (Buhimschi et al. 2003, Fidel et al. 1994, Kaga et al. 1996, Lee et al. 2003, Loftin et al. 2002, Mijovic et al. 2002, Mitsuhashi et al. 2000, Schwartz et al. 2003). The dams were observed every 4 hours during first the 12 hours after injection and then continuously until the time of delivery. After determination of the approximate time interval from LPS injection to delivery, the observation could be focused closer to the time of labor. LPS dose of 25 μg/mouse (~ 1 mg/kg) was used in subsequent experiments for both C57BL/6 and FVB mice. To establish the LPS dose leading to preterm birth of live-born pups in rSP-A and
rSP-D overexpressing lines, the dams received decreasing doses of LPS, starting from the dose used for wild-type mice (25 µg/mouse). 12.5 µg/mouse (~ 0.5 mg/kg) of LPS was chosen for both overexpressing lines.

To study the timing of delivery and pregnancy outcomes in C57BL/6, FVB, rSP-A and rSP-D mice, the dams were injected with LPS or with an equal volume of PBS as illustrated in Table 3. A few dams experiencing evident pain and/or prolonged labor were killed and excluded from the study. The viability of the prematurely born pups was observed immediately after birth and for up to 6 hours thereafter. However, the pups were euthanized in the case of clear weakening in the condition in order to prevent unnecessary suffering. The litters were called live-born when at least 50% of the pups were born alive.

Table 3. C57BL/6, FVB, rSP-A, and rSP-D mice injected with LPS or PBS to study the timing of delivery and pregnancy outcomes.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>LPS 25 µg</th>
<th>LPS 12.5 µg</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C57BL/6</td>
<td>FVB</td>
<td>rSP-A</td>
</tr>
<tr>
<td>N</td>
<td>45</td>
<td>44</td>
<td>12</td>
</tr>
<tr>
<td>16 dpc</td>
<td>13</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>17 dpc</td>
<td>32</td>
<td>26</td>
<td>12</td>
</tr>
</tbody>
</table>

¹C57BL/6 and FVB control mice were injected either at 16 dpc or at 17 dpc but handled as one group.

4.1.3 Sample collection (I-III)

To study the inflammatory response related to preterm birth in maternal and fetal compartments, another set of C57BL/6 and overexpressing mice were injected with LPS doses defined above (see section 4.1.2). In addition, C57BL/6 wild-type mice used as controls for rSP-A and rSP-D mice received 12.5 µg of LPS per mouse. FVB mice were not included in this part of the study. The dams were anesthetized 3 or 8 hours after the LPS or PBS injections with 0.08 ml/10 g of subcutaneous solution of ketamine (7 mg/ml) and medetomidine (40 µg/ml) in physiological NaCl. Maternal blood was obtained by orbital sinus puncture and the dams were killed with cervical dislocation thereafter. Maternal and fetal lungs, uterus (myometrium and endometrium), amniotic fluid, placenta, fetal membranes (amnion and yolk sack), blood from decapitated fetuses, and other fetal tissues (liver, heart, intestine and brain) were harvested. Tissues were frozen in liquid nitrogen and stored at -70 °C or fixed in 4% formaldehyde in PBS. Blood samples were allowed to clot overnight at +4 °C and sera were separated thereafter.
4.1.4 Determination of the stage of the estrous cycle

The stage of the estrous cycle of 16 female C57BL/6 mice, aged 8–9 weeks was defined. Mice were housed in the same cage and four mice were taken for analysis on consecutive days. Vaginal swabs and blood samples from orbital sinus were collected under ketamine-medetomidine anesthesia. The state of the estrous cycle was evaluated by the cytology of vaginal swabs and serum progesterone levels (Stelck et al. 2005). Mice were killed with cervical dislocation and uteri were harvested. Tissues were frozen in liquid nitrogen for the analysis of SP-C expression during the estrous cycle.

4.2 Collection of human tissue samples (III)

The study was approved by the Ethics Committee of the Oulu University Hospital. Human tissue samples were collected for analysis of SP-C mRNA and protein in nonpulmonary tissues. Placenta, amnion, and chorion were obtained from normal term delivery and samples of adult lung from surgical procedures with the permission of the donors. Tissues were frozen in liquid nitrogen and stored at -70 °C.

4.3 Protein analyses (I-III)

The primary antibodies used in the studies are listed in Table 4.

Table 4. The primary antibodies used in protein analyses.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Definition</th>
<th>Manufacturer</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB3420</td>
<td>Polyclonal rabbit anti-</td>
<td>Millipore, Billerica, MA, USA</td>
<td>Western analysis (1:200)</td>
</tr>
<tr>
<td></td>
<td>human SP-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB3434</td>
<td>Polyclonal rabbit anti-</td>
<td>Millipore, Billerica, MA, USA</td>
<td>Western analysis (1:200)</td>
</tr>
<tr>
<td></td>
<td>mouse SP-D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL-197</td>
<td>Polyclonal rabbit anti-</td>
<td>Santa Cruz Biotechnology,</td>
<td>Immunohistochemistry (1:200),</td>
</tr>
<tr>
<td></td>
<td>human proSP-C</td>
<td>Santa Cruz, CA, USA</td>
<td>Western analysis (1:200)</td>
</tr>
<tr>
<td>WRAB-SP-C</td>
<td>Polyclonal rabbit anti-</td>
<td>Seven Hills Bioreagents,</td>
<td>Immunohistochemistry (1:2000),</td>
</tr>
<tr>
<td></td>
<td>mouse proSP-C</td>
<td>Cincinnati, OH, USA</td>
<td>Western analysis (1:10000)</td>
</tr>
<tr>
<td>anti-TLR2</td>
<td>Polyclonal goat anti-</td>
<td>R&amp;D System Inc, Minneapolis, MN, USA</td>
<td>Immunohistochemistry (1:100)</td>
</tr>
<tr>
<td></td>
<td>mouse TLR2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-TLR4</td>
<td>Monoclonal rat anti-</td>
<td>R&amp;D System Inc, Minneapolis, MN, USA</td>
<td>Immunohistochemistry (1:100)</td>
</tr>
<tr>
<td></td>
<td>mouse TLR4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 The dilution of the antibody is expressed in parentheses.
4.3.1 Western analysis (II, III)

SP-A and SP-D proteins were detected in maternal and fetal lung (total protein of 15 µg and 20 µg, respectively) and in amniotic fluid (8.5 µl) of C57BL/6, rSP-A and rSP-D mice by Western analyses. In addition the presence of SP-C (detected as proSP-C) was examined in several tissues of C57BL/6 mice (maternal lung 15 µg, fetal lung 20 µg, uterus, fetal membranes and placenta 50 µg).

Prior to analyses, the harvested tissue samples were lysed in modified radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 5 mM benzamidine, 2 mM phenylmethanesulphonylfluoride, 10 µg/ml pepstatin A, 10 µg/ml aprotinin, 10 µg/ml antipain A, 10 µg/ml leupeptin, 10 µg/ml chymostatin). The supernatants were collected after centrifugation at low speed and the protein contents were quantified using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA). Protein samples were resolved on a 12% Bis-Tris gel (NuPAGE Novex, Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions and electrotransferred onto a Protran BA85 nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membranes were treated with 5% powdered milk in Tris-Buffered saline containing 0.1% Tween 20 overnight at +4 °C to prevent non-specific binding of the antibodies to membranes. After the blocking step, the primary antibodies (listed in Table 4) were visualized by chemiluminescence using an ECL-Plus Detection kit (GE Healthcare, Little Chalfont, UK).

4.3.2 Immunohistochemistry (I-III)

Immunohistochemistry was performed in order to study localization of TLR4 and TLR2 in fetal lung and in gestational tissues and SP-C in gestational tissues. After overnight fixing in 4% formaldehyde, the isolated tissue samples were embedded in paraffin and cut into 5 µm sections. Deparaffinized sections were heated either in citrate-phosphate buffer (pH 6) or in Tris-EDTA buffer (pH 9) according to the specific requirements for each antibody, and incubated in 0.03% H2O2 or Peroxidase-Blocking Solution (Dako, Glostrup, Denmark), respectively. After the inhibition of endogenous peroxidase, the sections were blocked with donkey serum in PBS-Tween to prevent non-specific staining. The sections were then incubated with the primary antibodies listed in Table 4. After addition of the secondary antibodies, the detections were carried out either with the Goat HRP-Polymer Kit (Biocare Medical, CA, USA) or with DAB substrate (Zymed...
Laboratories, San Francisco, CA, USA) according to the manufacturers’ instructions. Finally, the sections were counterstained with hematoxylin. The specificity of the TLR4 and SP-C antibodies was confirmed using the tissue sections from TLR4−/− (Hoshino et al. 1999) and SP-C−/− mice, respectively. The tissues of SP-C−/− mice were obtained from Dr. Stephan Glasser (Glasser et al. 2001, Glasser et al. 2003). The proper staining conditions for the TLR2 antibody had been established previously using tissue sections from TLR2−/− mice (provided by Prof. Birgitta Henriques-Normark, Karolinska Institutet, Solna, Sweden).

4.3.3 Cytometric Bead Array (I, II)

The Cytometric Bead Array (CBA) - Mouse Inflammation Kit (BD Biosciences San Diego, CA, USA) was used to quantify the levels of TNF-α, IL-6, IL-10 and monocyte chemo-attractant protein 1 (MCP-1) from maternal and fetal serum and amniotic fluid according to the manufacturer's instructions. For each sample (25 μl) antibody-coated capture beads and phycoerythrin (PE)–conjugated anti-mouse antibodies were added. After incubation the capture beads were washed to remove the unbound PE-reagent and the beads were suspended in PBS. The concentrations of the cytokines were measured using a FACSCalibur flow cytometer and the data were analyzed with the BD CBA analysis software (BD Biosciences). The detection limits for TNF-α, IL-6, IL-10, and MCP-1 were 7, 5, 17 and 53 pg/ml, respectively. In each PBS- or LPS-treated group, 5–10 dams and a total of 2–4 pups from every litter were analyzed individually.

4.4 RNA analyses

Total RNA from the harvested tissues was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. The extracted RNA was dried with vacuum and dissolved either in the RNase Protection Assay (RPA) hybridization buffer (BD Biosciences) or in RNA sample buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 10 mM NaOH, 50% formamide, 0.4 M formaldehyde) for Northern analysis. For reverse transcriptase-PCR (RT-PCR) RNA was further purified using RNeasy Micro Kit (Qiagen, Hamburg, Germany) according to the manufacturer’s protocol.
4.4.1 RNase Protection Assay (I-III)

The mRNA levels of several inflammatory mediators (SPs, TLR4, TLR2, IL-1β, TNF-α, MIP-2, IL-4, IL-6, and IL-10) in mouse tissues were quantified by the mouse custom-designed multi-probe RPA system (BD Biosciences). First the [α-32P]UTP-labeled antisense RNA probe was synthesized and total RNA (10 µg) was hybridized with the probe. After digestion of single-stranded RNA and free probe by RNase A and T1, the protected RNA fragments were purified using phenol extraction, precipitated, and resolved on a 5% denaturing polyacrylamide gel. The quantities of the protected RNAs were determined by PhosphorImager (Bio-Rad) and Quantity one software (version 4.6.0, Bio-Rad). The expressions of the housekeeping gene L32 were analyzed as a reference. The mRNA levels were expressed as fold increases compared with controls.

To study the LPS-induced inflammatory response leading to preterm birth in C57BL/6 mice, mRNAs from the tissues of 5–6 dams (PBS- or LPS-treated) or 6–8 of their pups (1–2 pups from every litter) were analyzed individually. All the samples from one tissue were analyzed on an individual gel (e.g. uteri of LPS- and PBS-treated mice; controls, 3 and 8 hour samples). For the analysis of rSP-A and rSP-D lines, two mRNA samples (5 µg each) from PBS-injected dams or their fetuses were pooled for analysis. Samples from PBS- or LPS-injected wild-type dams and their fetuses were pooled similarly as well. In contrast, LPS-injected rSP-A and rSP-D dams and their fetuses were analyzed individually. Pooling of the samples was performed due to limited space on gels. To make the comparisons between wild-type and over expressing mice possible, RNA samples of rSP-A or rSP-D mice were run on a gel with the samples of wild-type mice.

4.4.2 Semiquantitative reverse transcriptase-PCR (III)

RT-PCR was performed in order to amplify the target complementary DNAs (cDNAs) from mouse (fetal and maternal lung, uterus, placenta and fetal membranes) and human tissues (lung, placenta, amnion and chorion) to estimate SP-C expression levels in reproductive tissues and to investigate possible alternative splicing. In addition, probes for Northern analyses were prepared.

The first-strand cDNA synthesis reaction was catalyzed by Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) from two micrograms of total RNA according to the manufacturer’s instructions. Amplification of the target cDNAs was performed by PCR using specific primers.
for mouse and human SP-C (Table 5). The cycles in the PCRs were 15, 20, 25, 30 and 35 for mouse and 20, 25, 30 and 35 for human cDNAs. The amplified products were verified by sequencing and comparing to a known mouse or human SP-C sequences. An additional three primer pairs (Table 5) for mouse SP-C were used to verify the amplified PCR product from reproductive tissues and to evaluate the possible alternative splicing. The amplified PCR products were separated by electrophoresis on an agarose gel and the size of PCR products from reproductive tissues were compared with corresponding PCR products from lung.

### Table 5. The primer pairs used in RT-PCR.

<table>
<thead>
<tr>
<th>Definition</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mSP-C</td>
<td>5'-TGCACCTCAAACGCCTTCTC-3'</td>
<td>5'-TTCATGATG TAGCAGTAG TTTC-3'</td>
<td>280^a</td>
</tr>
<tr>
<td>mSP-C</td>
<td>5'-GAGGTCCTGATGGAGAGTC-3'</td>
<td>5'-CAGCTTCTGGCTTGTCTGAC-3'</td>
<td>640</td>
</tr>
<tr>
<td>mSP-C</td>
<td>5'-AGCAGACACCATCGCTACCT-3'</td>
<td>5'-CAGCAAGGCTAGAAAG-3'</td>
<td>280</td>
</tr>
<tr>
<td>mSP-C</td>
<td>5'-CAGCTCCAGGAACCTACTGC-3'</td>
<td>5'-CAGCTTCTGGCTTGTCTGAC-3'</td>
<td>320</td>
</tr>
<tr>
<td>hSP-C</td>
<td>5'-CTGCAAGCAAGATGGATGG-3'</td>
<td>5'-GTCTAGATGAGCG-3'</td>
<td>570^a</td>
</tr>
</tbody>
</table>

^a Primers of the primer pairs were located in different exons to exclude the amplification of similar-sized genomic DNA. ^PCR product was used as a probe in Northern analysis as well. GenBank accession number for mouse SP-C gene: NM_011359, GenBank accession number for human SP-C gene: NM_003018.

#### 4.4.3 Northern analysis (III)

The probes for the Northern analysis of SP-C were obtained by RT-PCR (see section 4.4.2) using mRNA from mouse or human lung. The amplified cDNA fragments were extracted from agarose gel and purified using GeneClean Turbo Kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer’s instructions and ligated to pGEM-T Easy vector (Promega, Madison, WI, USA). The \[^{[\alpha-32P]}\]dCTP-labeled mouse and human SP-C probes were synthesized using the Rediprime II Random Prime Labelling System (GE Healthcare) according to the manufacturer’s instructions.

For Northern analyses different amounts of total RNA from mouse and human tissues (mouse lung 10 µg, other mouse tissues 30 µg, human lung 5 µg, other human tissues 15 µg) were separated on agarose-formaldehyde gels. The RNA fragments were transferred overnight onto nylon membranes (Millipore Corporation, MA, USA) with 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7). The prehybridizations and hybridizations were performed in hybridization buffer.
(1 M NaCl, 1% SDS, 10% Dextran sulfate, 100 μg/ml herring sperm as carrier DNA, 5x Denhardt's Solution, 50% formamide) overnight at +42 °C followed by washes with 2xSSC, 0.1% SDS and 0.1xSSC, 0.1% SDS at +60 °C. After reaching the desired level of background activity, the detection of RNA intensities was performed using a PhosphorImager.

4.5 Genetic study (III)

This study was approved by the Ethics Committee of Oulu University Hospital. Participation in all studies was voluntary and written informed consent was obtained from the mothers. A detailed description of the genetic study is presented in original article III.

4.5.1 Study population and sample collection

The population of mothers with spontaneous preterm birth was selected from birth diaries of Oulu University Hospital between the years 1975 and 2005. The selection was performed prospectively from the year 2003. Blood samples or buccal cells were collected from the participants.

Spontaneous preterm birth was defined as a spontaneous onset of labor and birth before completion of 36 weeks of gestation. Mothers with one or more than one spontaneous preterm birth and only families of Finnish origin were included. However, only one of the at least two mothers who were first-, second- or third-degree relatives was included. Factors clearly increasing the risk of preterm birth (multiple gestation, chronic disease of the mother, narcotic or alcohol abuse, preeclampsia, polyhydramnios, accidents, septic infection, uterine malformation or fetus with severe disease or malformation syndrome) were used as exclusion criteria. In the families with more than one preterm delivery, only one preterm infant was included in the primary analysis. The infant was prospectively selected using the following low-risk criteria: 1) the age of the mother between 20 and 35 years at the time of the birth of the preterm infant, 2) preference of a girl over a boy, and 3) no deliveries within the preceding 2 years or the longest time interval from the previous delivery. The summary of the study population is presented in Table 6. The control population consisted of 202 mothers and 199 infants, who were prospectively recruited among mothers with at least three exclusively term deliveries with no pregnancy or labor-associated complications.
Cases of PPROM were stratified into two groups on the basis of the interval between PPROM and preterm birth: short (< 72 hours) and long duration (≥ 72 hours), as originally described (Richardson et al. 1974). For the post hoc study on the duration of PPROM, only mother-infant pairs with a complete set of SP-C genotypes were included (137 pairs). One case of PPROM from each mother with more than one preterm birth was allowed in the analysis. Among ten families with two or more spontaneous preterm births, the PPROM infant was a sibling of the preterm infant selected in the primary analysis.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Infants</th>
<th>Mothers</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>307</td>
<td>301</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>31.8±3.05 (22.7-35.9)</td>
<td>28.8±5.71 (16-46)</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>1867±635 (251-3375)</td>
<td>28.8±5.71 (16-46)</td>
</tr>
<tr>
<td>PPROM/no PPROM</td>
<td>131/176</td>
<td>139/162</td>
</tr>
<tr>
<td>Male/female</td>
<td>171/136</td>
<td></td>
</tr>
<tr>
<td>Single spontaneous preterm delivery</td>
<td>215</td>
<td>210</td>
</tr>
<tr>
<td>Two spontaneous preterm deliveries</td>
<td>69</td>
<td>68</td>
</tr>
<tr>
<td>3-6 spontaneous preterm deliveries</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Maternal age at time of birth, years</td>
<td></td>
<td>28.8±5.71 (16-46)</td>
</tr>
<tr>
<td>Smoking during pregnancy, yes/no/unknown</td>
<td>38/187/82</td>
<td></td>
</tr>
</tbody>
</table>

*a One infant per mother was included (some samples were excluded due to low-quality DNA), †Mean±SD (range).

### 4.5.2 DNA extraction and genotyping

Genomic DNA from whole blood specimens (n = 604) was extracted using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) or UltraClean DNA Blood Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Genomic DNA from buccal cells (n = 405) was extracted using Chelex 100 (Bio-Rad).

Two common nonsynonymous polymorphisms (rs4715, Thr138Asn and rs1124, Ser186Asn) are located within the SP-C gene as indicated in the dbSNP database (http://ncbi.nih.gov/SNP/). These two polymorphisms display strong linkage disequilibrium, with combined frequency of the 138Asn-186Asn and 138Thr-186Ser haplotypes being nearly 100% (Lahti et al. 2004). SNP Thr138Asn was thus selected as a candidate SNP to study the relevance of the SP-
C gene to spontaneous preterm birth. Genotyping of the SNP Thr138Asn was performed using the AcycloPrime II SNP Detection Kit (Perkin Elmer Life Sciences, MA, USA) according to the manufacturer’s instructions. The primers were as follows: forward PCR primer 5’-GCT GCT GAT CGC CTA CAA G-3’ and reverse 5’-AGG GAG ACA GCC CAC TCT TT-3’ and the SNP primer 5’-ATC CCC AGT CTT GAG GCT CTC A-3’. In the cases of inadequate DNA concentration, 1 µl of the PCR product was reamplified using nested primers 5’-CTG CTG ATC GCC TAC AAG C-3’ (forward) and 5’-GGG AGA CAG CCC ACT CTT TT-3’ (reverse) prior to genotyping with the SNP primer.

4.6 Statistical analyses (I-III)

The quantified mRNA values and protein concentrations were analyzed using Origin 7.0 or 8.0 (OriginLab Corp., Northampton, MA, USA) and SPSS versions 14.0.2 or PASW Statistics 17 (SPSS Inc., Chicago, IL, USA). The data were tested for normal distribution and statistical significances between experimental groups, and control groups were analyzed using either Student’s t-test or Mann-Whitney U test. The statistical tests for association of the SP-C SNP Thr138Asn with preterm birth in mothers and infants, including comparisons of allele and genotype frequencies by 2x2 and 2x3 contingency tables, respectively, and calculation of Hardy-Weinberg equilibrium, were performed using SPSS (version 14.0.2) and Arcus QuickStat software (Longman Software Publishing, Cambridge, UK). The differences in the duration of PPROM were analyzed using the non-parametric Kruskal-Wallis H test or the Mann-Whitney U test. Bonferroni correction was applied to control multiple testing in original article III.
5 Results

5.1 Mouse model of inflammation induced preterm birth - C57BL/6 and FVB mice (I)

LPS-induced inflammatory response leading to preterm birth was studied using mice of the C57BL/6 and FVB strains. First, the LPS dose needed to induce preterm birth of live-born offspring was established, and then the LPS-induced inflammatory response was assessed in maternal and fetal compartments of C57BL/6 mice.

5.1.1 Preterm birth of live-born pups following maternal LPS in C57BL/6 and FVB mice

In preliminary studies C57BL/6 dams received 10–50 µg/mouse of intraperitoneal LPS on day 16 or 17 of gestation. A dose of 25 µg/mouse resulted in the delivery of mostly (≥ 50%) live-born pups, whereas large doses (> 35 µg/mouse) induced delivery of dead fetuses within 24 hours. With doses lower than 20 µg/mouse, dams recurrently delivered live-born fetuses at term. The preterm birth inducing dose of LPS was found to be the same for the FVB strain (data not shown). PBS injection at 16 or 17 dpc resulted in term delivery in C57BL/6 and FVB wild-type mice at day 18–20 of pregnancy (Table 7).

Injection with 25 µg/mouse of LPS at 16 or 17 dpc induced preterm delivery of mostly live born pups (39% and 65%, respectively) within 17 h (± 4 h) in the C57BL/6 strain. The survival of the pups increased with advanced gestational age in the FVB strain as well (Table 7), but the interval between the LPS injections and delivery was longer (20 h ± 4 h). Despite the genetic background, all prematurely born pups died shortly after birth apparently as a consequence of respiratory failure. Length of gestation influenced the viability, because the pups born at 17 dpc died within 30 min, whereas the pups born at 18 dpc survived up to several hours. The LPS-induced preterm birth in C57BL/6 mice was more constant (Table 7), and therefore the FVB strain was excluded from subsequent studies.
Table 7. Induction of preterm delivery by maternal LPS (25 μg/mouse) in C57BL/6 and FVB mice.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>C57BL/6</th>
<th>FVB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPS at 16 dpc</td>
<td>LPS at 17 dpc</td>
</tr>
<tr>
<td>N litters/pups</td>
<td>13/84</td>
<td>32/211</td>
</tr>
<tr>
<td>17 dpc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litters born</td>
<td>13 (100%)</td>
<td>-</td>
</tr>
<tr>
<td>Pups born</td>
<td>84 (100%)</td>
<td>-</td>
</tr>
<tr>
<td>Live-born litters²</td>
<td>5 (38%)</td>
<td>-</td>
</tr>
<tr>
<td>Live-born pups</td>
<td>33 (39%)</td>
<td>-</td>
</tr>
<tr>
<td>18 dpc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litters born</td>
<td>-</td>
<td>29 (91%)</td>
</tr>
<tr>
<td>Pups born</td>
<td>-</td>
<td>189 (90%)</td>
</tr>
<tr>
<td>Live-born litters²</td>
<td>-</td>
<td>21 (72%)</td>
</tr>
<tr>
<td>Live-born pups</td>
<td>-</td>
<td>122 (65%)</td>
</tr>
<tr>
<td>19 dpc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litters born</td>
<td>-</td>
<td>3 (9%)</td>
</tr>
<tr>
<td>Pups born</td>
<td>-</td>
<td>22 (10%)</td>
</tr>
<tr>
<td>Live-born litters²</td>
<td>-</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>Live-born pups</td>
<td>-</td>
<td>22 (100%)</td>
</tr>
<tr>
<td>20 dpc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litters born</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Live-born litters²</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Live-born pups</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ Only the litters are shown. ² Controls injected with PBS at 16 and 17 dpc. ³ Litter was called live-born when ≥ 50% of the pups were born alive.

### 5.1.2 Characterization of the inflammatory responses in maternal, gestational, and fetal tissues of C57BL/6 mice after maternal LPS challenge

The LPS-induced inflammatory response leading to preterm birth of live-born pups was quantified in maternal and fetal compartments 3 and 8 hours after the injections by RPA and CBA. PBS-injected dams and their pups were used as controls. The length of gestation did not significantly \( P < 0.05 \) influence the basal levels of cytokines in maternal or fetal serum or in amniotic fluid between
the two different gestational ages (16 and 17 dpc). In general the LPS-induced responses in fetal compartments were lower by more than one order of magnitude compared with the maternal response. There were either no differences between the mice injected at 16 or 17 dpc or the response was more marked at the later stage of gestation.

**Maternal response**

Maternal response to intraperitoneal LPS was rapid and robust. Concentrations of TNF-α, MCP-1, IL-10, and IL-6 in serum were significantly \((P < 0.05)\) increased at 3 and 8 hours after the injections compared with controls (Figure 1A in the original article I). In lung there was a transient increase in TLR2 expression. TLR4 expression increased as well, but the induction was clearly weaker (Figure 4A and B in I). The responses of proinflammatory cytokines IL-1β and MIP-2 were strong, whereas inductions of IL-4 and TNF-α were barely detectable (Figure 3D in I).

**Response in gestational tissues**

Maternal systemic inflammation induced by intraperitoneal LPS caused a rapid induction in the expression of proinflammatory cytokines in gestational tissues as well. The response was most intense in uterus, where the levels of IL-1β, MIP-2, IL-4, TNF-α, IL-6, and IL-10 mRNAs were significantly \((P < 0.05)\) upregulated (Figure 3C in I). In addition, the levels of IL-1β, MIP-2, and IL-6 in placenta and IL-1β and MIP-2 in fetal membranes were increased (Figure 3A and B in I).

Despite the marked proinflammatory response in gestational tissues, there were only minor differences in the expression levels of TLR4, the component of the LPS receptor complex (Figure 4A in I). In contrast, the expression of TLR2 transiently increased in placenta and uterus (Figure 4B in I). TLR4 and TLR2 proteins in placenta were localized to trophoblasts including giant trophoblasts by immunohistochemistry. TLR2 was additionally detected in placental macrophages. Maternal LPS increased the number of TLR2-positive macrophages in the labyrinthine placenta, whereas no remarkable changes in the TLR4 or TLR2 staining of placental trophoblasts were detected (Figure 6C-H in I).

SP-D mRNA was evident in the uterus, placenta, and fetal membranes, whereas weak expression of SP-A was detected only in the uterus. In placenta and fetal membranes, SP-D expression was significantly \((P < 0.05)\) decreased at 16
dpc in response to maternal LPS. In addition, LPS tended to increase the expression of SP-A in the uterus (Figure 5A and B in I).

**Fetal response**

Maternal LPS challenge significantly \((P < 0.05)\) increased the concentrations of proinflammatory cytokines (TNF-\(\alpha\), MCP-1 and IL-6) in amniotic fluid, whereas the changes in IL-10 levels were small (Figure 1B in I). The increases in the concentrations of MCP-1, IL-10, and IL-6 correlated with those seen in dam serum, whereas the TNF-\(\alpha\) response was slower (Figure 1A and B in I). In fetal serum the concentrations of TNF-\(\alpha\) and IL-10 were higher compared to amniotic fluid, whereas the levels of MCP-1 and IL-6 were clearly lower (Figure 1C in I and data not shown).

In fetal lung, only minor changes in the expression levels of IL-1\(\beta\), MIP-2, TNF-\(\alpha\), and IL-6 were detected (Figure 2 in I). However, the expression levels of genes coding proteins that recognize LPS (SP-A, SP-D, and TLR4) showed a barely detectable increase by 3 hours, and significantly \((P < 0.05)\) downregulated expression was detected thereafter. A similar expression pattern was evident for TLR2 mRNA (Figure 4A, 4B, 5A and 5B in I). SP-D expression was not detected at 16 dpc, otherwise the downregulation of these inflammatory mediators was stronger at later gestational age. TLR2 protein localized to interstitial macrophages and maternal LPS had no detectable effect in their number. However, after maternal LPS challenge macrophages frequently appeared to be concentrated around the pulmonary vessels. TLR4 protein was barely detectable in fetal lung, regardless of LPS (Figure 6A and B in I).

Because the inflammatory response to maternal LPS in fetal lung was so low, the fetal response was further evaluated in liver, heart, brain, and intestine at 17 dpc (Figure 2 in I). Similarly only minor changes in the expression rates of cytokines were detected, with the exception of MIP-2 expression that significantly \((P < 0.05)\) increased in liver, and IL-1\(\beta\) that decreased in liver and intestine. The changes in the levels of other inflammatory mediators were not significant (Figure 2, 4C and 4D in I). The low number of fetuses \((N = 4)\) in these experiments may partly explain the absence of significant changes.
5.2 Mouse model of inflammation induced preterm birth - rSP-A and rSP-D overexpressing mice (II)

SP-A and SP-D produced by fetal lung can be proposed to contribute to intrauterine inflammatory processes related to initiation of labor, because the surfactant components are secreted from fetal lungs into amniotic fluid. This hypothesis has been supported by the finding that injection of SP-A into mouse amniotic fluid initiates labor (Condon et al. 2004). Here we used mice overexpressing either rSP-A or rSP-D under the control of the hSP-C promoter to study the role of SP-A and SP-D in the process of LPS-induced preterm birth.

5.2.1 Characterization of rSP-A and rSP-D overexpressing mice

The presence of SP-A and SP-D at 17 dpc was evaluated in the tissues of mice overexpressing rSP-A or rSP-D, respectively. In addition, the basal concentrations of cytokines in sera and in amniotic fluid and the basal expression levels of cytokines, TLR4, and TLR2 in several tissues were quantified.

Presence of rSP-A and rSP-D in fetal lung, gestational tissues and in amniotic fluid

SP-D protein was detected in the uterus, placenta, fetal membranes, and in amniotic fluid in addition to fetal lung by Western analysis. Higher levels of SP-D were evident in all tissues and amniotic fluid of rSP-D mice compared with C57BL/6 wild-type mice (Figure 6A). The size of SP-D protein (43 kDa) in gestational tissues and in amniotic fluid was identical with SP-D from lung. Detectable levels of SP-A protein were evident only in lungs and amniotic fluid, the levels being higher in rSP-A overexpressing animals (Figure 6B). The sizes of the detected SP-A proteins were 32–38 kDa and 26 kDa, representing the glycosylated and non-glycosylated SP-A, respectively (Elhalwagi et al. 1999). Low levels of SP-A protein in gestational tissues and low sensitivity of the antibody may explain why there was no difference in the levels of SP-A between rSP-A and wild-type mice. Therefore, the expression of SP-A mRNA in these tissues was additionally analyzed by RPA (Figure 6C). SP-A expression was 4-fold higher in uteri of rSP-A mice compared with wild-type (data not shown). In addition there was a clear expression of SP-A in fetal membranes and placentas of rSP-A mice, whereas no mRNA was detected in corresponding wild-type tissues.
Collectively these data indicate that in addition to lung, hSP-C promoter drives the expression of rSP-A and rSP-D in the gestational tissues of overexpressing animals.

Fig. 6. A) SP-D protein in fetal lung, amniotic fluid, and gestational tissues. B) SP-A protein in fetal lung and amniotic fluid. C) SP-A mRNA in gestational tissues.

**Effect of rSP-A or rSP-D overexpression on the levels of cytokines and TLRs**

The overexpression of either rSP-A or rSP-D had some effect on the basal levels of cytokines and TLRs. Most notably the levels of inflammatory mediators were lower in placentas of overexpressing mice compared with controls, and a similar trend was observed in fetal membranes. In sera of overexpressing animals, the concentrations of cytokines were somewhat lower compared with wild-type mice, whereas in amniotic fluid of rSP-A and rSP-D fetuses the levels were elevated. The differences between rSP-A, rSP-D, and wild-type mice are presented in Tables 8 and 9.
Table 8. Relative basal levels of inflammatory mediators in rSP-A or rSP-D mice compared with wild-type mice.

<table>
<thead>
<tr>
<th>Inflammatory mediator</th>
<th>Fetal lung</th>
<th>Maternal lung</th>
<th>Uterus</th>
<th>Placenta</th>
<th>Fetal membranes</th>
<th>Maternal serum</th>
<th>Fetal serum</th>
<th>AF²</th>
</tr>
</thead>
<tbody>
<tr>
<td>rSP-A vs. wild-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>1.3</td>
<td>1.2</td>
<td>1.2</td>
<td>0.44*</td>
<td>0.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>1.3</td>
<td>1.2</td>
<td>0.92</td>
<td>0.52*</td>
<td>0.67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.4</td>
<td>0.72</td>
<td>1.4</td>
<td>0.63*</td>
<td>0.50</td>
<td>0.69*</td>
<td>0.86</td>
<td>1.4*</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.8</td>
<td>0.94</td>
<td>1.2</td>
<td>0.56</td>
<td>0.44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.6</td>
<td>0.86</td>
<td>0.69</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-2</td>
<td>1.9*</td>
<td>1.3</td>
<td>0.33*</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>2.2*</td>
<td>0.42</td>
<td>0.63</td>
<td>0.69</td>
<td>0.90*</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>1.3</td>
<td>0.73</td>
<td>2.3*</td>
<td>0.96</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td></td>
<td>0.78*</td>
<td>1.2</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rSP-D vs. wild-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>0.97</td>
<td>0.99</td>
<td>0.89</td>
<td>0.43*</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>1.1</td>
<td>1.5</td>
<td>1.1</td>
<td>0.66</td>
<td>0.95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.78*</td>
<td>0.43</td>
<td>0.72</td>
<td>0.56*</td>
<td>1.0</td>
<td>0.74</td>
<td>1.0</td>
<td>1.2*</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.66</td>
<td>0.44</td>
<td>0.93</td>
<td>0.70</td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.48</td>
<td>0.70</td>
<td>0.77</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-2</td>
<td>1.4</td>
<td>1.1</td>
<td>0.4</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>1.2*</td>
<td>1.0</td>
<td>0.83</td>
<td>1.1</td>
<td>1.9</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>1.0</td>
<td>1.1</td>
<td>0.80*</td>
<td>0.65*</td>
<td>2.1*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.1</td>
<td>1.3</td>
<td>1.2*</td>
<td>1.2*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* $P < 0.05$, ¹ Detected as mRNA, ² Detected as protein. AF = Amniotic fluid. Difference < 1.5 fold is indicated in italics. Inflammatory mediators with no detectable expression are not listed.

Table 9. Relative basal levels of cytokines in rSP-A mice compared with rSP-D mice.

<table>
<thead>
<tr>
<th>Cytokine¹</th>
<th>Maternal serum</th>
<th>Fetal serum</th>
<th>Amniotic fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>0.93</td>
<td>0.86*</td>
<td>1.2</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.9*</td>
<td>0.65*</td>
<td>0.67*</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.83*</td>
<td>0.47*</td>
<td>0.84</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.72*</td>
<td>1.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ¹ Quantified as protein; due to the limited sample space on RPA gels, it was not possible to compare the basal expression levels between rSP-A and rSP-D mice. Difference < 1.5 fold is indicated in italics.
5.2.2 Preterm birth of live born pups following maternal LPS in rSP-A and rSP-D overexpressing mice

The normal length of pregnancy in rSP-A and rSP-D mice was similar to that of C57BL/6 wild-type mice (Tables 7 and 10). To define the LPS dose needed to induce preterm birth of live-born offspring, the dams received decreasing doses of LPS (Table 10) at 17 dpc, starting from the dose that was used for wild-type mice (25 µg/mouse). Unexpectedly over 50% of the pups were stillborn with this dose, therefore the tested doses needed to be decreased. In both transgenic lines the highest amount of live-born litters (≥ 50% of the pups born alive) was obtained using an LPS dose of 12.5 µg/mouse. Injection with 12.5 µg of LPS at 17 dpc induced preterm delivery of mostly live-born fetuses both in rSP-A (76%) and rSP-D (66%) overexpressing mice within 19 h (± 4 h). All the pups born at 18 dpc clearly had breathing difficulties and they died within a few minutes, indicating that the overexpression of either rSP-A or rSP-D did not improve the viability of the prematurely born pups.
Table 10. Induction of preterm delivery by maternal LPS at 17 dpc in rSP-A and rSP-D mice.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>rSP-A line</th>
<th>rSP-D line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPS 12.5 µg</td>
<td>LPS 15 µg</td>
</tr>
<tr>
<td>18 dpc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litters born</td>
<td>8 (67%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>Pups born</td>
<td>58 (66%)</td>
<td>35 (100%)</td>
</tr>
<tr>
<td>Live-born litters²</td>
<td>8 (100%)</td>
<td>2 (33%)</td>
</tr>
<tr>
<td>Live-born pups</td>
<td>44 (76%)</td>
<td>5 (14%)</td>
</tr>
<tr>
<td>19 dpc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litters born</td>
<td>4 (33%)</td>
<td>-</td>
</tr>
<tr>
<td>Pups born</td>
<td>30 (34%)</td>
<td>-</td>
</tr>
<tr>
<td>Live-born litters²</td>
<td>4 (100%)</td>
<td>-</td>
</tr>
<tr>
<td>Live-born pups</td>
<td>30 (100%)</td>
<td>-</td>
</tr>
<tr>
<td>20 dpc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litters born</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pups born</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Live-born litters²</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Live-born pups</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ Only the litters are shown, ² rSP-A and rSP-D control mice injected with PBS, ³ Litter was called live-born when ≥ 50% of the pups were born alive.
5.2.3 Characterization of the inflammatory response in maternal, gestational, and fetal tissues of rSP-A and rSP-D mice after maternal LPS challenge

To further characterize the differences in the LPS-induced inflammatory response between overexpressing and wild-type mice, the levels of inflammatory mediators in maternal and fetal compartments were quantified by RPA and CBA. Here the LPS-injections were performed only at 17 dpc. Because the inflammatory response leading to preterm delivery of live-born pups in rSP-A and rSP-D mouse lines was evoked by 12.5 μg/mouse of LPS, the wild-type mice used as controls in this study were injected with the same dose. In general the LPS-induced inflammatory response in C57BL/6 wild-type mice after 12.5 μg of LPS resembled that after the higher (25 μg/mouse) dose (see section 5.1.2), but the levels of the inflammatory mediators were lower. Therefore, only the comparisons between rSP-A, rSP-D, and wild-type mice at 3 and 8 hours after the LPS challenge are presented. In addition, only the responses of TNF-α, IL-1β, IL-4, IL-10, TLR2, TLR4, SP-A, and SP-D are reported.

Maternal response

The overexpression of rSP-A led to decreased LPS-induced maternal inflammatory response, evidenced with lower levels of TNF-α and IL-10 in sera of rSP-A females compared with wild-type mice. In the lungs of rSP-A dams, the levels of inflammatory mediators tended to be lower, too, but many of these differences were not statistically significant ($P < 0.05$). The effect of rSP-D overexpression was more dispersed. Compared with rSP-A mice, the levels of TNF-α, IL-1β, and IL-4 were more marked in rSP-D mice, whereas the levels of TLR2, TLR4, SP-A, and SP-D were more constant. The comparisons of the maternal inflammatory response to intraperitoneal LPS challenge between rSP-A, rSP-D and wild-type mice are summarized in Table 11.
Table 11. Relative levels of inflammatory mediators in maternal serum and lung between rSP-A, rSP-D, and C57BL/6 mice after intraperitoneal LPS (12.5 μg/mouse) challenge at 17 dpc.

<table>
<thead>
<tr>
<th>Inflammatory mediator</th>
<th>rSP-A vs. WT</th>
<th>rSP-D vs. WT</th>
<th>rSP-A vs. rSP-D</th>
<th>3h</th>
<th>8h</th>
<th>3h</th>
<th>8h</th>
<th>3h</th>
<th>8h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal serum¹</td>
<td></td>
<td></td>
<td></td>
<td>0.78</td>
<td>0.54</td>
<td>1.0</td>
<td>0.61</td>
<td>0.77</td>
<td>0.90</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.73</td>
<td>0.56*</td>
<td>0.71</td>
<td>0.49*</td>
<td>1.0</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
<td></td>
<td>0.73</td>
<td>0.56*</td>
<td>0.71</td>
<td>0.49*</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Maternal lung²</td>
<td></td>
<td></td>
<td></td>
<td>0.68</td>
<td>0.92</td>
<td>1.5</td>
<td>1.6</td>
<td>0.45*</td>
<td>0.33*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.28</td>
<td>0.80</td>
<td>2.1*</td>
<td>2.8</td>
<td>0.13*</td>
<td>0.66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.70</td>
<td>0.75</td>
<td>1.5</td>
<td>3.7</td>
<td>0.43</td>
<td>0.31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>0.77</td>
<td>1.1</td>
<td>0.74</td>
<td>0.81</td>
<td>1.0</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>0.56</td>
<td>0.86</td>
<td>0.45*</td>
<td>0.45*</td>
<td>1.3</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>1.2</td>
<td>0.86</td>
<td>1.1</td>
<td>2.3</td>
<td>1.0</td>
<td>0.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP-A</td>
<td>1.6</td>
<td>1.1</td>
<td>1.9</td>
<td>2.4</td>
<td>0.87</td>
<td>0.58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP-D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05, ¹ Quantified as protein, ² Quantified as mRNA, rSP-A and rSP-D tissues were analyzed on separate gels which causes the occasional mismatches. Difference < 1.5 fold is indicated in italics.

Response in gestational tissues

The overexpression of either rSP-A or rSP-D modulated the inflammatory response to maternal LPS also in gestational tissues to some extent. For example, the expression levels of IL-4 and IL-10 in the uteri and IL-10 in fetal membranes of overexpressing animals tended to be lower compared with the corresponding wild-type tissues. In contrast, in fetal membranes the levels of IL-4 in overexpressing animals were clearly higher. There were hardly any differences in the levels of proinflammatory cytokines TNF-α and IL-1β in uteri and placentas among these three different mouse lines, whereas in fetal membranes the differences were more marked. The comparisons of the inflammatory responses in gestational tissues to intraperitoneal LPS challenge between rSP-A, rSP-D and wild-type mice are summarized in Table 12.
Table 12. Relative expression levels of inflammatory mediators in gestational tissues between rSP-A, rSP-D and C57BL/6 mice after maternal LPS (12.5 μg/mouse) challenge at 17 dpc.

<table>
<thead>
<tr>
<th>Inflammatory mediator</th>
<th>rSP-A vs. WT</th>
<th>rSP-D vs. WT</th>
<th>rSP-A vs. rSP-D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3h</td>
<td>8h</td>
<td>3h</td>
</tr>
<tr>
<td>Uterus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.0</td>
<td>0.60</td>
<td>1.3</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.73</td>
<td>0.81</td>
<td>0.62</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.48</td>
<td>0.50*</td>
<td>0.66</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.50</td>
<td>0.76*</td>
<td>0.55*</td>
</tr>
<tr>
<td>TLR4</td>
<td>1.10</td>
<td>0.77</td>
<td>0.96</td>
</tr>
<tr>
<td>TLR2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.3</td>
</tr>
<tr>
<td>SP-A</td>
<td>0.67</td>
<td>3.20</td>
<td>0.99</td>
</tr>
<tr>
<td>SP-D</td>
<td>0.92</td>
<td>0.81</td>
<td>1.0</td>
</tr>
<tr>
<td>Placenta</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
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<td>1.00</td>
<td>1.3</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.45</td>
<td>0.80</td>
<td>0.70</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.8*</td>
<td>1.10</td>
<td>1.6*</td>
</tr>
<tr>
<td>TLR4</td>
<td>0.91</td>
<td>0.66*</td>
<td>1.1</td>
</tr>
<tr>
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<td>1.2</td>
</tr>
<tr>
<td>SP-D</td>
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<td>1.10</td>
<td>0.92</td>
</tr>
<tr>
<td>Fetal membranes</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>3.60</td>
<td>7.0*</td>
<td>1.8</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.28*</td>
<td>0.73</td>
<td>1.4</td>
</tr>
<tr>
<td>IL-4</td>
<td>8.5*</td>
<td>18*</td>
<td>4.5</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.67</td>
<td>0.99</td>
<td>0.51*</td>
</tr>
<tr>
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<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>TLR2</td>
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<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>SP-D</td>
<td>1.10</td>
<td>0.89</td>
<td>0.80</td>
</tr>
</tbody>
</table>

* P < 0.05, 1 Quantified as mRNA, 2 rSP-A and rSP-D tissues were analyzed on separate gels which causes the occasional mismatches. Difference < 1.5 fold is indicated in italics.

Fetal response

After the maternal LPS challenge, the concentrations of TNF-α and IL-10 in the amniotic fluid of rSP-A and rSP-D fetuses were higher compared with wild-type fetuses, whereas in sera the response was reversed. In fetal lung the overexpression of either rSP-A or rSP-D did not notably influence the expression levels of cytokines, TLRs, or SPs after maternal LPS. The accumulation of TLR2-positive macrophages around the pulmonary vessels that was seen in the lungs of
wild-type fetuses (see 5.1.2) was not evident in rSP-A or rSP-D tissues (data not shown). The inflammatory response in other fetal tissues was not evaluated. The comparisons of the inflammatory responses in fetal compartments to maternal LPS challenge at 17 dpc between rSP-A, rSP-D, and wild-type mice are summarized in Table 13.

Table 13. Relative levels of inflammatory mediators in fetal compartments between rSP-A, rSP-D, and C57BL/6 mice after maternal LPS (12.5 μg/mouse) challenge at 17 dpc.

<table>
<thead>
<tr>
<th>Inflammatory mediator</th>
<th>rSP-A vs. WT 3h</th>
<th>rSP-A vs. WT 8h</th>
<th>rSP-D vs. WT 3h</th>
<th>rSP-D vs. WT 8h</th>
<th>rSP-A vs. rSP-D 3h</th>
<th>rSP-A vs. rSP-D 8h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amniotic fluid¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.3*</td>
<td>1.7*</td>
<td>1.6*</td>
<td>2.7*</td>
<td>1.5</td>
<td>0.62</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.7</td>
<td>1.6*</td>
<td>2.2*</td>
<td>4.3*</td>
<td>0.76</td>
<td>0.37*</td>
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<tr>
<td>Fetal serum¹</td>
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<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.7</td>
<td>0.96</td>
<td>0.95</td>
<td>0.67*</td>
<td>1.8</td>
<td>1.4*</td>
</tr>
<tr>
<td>IL-10</td>
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<td>0.82</td>
<td>0.54*</td>
<td>0.58*</td>
<td>1.4</td>
<td>1.4*</td>
</tr>
<tr>
<td>Fetal lung²</td>
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</tr>
<tr>
<td>TNF-α</td>
<td>1.8</td>
<td>2.2</td>
<td>1.3</td>
<td>1.2</td>
<td>1.5</td>
<td>2.8*</td>
</tr>
<tr>
<td>TLR4</td>
<td>1.2</td>
<td>1.2</td>
<td>1.8</td>
<td>2.1</td>
<td>0.70*</td>
<td>0.89</td>
</tr>
<tr>
<td>TLR2</td>
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<td>1.3</td>
<td>1.4</td>
<td>1.5</td>
<td>0.95</td>
<td>0.97</td>
</tr>
<tr>
<td>SP-A</td>
<td>1.0</td>
<td>2.1</td>
<td>1.1</td>
<td>0.93</td>
<td>0.91</td>
<td>2.6</td>
</tr>
<tr>
<td>SP-D</td>
<td>0.83</td>
<td>2.0</td>
<td>1.3</td>
<td>1.5</td>
<td>0.66</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* P < 0.05, ¹ Quantified as protein, ² Quantified as mRNA, ³ rSP-A and rSP-D tissues were analyzed on separate gels which causes the occasional mismatches. Difference < 1.5 fold is indicated in italics.

5.3 Nonpulmonary expression of SP-C (III)

While the LPS-induced inflammatory response in C57BL/6 mice was characterized, a weak but definite SP-C expression was detected in gestational tissues. Here the expression was further characterized and the SP-C expression in human gestational tissues was defined. In addition, possible alternative splicing of the SP-C transcript and tissue distribution and processing of proSP-C in mouse and human tissues were studied. In contrast to SP-C, the expression of SP-B was not detected in the initial assays. Therefore, the possible involvement of SP-B in the innate immunity reactions related to preterm birth was not evaluated.
5.3.1 Expression of SP-C in mouse and human gestational tissues

In addition to strong expression of SP-C in both maternal and fetal lung, a weak but definite expression was detected in the uterus, placenta, and fetal membranes of C57BL/6 mice by RPA. Maternal LPS had no statistically significant ($P < 0.05$) effect on mRNA levels of SP-C in any tissues studied (Figure 1A-E in III). SP-C mRNA was not evident in the uteri of young nulliparous females and no changes were thus detected during the estrus cycle. The expression of SP-C in mouse gestational tissues was further confirmed using RT-PCR. The expression level of SP-C in uterus was higher compared with expressions in placenta and fetal membranes, but notably lower compared to that in lung (Figure 1F in III). SP-C mRNA was detected in human tissues (lung, placenta, and fetal membranes) by RT-PCR as well, with the exception of chorion (Figure 4B in III).

5.3.2 Processing of SP-C mRNA in gestational tissues

Mouse SP-C cDNA was amplified with four different primer pairs (Table 5) to study the possible alternative splicing of SP-C mRNA in gestational tissues. The sizes of the PCR-fragments were identical to corresponding PCR-products from lung cDNA (Figure 2B in III). The authenticity of the amplified products was confirmed by sequencing. By Northern analysis the expression of SP-C was evident only in mouse uterus and lung and in human lung (Figure 2A and 4A in III). No evidence of alternative splicing was observed.

5.3.3 ProSP-C in gestational tissues

The proforms of SP-C were detected by Western blots in mouse uterus, fetal membranes, and placenta in addition to maternal and fetal lung by using two different SP-C-specific antibodies (Table 4). ProSP-C appeared as 16 kDa proform and a doublet with an estimated molecular weight of 21 kDa. The sizes of the detected proteins in fetal lung and reproductive tissues were similar but not identical to that in maternal lung (Figure 3A in III). In fetal membranes proSP-C localized to certain epithelial yolk sac cells and this staining was lacking in the tissues deficient in SP-C (Figure 3B-E in III). Due to the high background staining, proSP-C was not detectable in uterus or placenta. In addition, Western analysis from human gestational tissues (placenta, amnion, and chorion) provided some evidence about the presence of proSP-C in these tissues (data not shown).
5.4 SP-C SNP Thr138Asn in spontaneous preterm birth (III)

Previously, an association of SP-C SNP Thr138Asn with very preterm birth has been reported. In that study the cause of preterm birth (spontaneous labor versus all cases) was not taken into account (Lahti et al. 2004). The purpose of the present study was to analyze whether the same SNP associates with preterm birth after spontaneous onset of labor as well.

Here the association of SP-C SNP Thr138Asn with either preterm birth or spontaneous preterm delivery was not observed (Table II in III). Additional stratification by gestational age did not reveal any differences in the distribution of the alleles. In addition, there was no association between the same SNP and PPROM as a whole in the fetuses or the mothers. However, when the fetuses and mothers were further grouped on the grounds of the interval between PPROM and preterm birth, SNP Thr138Asn associated with the duration of PPROM in the fetuses but not in the mothers (Figure 5 in III). The duration of PPROM was significantly ($P < 0.0001$) different among fetuses with CC (median 6.5 days), AA (median 1.3 days) or CA genotypes (median 2.0 days) genotypes. The maternal genotypes neither associated with the duration of PPROM nor influenced the duration together with the fetal genotype (Table III in III).
6 Discussion

Prematurity is the main cause of perinatal mortality and morbidity in developed countries. In many cases preterm parturition is associated with intrauterine bacterial infection and consequent inflammatory processes. However, despite years of research, factors involved in the initiation of labor are incompletely understood and much effort is still needed before preterm birth can be effectively prevented. It should be noted that preterm parturition is most likely the end result of the complex interplay of various factors that may even have overlapping functions. Therefore, the determination of the roles of single components of the pathway may not provide clear answers for the underlying question about the initiation of labor.

Here the inflammatory factors related to preterm labor were studied in mice after maternal systemic LPS challenge. In addition, the possible involvement of SP-C in the process of preterm birth was evaluated.

6.1 Experimental mouse model of inflammation-induced preterm birth

Mice are among the most popular model organisms to study the mechanism of parturition. However, in many previous prematurity studies, the administration of LPS, mainly at 14–15 dpc, has resulted either in fetal death or delivery of dead fetuses rather than preterm birth, or the outcome of the fetuses has not even been described (reviewed in Elovitz & Mrinalini 2004). The most notable advantage in our model of LPS-induced preterm birth is that over 50% of the pups are born alive. This allows us to study the activation of the innate immune system truly related to the initiation of labor. The main difference between the current model and the other preterm birth mouse models is the gestational age used. The gestational ages used here (16 and 17 dpc) coincide with the transition from the canalicular to the saccular stage of human lung development, corresponding to 23 to 27 weeks of pregnancy, whereas 14–16 dpc represent the pseudoglandular stage (7–16 weeks in human pregnancy) (for reviews, see Vorbroker et al. 1995 and Warburton et al. 2000). Maturation of fetal lungs is an important issue because the lung is the organ that limits the survival after preterm birth.

In the present model, preterm birth of live-born pups was induced by intraperitoneal administration of LPS at 16 or 17 dpc. LPS resulted in rapid maternal inflammation characterized as increased levels of inflammatory

81
mediators in serum and lung. From the maternal compartments, the inflammatory signal was rapidly transmitted to the fetal side as the cytokine levels increased in amniotic fluid and fetal serum. In general the LPS-induced cytokine responses in maternal, fetal, and feto-maternal compartments were lower at 16 dpc compared with 17 dpc. Incidence of stillbirth decreased with the advanced gestation, therefore the magnitude of intrauterine proinflammatory responses alone may not explain the LPS-induced fetal deaths. In humans the mortality rate of extremely preterm fetuses is higher compared with fetuses with more advanced gestation (Rautava et al. 2007), which is consistent with the present data. As expected, fetal outcome was dependent on LPS dose as well, because the incidence of stillbirth increased with large doses. In addition, there were differences in LPS responsiveness between mouse strains suggesting the influence of genetic constitution.

6.1.1 Role of cytokines

Gestational tissues are sources of a large number of cytokines that contribute to the initiation of preterm labor or PPROM. Initiation of the inflammatory cytokine cascade in uterus or fetal membranes is thought to lead to uterine contractions and the rupture of membranes, respectively (Goldenberg et al. 2000, Romero et al. 2006). Several inflammation-associated genes, including IL-1, TNF-α, MCP-1, and IL-10 have been shown to be upregulated in amnion and choriodecidua at preterm labor. Several studies have also demonstrated the release of these cytokines by fetal membranes, decidua, and placenta in response to an inflammatory stimulus, such as LPS (reviewed in Keelan et al. 2003). The importance of TNF-α and IL-1 in the initiation of labor has been demonstrated experimentally, since both IL-1 and TNF-α can be used to induce preterm birth in animal models (Bry & Hallman 1993, Romero et al. 1991, Sadowsky et al. 2006). However, due to the redundancy of the cytokine network, blockade of a specific cytokine is not sufficient to prevent preterm delivery (Fidel et al. 1997, Hirsch et al. 2002, Hirsch et al. 2006, Wang et al. 2006, Yoshimura & Hirsch 2005). In addition to proinflammatory cytokines, anti-inflammatory cytokines such as IL-10 and IL-4 are produced by gestational tissues as well (Keelan et al. 2003). The role of these cytokines may be in to control the actions of proinflammatory cytokines. In addition, increased concentrations of pro- and anti-inflammatory cytokines have been detected in the amniotic fluid of women in preterm labor and in experimental animal models (e.g. Gotsch et al. 2008, Gravett et al. 1994, Greig

In our study maternal LPS challenge increased the levels of both pro- and anti-inflammatory cytokines in the uterus, whereas in placenta and fetal membranes the changes were restricted to proinflammatory cytokines. The concentrations of the cytokines increased in maternal serum and in amniotic fluid, which is consistent with the previous data.

### 6.1.2 Role of TLR4 and TLR2

The inflammatory cascade leading to parturition can be assumed to be initiated via the activation of different TLRs at the maternal-fetal interface. TLR4 has an important role in the firstline recognition of LPS, and genetic variation in the TLR4 gene has been reported to be associated with increased risk of preterm birth in infants (Lorenz et al. 2002). The expression of TLR4 has been reported in the placenta, uterus, and cervix (Herath et al. 2006, Holmlund et al. 2002, Kumazaki et al. 2004, Watari et al. 2000), and there is even some evidence about the increased expression in human preterm placentas with chorioamnionitis and in mouse uterus in response to intrauterine inflammation (Ellovitz et al. 2003, Kumazaki et al. 2004). In mice, functional TLR4 has been shown to be essential for E.coli-induced preterm labor (Wang & Hirsch 2003).

In the present study only minor changes in TLR4 expression in gestational tissues were detected after maternal LPS challenge, despite a marked induction of the cytokine response. In contrast the level of TLR2 increased in several tissues. The observed upregulation of TLR2 by LPS has been described before (Ojaniemi et al. 2006). The hypothesis is that TLR2 induction occurs through TLR4 mediated activation of NF-κB. TLR2 mRNA can be upregulated by TNF-α and IL-1 (Liu et al. 2000) and this induction occurs probably through the binding of NF-κB to the TLR2 promoter, which contains binding sites for NF-κB (Wang et al. 2001). In addition, Ojaniemi et al. (2006) detected an in vivo activation of NF-κB in mouse Kupffer cells and hepatocytes following LPS treatment, and TLR2 protein upregulation in the same cells. Furthermore, TLR2 expression was not induced in the tissues of TLR4−/− mice challenged with LPS, suggesting that the TLR4 gene and signaling are necessary for the LPS-induced TLR2 upregulation. Concomitant activation of the TLR2 signaling pathway may be important for the efficient elimination of LPS or for the preparation of the immune system for a more extensive infection.
6.1.3 Role of SP-A and SP-D

In the present study the roles of either SP-A or SP-D in the modulation of the LPS-induced inflammatory responses related to preterm birth were evaluated for the first time by using mice overexpressing rSP-A or rSP-D. In addition, the effect of rSP-A overexpression on the inflammatory response has not been previously defined.

SP-A and SP-D in nonpulmonary tissues

Surfactant proteins A and D are present in the female reproductive tract and in amniotic fluid. However, the origin of SP-A and SP-D in intrauterine tissues has been under the question because in many tissues the presence of protein but not mRNA has been reported. For example, Garcia-Verdugo et al. (2007) were not able to detect SP-A mRNA in myometrium, suggesting that the presence of SP-A protein in this tissue may be due to a paracrine process. In addition, amniotic fluid has been demonstrated to be the source of at least part of the SP-A protein found in fetal membranes (Lee et al. 2010). Here SP-A and SP-D mRNAs were detected for the first time in the uterus and in fetal membranes, respectively. In addition, the expression of SP-D was evident in the placenta and uterus as previously reported (Akiyama et al. 2002, Herias et al. 2007, Madsen et al. 2000). Considering the well established roles of SP-A and SP-D in innate immunity, it can be assumed that these two proteins play a role in regulating the intrauterine inflammatory responses related to parturition.

SP-A and SP-D as modulators of intrauterine inflammatory responses related to parturition

In 2004 Condon et al. were able to induce preterm birth by injection of SP-A into mouse amniotic fluid. In the subsequent studies they demonstrated that SP-A elevates the expression of NF-κB and IL-1β by amniotic fluid macrophages and increases their migration to myometrium. According to this data SP-A secreted from fetal lungs in increasing amounts towards the term can be proposed to act as a hormone that initiates labor. However, the migration of macrophages to the myometrium towards term pregnancy was not confirmed in another study using a different mouse strain (Lee et al. 2010), and even a decline in the number of mouse myometrial macrophages prior to birth has been reported (Mackler et al.
In humans the macrophages of fetal origin have not been detected in the myometrium of women with labor at term (Kim et al. 2006, Leong et al. 2008) or in amniotic fluid (Lee et al. 2010). In addition, a decrease in the concentration of SP-A in the amniotic fluid of women at term in labor compared with those not in labor has been observed (Chaiworapongs a et al. 2008). Furthermore, SP-A−/− mice go into labor spontaneously at term, similarly as wild-type mice (Korfhagen et al. 1996). Taken together this data clearly demonstrates that the model reported in mice is inadequate and it is not applicable to humans as such. However, the contribution of SP-A to the initiation of labor has been supported differently. Fetal membranes and myometrial cells contain binding sites for SP-A. In fetal membranes SP-A has been proposed to mediate the anti-inflammatory responses during pregnancy (Lee et al. 2010), whereas in the uterus SP-A may contribute to the regulation of uterine contractions (Breuiller-Fouche et al. 2010, Garcia-Verdugo et al. 2007, Garcia-Verdugo et al. 2008). Based on the structural and functional differences between SP-A and SP-D, it can be proposed that SP-D plays a role in the initiation of labor as well. Indeed, there is evidence that SP-D has a role in regulating the intrauterine inflammatory responses (Oberley et al. 2004, Oberley et al. 2007a).

Our findings do not support the role of SP-A as a preterm labor initiating factor. Firstly, the expression of SP-A in the lungs of wild-type fetuses was downregulated prior to preterm labor induced by maternal LPS challenge. Secondly, susceptibility to preterm birth was not increased in mice overexpressing rSP-A. Instead, our results raise the possibility that highly elevated levels of SP-A and SP-D increase fetal mortality in LPS-induced preterm birth, because a higher proportion of overexpressing dams gave birth to dead fetuses with the dose that was used to induce preterm birth of live-born pups in wild-type mice. Based on the well established role of SP-A and SP-D as anti-inflammatory factors, this result was not expected. However, in a study by Vuk-Pavlovic et al. (2006) the overexpression of rSP-D enhanced Pneumocystis infection in CD4-depleted mice. They speculated that augmented levels of SP-D facilitate the establishment of the infection that may partly be due to aggregation of Pneumocystis into structures that are too large for efficient macrophage uptake. Additional studies by Gardai et al. (2003) have provided further evidence that SP-A and SP-D can act as both pro- and anti-inflammatory mediators depending on the binding orientation and the cell surface receptors.
Modulation of the LPS-induced inflammatory response by SP-A and SP-D

Modulation of the LPS-elicited inflammatory response by SP-A and SP-D has been extensively studied, especially for SP-A. The results have been somewhat confusing because both upregulation and downregulation of the inflammatory response has been reported (reviewed in Kuroki et al. 2007 and Wright 2005). Differences in the stimulatory effects of SP-A may be explained by various factors, including the state of activation of the cell, type of cell, type of pathogen, the period of exposure, type of the cellular receptor, and the binding orientation of the collectin (Gardai et al. 2003). In addition, the origin of collectins (Doyle et al. 1998) and purification methods (van Iwaarden et al. 1995) may also have an effect on the outcome. Contamination of the SP-A preparations with e.g. LPS and transforming growth factor β has also influenced the outcome, especially in the primary studies (Kunzmann et al. 2006).


In the present study, smooth LPS was used and both SP-A and SP-D were demonstrated to modulate the LPS-induced inflammatory response in vivo. The overexpression of either rSP-A or rSP-D in fetal lung and in gestational tissues led to a diverse LPS-induced inflammatory response in maternal and fetal compartments. The concentrations of TNF-α and IL-10 in the amniotic fluid of overexpressing fetuses after maternal LPS challenge were clearly higher compared with wild-type fetuses, whereas the levels of the same cytokines in maternal serum were lower in overexpressing animals. The pattern of the inflammatory response in the tissues of overexpressing mice was different from that of wild-type mice. Particularly the levels of IL-4 in the fetal membranes of
overexpressing animals were clearly higher. In contrast, the expression of IL-4 and IL-10 in the uteri and IL-10 in fetal membranes was lower in overexpressing mice. In addition, there were some differences between rSP-A and rSP-D mice. For example, the expression of IL-10 in uterus and TNF-α, IL-4, and IL-10 in fetal membranes after LPS was more prominent in the tissues of rSP-A mice. However, there was no detectable difference in the pregnancy outcomes between the overexpressing mouse lines.

The differences in the cytokine responses between overexpressing and wild-type mice demonstrated here may be due to the ability of SP-A or SP-D to modulate the expression or function of TLRs. In human macrophages SP-A has been shown to regulate TLR2 but not TLR4 expression. However, in the same study TNF-α secretion induced by TLR4 or TLR2 ligands was diminished by SP-A indicating that SP-A can alter the biological activity of both TLRs (Henning et al. 2008). In addition, the phosphorylation of several proteins in the TLR4 and TLR2 signaling pathways is affected by SP-A leading to altered activation of NF-κB (Gardai et al. 2003, Henning et al. 2008). We demonstrated that the overexpression of either rSP-A or rSP-D leads to a decreased basal expression level of both TLR4 and TLR2 in placenta and TLR4 in fetal membranes compared with corresponding wild-type tissues. After the maternal LPS challenge, the expressions of TLR2 and TLR4 in gestational tissues of overexpressing animals tended to be lower.

Taken together, our data indicate that overexpression of either rSP-A or rSP-D modulates the innate immunity responses related to preterm birth. These findings support the postulated roles of SP-A and SP-D in the controlling the parturition process, but the exact roles remain to be studied.

6.1.4 Role of SP-C

For a long time SP-C was thought to be a completely lung-specific protein and have roles merely in surfactant metabolism. The tissue distribution of SP-C has been studied by means of overexpressing mice in which the overexpression of SP-C is driven by mSP-C or hSP-C promoters (Glasser et al. 1990, Glasser et al. 2005). However, the selection of the tissues in the expression studies was rather narrow and did not include the tissues (eye, salivary glands, skin, kidney and placenta), from which either SP-C mRNA or protein has been detected recently (Brauer et al. 2007a, Brauer et al. 2009, Eikmans et al. 2005, Mo et al. 2007, Sati et al. 2010). In the present study, the presence of SP-C mRNA and proprotein in
mouse placenta, fetal membranes, and uterus of pregnant females and SP-C mRNA in human placenta and amnion was demonstrated for the first time. Interestingly, SP-C expression was not detected in the uteri of nulliparous mice. The levels of SP-C in gestational tissues were notably lower compared with the levels in lung, and maternal LPS challenge did not have an effect on the expression of SP-C in mouse tissues. Overexpression of rSP-A and rSP-D in mouse gestational tissues driven by the hSP-C promoter further supports the nonpulmonary expression of SP-C.

In addition to participation in lowering the alveolar surface tension, SP-C has been suggested to modulate pulmonary innate immunity responses (Augusto et al. 2001, Augusto et al. 2002, Augusto et al. 2003, Glasser et al. 2001, Glasser et al. 2003, Glasser et al. 2008, Glasser et al. 2009). Furthermore, mutations in the SP-C gene predispose patients to interstitial lung disease with recurrent infections (Beers & Mulugeta 2005, Brasch et al. 2004, Hamvas et al. 2004, Mulugeta & Beers 2006, Thomas et al. 2002). The functions of SP-C in nonpulmonary tissues are still unsolved and they can only be speculated. Since ATII cells are specialized to secrete mature SP-C (Mulugeta & Beers 2006, Weaver & Conkright 2001), it can be proposed that processing of SP-C proprotein in reproductive tissues may be different from lung and leads to secretion of an alternative form of SP-C. It was not possible to study the processing of proSP-C in these tissues because the available antibodies do not recognize the mature SP-C. Even so, it can be reasonably postulated that at least some of the functions of SP-C in gestational tissues are related to innate immunity responses during pregnancy. In addition, the association of the SP-C polymorphism with the length of PPROM was proposed as discussed in section 6.2.

**6.1.5 Role of the fetus**

There is increasing evidence that the fetus itself may generate the signals needed to initiate labor (Challis et al. 2005, Condon et al. 2004, Romero et al. 1998). The contribution of fetal lung has been proposed because fetal lung is the source of surfactant proteins that are secreted into amniotic fluid. Notably the role of SP-A as a labor producing factor has been demonstrated in mice (Condon et al. 2004), but the hypothesis is not supported by the other findings as discussed in section 6.1.3. According to our data, the expression levels of SP-A, SP-D, TLR4, and TLR2 in fetal lungs were significantly decreased after maternal LPS challenge, suggesting an acute inactivation of the fetal host defense. The present results do
not refute the theory of the involvement of fetal lungs in the timing of delivery, and the relevance of the downregulated fetal response remains to be studied. In addition to lung, only minor changes in the expression levels of inflammatory mediators were evident in other fetal tissues after maternal LPS challenge, with the exception of a marked increase in MIP-2 expression in fetal liver and downregulation of IL-1β in liver and intestine. This is consistent with the earlier findings by Rougioja et al. (2005), who demonstrated that maternal LPS challenge at 14 dpc does not induce inflammation in fetal tissues. It can be speculated that the absence/downregulation of the fetal inflammatory response may be an attempt to prevent preterm parturition or to protect the fetus during maternal inflammation.

Our model of systemic inflammation differs from the most common inflammatory pathway in human pregnancies, ascending inflammation via the cervix and decidual space (Romero et al. 2006). Intra-amniotic inflammation is considered to be the extension of the ascending inflammation that is characterized by activation of proinflammatory cytokines and accumulation of inflammatory cells in fetal lung (Jobe et al. 2000, Kallapur et al. 2005, Newnham et al. 2002). According to models of ascending inflammation in rabbits and sheep, the administration of LPS or IL-1α into the amniotic fluid results in acceleration of SP-A expression and improvement of lung function in fetuses born prematurely (Bry et al. 1997, Bry & Lappalainen 2001, Jobe et al. 2000, Newnham et al. 2002). Analogously in chorioamnionitis, acceleration of fetal lung maturity is evident as demonstrated by the decreased incidence of RDS after preterm birth (Andrews et al. 2006, Watterberg et al. 1996). Based on the earlier findings and data presented here, it can be proposed that the route of entry and the identity of the labor-inducing inflammatory signal may influence the fetal outcome.

6.1.6 Transmission of the inflammatory signal from maternal to fetal compartments

Although the influence of LPS on the initiation of labor has been convincingly described in many previous studies (reviewed in Elovitz & Mrinalini 2004) and in the present study, it is not clear if LPS can be transferred through the placenta. By mating the LPS-hyporesponsive females of the C3H/HeJ mouse strain with LPS-responsive males, Silver et al. (1994) demonstrated that LPS-induced fetal death is due to a maternal rather than fetal response to LPS. However, there is also evidence that 125I-labelled LPS passes through the mouse placenta and reaches the
fetus (Kohmura et al. 2000). We tried to evaluate the presence of LPS in amniotic fluid and fetal blood by the Limulus amebocyte lysate (LAL) assay and Western analysis, but only traces, if any of LPS were detected (unpublished observations) suggesting a cytokine/chemokine-mediated signal transmission. However, LPS can cause an inflammatory response in very low doses, therefore the LPS-mediated inflammatory response in fetus cannot be completely ruled out.

In human placenta perfusion models, the transfer of TNF-α or IL-1 to the fetal side does not occur (Aaltonen et al. 2005, Zaretsky et al. 2004). In contrast, Zaretsky et al. (2004) were able to detect the transfer of IL-6, whereas in the study by Aaltonen et al. (2005) the increase of the IL-6 concentration in the perfusate was demonstrated to be due to the endogenous release. It should be noted that placentas in these studies were obtained from uncomplicated term pregnancies. The permeability of preterm placenta may be different from the term placenta and it may even change during gestation and due to infection. Furthermore, currently there is no data available about the transfer of cytokines through the mouse placenta. In our model maternal and fetal responses differed from each other supporting the cytokine mediated signal transmission to the fetal compartments.

6.2 SP-C SNP Thr138Asn in spontaneous preterm birth

It has been observed previously that SP-C SNP Thr138Asn associates with very preterm birth in a population consisting of preterm births of different causes (Lahti et al. 2004). Here the association of the same SNP with preterm birth was extended by submitting a homogeneous Northern European population of mothers and singleton infants for a detailed study of spontaneous preterm labor and preterm birth. The SP-C alleles or genotypes did not associate with prematurity or the risk of PPROM. However, fetuses delivered after the short duration of PPROM (< 72 hours) had significant over-representation of the minor SP-C allele A, whereas the frequency of minor allele A was decreased in fetuses delivered after prolonged PPROM (≥ 72 hours). In contrast, maternal alleles did not associate with the duration of PPROM. Lack of a similar association with the maternal alleles suggests that the fetus or the extraembryonic fetal tissues (principally fetal placenta and chorio-amnion) may have a role in delaying the preterm labor process. This supports the proposed role of the fetus in influencing the susceptibility to preterm birth in humans.
Many of the SP-C mutations predisposing to pneumonitis and interstitial lung diseases with recurrent infections have been found in the BRICHOS domain (Beers & Mulugeta 2005). This is the same region where SNP Thr138Asn is located, suggesting that the fetal SP-C gene is involved in protection against infection or inflammatory response after PPROM. The prolongation of the duration of PPROM allows the development of surfactant secretion decreasing the risk of RDS. On the other hand, early rupture of fetal membranes and very long duration of oligohydramnion due to prolonged PPROM may predispose to various conditions, including pulmonary hypoplasia (Uotila & Sikkinen 2009).

6.3 Relevance of the mouse model to the study of human parturition

Although reproductive events are species-specific, animal models are essential research tools to study the pathways leading to preterm birth. The use of mice as model organisms has several advantages and disadvantages. Mice are relatively cheap and easy to handle, breed, and house. They have a short gestational period (19–20 days) and they reach sexual maturity rapidly, approximately at the age of five weeks. The mouse genome and immune system are well-characterized and similar patterns of inflammatory cytokines are evident between mice and humans during infection-related preterm birth. Moreover, the mouse genome can be modified through transgenic and gene knockout methodologies. In addition, the availability of a wide range of commercial antibodies, small interfering RNAs, cDNA libraries, and techniques such as microarray has enhanced the use of mice in parturition studies (reviewed in Hirsch & Wang 2005 and Kemp et al. 2010).

Perhaps the major disadvantage in using mice as a model for human parturition is the importance of progesterone in the parturition process. In mice the main source of progesterone throughout the pregnancy is the corpus luteum and progesterone withdrawal from the maternal circulation precedes labor. In contrast, placenta is the primary site of progesterone production in humans, and maternal serum progesterone levels remain constant or even continue to increase towards the end of gestation and throughout labor. However, it has been suggested that human parturition is characterized by a functional withdrawal of progesterone (for reviews, see Kemp et al. 2010 and Mitchell & Taggart 2009). Other disadvantages include significant variations in the inflammatory response to various inflammatory factors (e.g. LPS) among different strains of mice and
multiple pregnancy, which limit the possibilities to study the role of the fetus (Hirsch & Wang 2005, Kemp et al. 2010).

The localization of fetal membranes and placenta in the vicinity of the myometrium suggest that these tissues have the capacity to take part in the inflammatory responses or even be the source of labor-inducing signals between mother and fetus. The function of mouse and human placentas is remarkably similar, but the differences in the inner structure of the placenta may contribute to the distinct transfer of substances from the mother to the fetus between these two species (Georgiades et al. 2002). In addition, the differences in the anatomic constitution of human and mouse fetal membranes may also reflect their physiological functions. Mouse fetal membranes are primarily composed of the amnion and yolk sac, whereas in humans the yolk sac is a transient structure that disappears early in the pregnancy and the fetus is surrounded by the amnion and chorion (Cross 1998, Perry 1981).

6.3.1 Use of rSP-A and rSP-D overexpressing mice

In the present study mice overexpressing either rSP-A or rSP-D under the control of the hSP-C promoter were used (Elhalwagi et al. 1999, Fisher et al. 2000). In general, the transgene copy number and zygosity of transgenic mice can have an effect on the function of the gene. Multiple transgene copies can lead to extremely high expression, and sometimes result in transgene silencing. In the present study the copy number of rSP-A or rSP-D overexpressing mice was not evaluated. However, according to the initial characterization of rSP-D mice, the copy number of rSP-D varies between 2–3 per genome (Fisher et al. 2000). For our experiments the rSP-A or rSP-D overexpressing females were mated to overexpressing males of the same line. The pups were genotyped and only the pups expressing the rat transgene were used in the experiments. However, the zygosity of the pups was not determined. In some cases the transgene can display position effects, such as interference with the function of the endogenous gene leading to a phenotype that is not due to the intrinsic properties of the transgene itself. The use of insulator sequences has been shown to overcome these position effects. These regulatory elements are characterized by their capacity to establish genomic barriers that protect DNA sequences from the spreading of neighboring heterochromatin, and their potential to interfere with the activity from distally located enhancers (Giraldo et al. 2003, Montoliu 2002). The insulator sequences have not been used in the generation of mice overexpressing rSP-A or rSP-D.
However, the undesired side effects resulting from the site of introduction of the transgene may not be the case here, since two different overexpressing mouse lines were used and the results were comparable between the lines.

Rat SP-A and SP-D constructs have been used to restore the structural and functional defects of surfactant in SP-A\(^{-/}\) and SP-D\(^{-/}\) mice, respectively (Fisher et al. 2000, Ikegami et al. 2001). This indicates that the function of rat proteins in overexpressing mice is similar to that of endogenous proteins, at least in the lung. Furthermore, the degree of oligomerization can significantly affect the function of collectins (Kishore et al. 1996, Sanchez-Barbero et al. 2005, Sanchez-Barbero et al. 2007, Sorensen et al. 2009, White et al. 2008, Yamada et al. 2006, Zhang et al. 2001, Zhang et al. 2006). Multimerization of rSP-A or rSP-D can be assumed to be very similar to that of corresponding mouse proteins, because at the amino acid level there is a 91% identity between mouse and rat SP-A and 92% identity between mouse and rat SP-D. However, it cannot be completely ruled out that the functions of rat proteins are different in mouse. Notably, the placenta plays a critical role in immunological tolerance that protects the fetus from rejection (Kanellopoulos-Langevin et al. 2003). Therefore, it is possible that the abnormal expression of SP-A or SP-D may compromise the immunological tolerance of the fetus. However, in my opinion the use of rSP-A and rSP-D overexpressing mouse lines in the studies of inflammation induced preterm birth is relevant and provides additional knowledge about the roles of these proteins in the process of preterm parturition.

6.4 Strengths and limitations of the study

In the present study, the activation of innate immunity responses related to preterm birth was studied using mouse as a model organism. Usage of mice in parturition studies has several advantages and disadvantages as discussed in section 6.3. In our model preterm birth of live-born pups was induced by intraperitoneal injection of LPS. This mimics the maternal systemic inflammation that is not the most common pathway of inflammation-induced preterm labor in humans. The administration of live bacteria to the cervix would better resemble the ascending route of infection. However, the use of live bacteria was not possible due to lack of suitable facilities to infect mice with live pathogens. The LPS used in this study was purified with gel-filtration chromatography. Further purification using for example ion exchange chromatography would lead to a
higher degree of purity. However, to better reflect the naturally occurring inflammation, the lower level of purification was selected.

The LPS-induced inflammatory response in these studies was quantified by means of RPA and CBA. RPA is a highly sensitive and specific method that does not include amplification of the target mRNA. However, the number of the inflammatory mediators quantified in these assays was limited and additional inflammatory mediators might provide important information about the initiation of labor. Therefore, further studies using for example expression arrays would be needed. In addition, the small number of animals especially in the expression studies of overexpressing mice may limit the power of the findings. Our findings provide additional knowledge about the roles of SPs, cytokines, and TLRs in the LPS-induced preterm labor, but the exact mechanism remains unknown.

The association of SP-C SNP Thr138Asn with spontaneous preterm birth was examined in a homogenous northern European population. However, the subpopulation in the PPROM association studies was rather small. The presence of SP-C in gestational tissues was a novel finding. The expression of SP-C in these tissues was verified with three different methods (RPA, RT-PCR, and Northern analysis). Additionally, the presence of SP-C protein was detected with Western analysis and immunohistochemistry.
7 Summary and conclusions

Preterm birth continues to be the leading cause of mortality and morbidity in newborn infants. In many cases the primary cause of preterm birth remains unclear, but inflammatory processes in maternal and/or fetal tissues have been implicated as one major factor.

We have established a mouse model of the maternal systemic inflammation induced preterm birth that leads to delivery of mostly live-born pups. In this model the maternal inflammatory signal was rapidly transmitted to fetal compartments. However, the early maternal and fetal inflammatory responses differed remarkably from each other, as the maternal increases in cytokine and TLR expression after LPS challenge were much more robust than those in fetal assays. The unexpected downregulation of SPs and TLRs in fetal lung suggest an acute inactivation of the fetal host defense. The pregnancy outcomes were dependent on the LPS-dose and also on the mouse strain, suggesting the influence of genetic constitution.

Surfactant proteins A and D are important for efficient local host defense including neutralization and opsonization of pathogens and modulation of the LPS-induced inflammatory cell responses. Depending on various factors, SP-A and SP-D can exhibit both anti- and proinflammatory features. In the present model the overexpression of either rSP-A or rSP-D modulated the LPS-induced inflammatory response related to preterm birth, evidenced as differences in cytokine and TLR expression compared with wild-type mice. Regardless of the observed differences among overexpressing and wild-type mice, the precise role of either SP-A or SP-D in the process of labor remains to be studied.

Surfactant protein C was originally thought to be expressed only in the lung and its functions in pulmonary surfactant are well characterized. In addition, the immunomodulatory functions of SP-C have been demonstrated. Here the presence of SP-C was evidenced in placenta, fetal membranes, and in pregnant uterus. Maternal inflammation had no effect on the expression. However, it can be proposed that SP-C expressed in gestational tissues is involved in the intrauterine host defense during pregnancy. In addition, an association of fetal SP-C SNP Thr138Asn with the duration of PPROM was observed in humans, whereas the maternal SP-C genotype had no influence. This supports the proposed role of the fetal tissues in producing the signals needed to initiate labor.

In conclusion, the present study enhances our understanding about the molecular consequences of serious infections and consequent inflammatory
processes that threaten pregnancies and are risk factors for preterm birth and long-
term morbidity or death of infants. Unfortunately our mouse model fails to provide a
precise mechanism of inflammation-induced preterm birth. The LPS-induced cytokine
responses in many tissues were ambiguous, because some inflammatory mediators
were downregulated in some tissues and upregulated in other tissues. However,
estational tissues are not just a one block but rather separate tissues that may
influence different facets of preterm parturition syndrome.

By utilizing the established mouse model and exploiting the current data, new
treatments for the prevention and management of preterm births may be developed
in the future. In addition, better understanding of the genotypes and gene-
environment interactions predisposing to prematurity may contribute to
individualized treatment strategies.
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Experimental Mouse Model and Study of Human Tissues

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