Influence of Inflammation and of Stage of Lung Development on the Development of Neonatal Lung Injury

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ABSTRACT

Bronchopulmonary dysplasia (BPD) is a major cause of mortality and long-term morbidity in prematurely born infants. Pulmonary inflammation, and abnormal alveolar and vascular development of the lung are histological characteristics of BPD. Interleukin (IL)-1β is a central cytokine in inflammation. Increased concentration of IL-1β in amniotic fluid or postnatally in the lungs of newborn infants is associated with the development of BPD. Bitransgenic mice expressing human (h)IL-1β in the lung epithelium develop a BPD-like illness. The aims of this thesis were to study the development of hIL-1β-induced lung disease in this transgenic mouse model in order to find factors regulating the development of the disease and to analyze gastric fluid in order to identify premature infants who are at high risk of developing BPD.

Since preterm labor is often preceded by intrauterine infection, the majority of infants born at less than 30 weeks of gestation have been exposed to antenatal inflammation. To study the effect of maternal inflammation on fetal inflammatory responses, hIL-1β expression was induced in pregnant dams and their hIL-1β-expressing offspring were compared to those of control dams. In bitransgenic dams, the production of hIL-1β starts before the fetuses start producing hIL-1β. The results show that maternal hIL-1β production preceding fetal hIL-1β production causes silencing of inflammatory genes in the lungs of bitransgenic offspring and protects them against hIL-1β-induced lung injury.

The mammalian lung undergoes five distinct developmental stages, the embryonic, the pseudoglandular, the canalicular, the saccular, and the alveolar stage. Children developing BPD are typically born in the early saccular stage. Expression of hIL-1β was induced in fetal and newborn mice at different time points in order to study the sensitivity of the lung to hIL-1β-induced injury during the different developmental stages. The results show that hIL-1β production in the lungs during the mid-saccular stage, but not in the late canalicular-early saccular or late saccular-alveolar stages, is sufficient to cause a BPD-like illness with abnormal lung development, inflammation, and increased mortality.

Usually tracheal aspirates are used to detect inflammation in the newborn lung. Obtaining tracheal aspirates from premature infants requires intubation, an invasive procedure that may promote the development of BPD. Gastric aspirate samples can be retrieved from premature infants at the time of routine placement of a nasogastric tube shortly after birth. The results show that levels of inflammatory proteins in the gastric aspirates are strongly increased in fetuses exposed to clinical chorioamnionitis and are associated with the development of BPD. These results suggest that gastric aspirate can be used instead of more invasive methods to assess the exposure of premature infants to inflammation and to assess the impact of perinatal inflammation on neonatal outcome.

Keywords: premature birth, BPD, inflammation, IL-1β, lung development, gastric aspirate

POPULÄRVETENSKAPLIG SAMMANFATTNING

Denna avhandling handlar om en inflammatorisk lungsjukdom som heter bronkopulmonell dysplasi (BPD). Barn får diagnosen BPD om de har behov av extra syrgas då de är 28 dagar gamla. BPD graderas sedan som mild, medel, eller svår beroende på syrgasbehovet vid 36 veckor postmenstruell ålder, dvs den tidpunkt då mamman skulle ha varit gravid i vecka 36 om barnet ej fötts för tidigt. BPD drabbar ofta spädbarn som är mycket för tidigt födda. En normal graviditet varar i 40 veckor, men de barn som får BPD är oftast födda redan efter 23-26 veckors graviditet. Lungutveckling hos människa och däggdjur sker i fem olika stadier, det embryonala, det pseudoglandulära, det kanalikulära, det sakulära, och det alveolära stadiet. De barn som föds för tidigt och sedan utvecklar BPD är ofta födda i det sakulära lungvecklingsstadiet. Lungorna hos människor utvecklas förhållandevis ganska sent under graviditeten och bildningen av lungblåsor, alveoler, är komplett först vid 2-3 års ålder. Då barn föds för tidigt avbryts den normala lungutvecklingen. Lungorna är omogna och därmed får barnet svårt att syresätta sig. En för tidig förlossning föregås ofta av någon slags infektion, antingen i eller utanför livmodern, hos mamman. Interleukin (IL)-1β är ett inflammatoriskt protein. Förhöjda nivåer av IL-1β i fostervatten är associerat med för tidig födsel. Inflammation är ett av kännetecknen för BPD och IL-1β är ett protein som är av stor betydelse vid inflammation. En musmodell har utvecklats för att efterlikna BPD hos människor. Dessa möss som producerar IL-1β i sina lungor får en BPD-liknande sjukdom med onormal lungutveckling, inflammation, försämrad tillväxt, samt ökad dödlighet.

Musmodellen användes för att studera hur en pågående inflammation hos mamman påverkar en inflammation hos fostren. Detta undersöktes genom att inflammationen hos mamman startades redan vid början av graviditeten medan inflammationen hos ungarna startades först dag 15 (möss är dräktiga i 19 dagar) i graviditeten. Resultatet visar att en inflammation som startar hos mamman innan den startar hos ungarna skyddar de nyfödda ungarna mot inflammation framkallad av IL-1β. Ungarna utvecklar en form av tolerans mot IL-1β. I en situation då inflammationen startar hos både mamma och ungar samtidigt så finns toleransen inte och ungarna får en BPD-liknande sjukdom. Mammans inflammation måste alltså starta innan ungarnas för att den ska kunna verka skyddande.

För att undersöka vilket lungutvecklingsstadie som är mest känsligt mot inflammation framkallad av IL-1β startades produktionen av IL-1β under korta perioder i de olika

utvecklingsstadierna. Resultatet visar att en kortvarig produktion av IL-1β i det sakulära stadiet orsakar en liknande sjukdom som när IL-1β produceras under en längre tid under hela dräktigheten. Då IL-1β produceras i det kanalikulära-tidiga sakulära eller det sena sakulära-alveolära stadierna så utvecklar inte ungarna samma svåra sjukdom. Det visar att det sakulära stadiet är det som är mest känsligt för inflammation.

När fostret är i livmodern så sväljer det bland annat fostervatten och vätska utsöndrat från lungorna. Magsäcksvätska sugs upp då en sond till magen, genom vilken barnet får sin näring, sätts på plats. Denna vätska innehåller bland annat fostervatten och lungsekret. Vi utvärderade om det fanns något samband mellan nivåerna av inflammatoriska proteiner, bland annat IL-1β, i magsäcksvätska och infektion i fosterhinnorna, utveckling av BPD, behov av respirator, eller för tidig födsel. Resultaten visar ett starkt samband mellan inflammatoriska proteiner och infektion i fosterhinnorna. De barn som utvecklar medel eller svår BPD var födda tidigare, vägde mindre, och behövde oftare respirator för att kunna syresätta sig än de som inte har BPD eller har mild BPD. Resultaten visar även att de barn som senare utvecklar BPD redan vid födseln har förhöjda nivåer av inflammatoriska proteiner i magsäcksvätskan. Detta samband försvinner dock om man tar hänsyn till gestationsåldern.

Sammanfattningsvis visar denna avhandling att en inflammation hos mamman kan framkalla en form av tolerans mot inflammation i ungarnas lungor, att det sakulära lungutvecklingsstadiet är det stadie där lungan är mest känslig mot inflammations-inducerad skada, och att magsäcksvätska från för tidigt födda barn kan användas för att studera hur perinatal inflammation påverkar barnets prognos.

LIST OF PAPERS

The thesis is based on the following papers:

I. Maternal IL-1beta Production Prevents Lung Injury in a Mouse Model of Bronchopulmonary Dysplasia. Bäckström, E., Lappalainen, U., and Bry, K. Am J Respir Cell Mol Biol Vol 42, pp 149–160, 2010.

II. Developmental Stage is a Major Determinant of Lung Injury in a Murine Model of Bronchopulmonary Dysplasia. Bäckström, E., Hogmalm, A., Lappalainen, U., and Bry, K. Accepted, Pediatric Research, 2010.

III. Elevated Gastric Aspirate Levels of Inflammatory Cytokines at Birth Are Associated with the Development of Bronchopulmonary Dysplasia.
Stichel, H., Bäckström, E., Hafström, O., Nilsson, S., Lappalainen, U., and Bry, K. Submitted, Acta Pediatrica, 2010.

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ABBREVIATIONS

Areg – amphiregulin
BAL – bronchoalveolar lavage
BCA – bicinchoninic acid
BPD – bronchopulmonary dysplasia
CCSP – Clara cell secretory protein
CLD – chronic lung disease
CMV – cytomegalovirus
COPD – chronic obstructive pulmonary disease
CPAP – continuous positive airway pressure
CRP – C-reactive protein
E – embryonic day
EGF – epidermal growth factor
ELISA – enzyme-linked immunosorbent assay
ENA-78 – epithelial-derived neutrophil activating-78
FiO_2 – fraction inspired oxygen
CSF –colony stimulating factor
Gro-α – growth related oncogene- α
ICE – IL-1β-converting enzyme
IL – interleukin
IL-1R – IL-1 receptor
IL-1Ra – IL-1 receptor antagonist
i.p. – intraperitoneal
LPS – lipopolysaccaride
MCP – monocyte chemoattractant protein
MIP – macrophage inflammatory protein
MMP – matrix metalloprotease
NK – natural killer
PAS – periodic acid Schiff
PDA – patent ductus arteriosus
PRR – pattern recognition receptor
PN – postnatal day
RDS – respiratory distress syndrome
ROM – rupture of membranes
ROP – retinopathy of prematurity
rtTA – reverse tetracycline transactivator
SAA – serum amyloid A
TA – tracheal aspirate
tetO – tetracycline operator
TLR – toll-like receptor
TNF – tumor necrosis factor
TUNEL – TdT-mediated dUTP nick-end labeling

INTRODUCTION

Respiratory system

Lung structure

In humans, the left lung is divided into two lobes and the right lung into three lobes (1). In the murine lung, the left lung form a single lobe and the right lung is divided into four lobes (superior, middle, postcaval, and inferior) (2). The airways are divided into two zones, the conducting zone and the respiratory zone. The conducting zone extends from the top of the trachea, the bronchi, the bronchioles, and all the way to the terminal bronchioles. The respiratory zone consists of the respiratory bronchioles, the alveolar ducts, and the alveoli. The airway epithelium is composed of basal cells, cilated cells, and mucus-producing goblet cells. Mucus can trap invading microorganisms and the ciliated cells transport the foreign matter out of the trachea along with the mucus (1). The bronchiolar epithelium also contains nonciliated, nonmucus secretory Clara cells that secrete Clara cell secretory protein (CCSP) (3). The main functions of the conducting zone are to provide a low-resistance pathway for air flow, to defend the lung against microbes, toxic chemicals and other foreign matter, and to warm and moisten the inhaled air (1).

More than 95% of the alveolar surface area is covered with large, thin, squamous type I pneumocytes, which are responsible for gas exchange between the alveolus and the pulmonary capillaries (4). Chen and co-workers suggested an additional biological function for type I cells, as protectors of the alveolar epithelium against oxidative injury through up-regulation of apolipoprotein E and transferrin (5). The cuboidal type II pneumocytes covering about 5% of the surface area have multiple functions. They secrete surfactant that lowers the surface tension in the alveoli, they are important in the fluid balance across the alveolus, and they are also able to secrete various cytokines in the immunological defence of the alveolus. Type II cells can differentiate into type I cells when needed, as type I cells are unable to replicate themselves (6).

Lung development

Normal mammalian lung development, which occurs as a series of tightly regulated events, can be divided into five different stages, the embryonic, the pseudoglandular, the canalicular, the saccular, and the alveolar stage (Fig. 1) (7, 8).

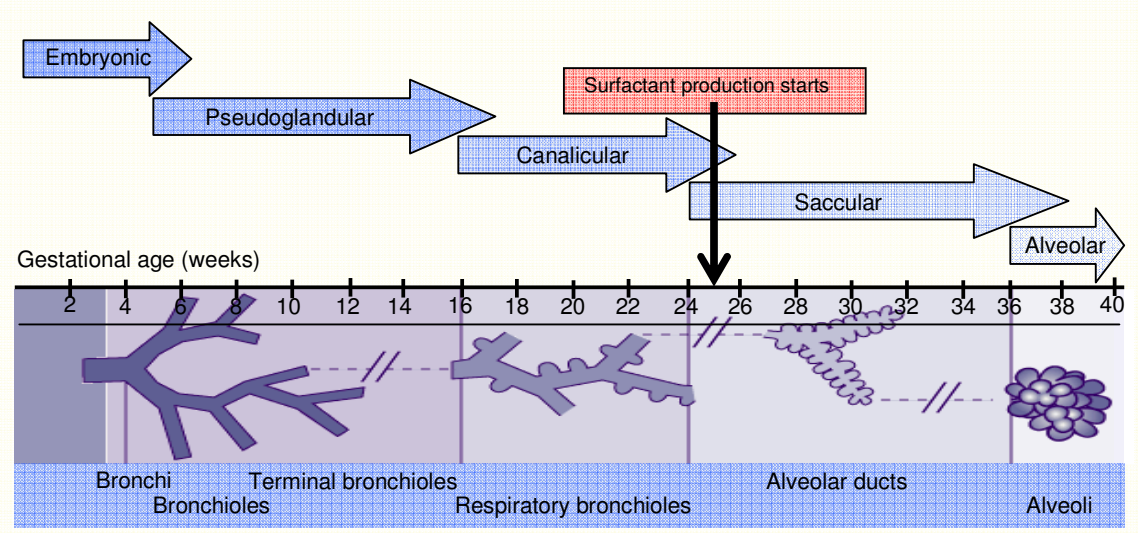

Figure 1. Developmental stages of the human lung.

Embryonic stage

Mammalian lung development starts as an outgrowth of the ventral wall of the primitive foregut endoderm (9). When the lung bud branches for the first time, it gives rise to the proximal structures of the tracheo-bronchial tree. By the fifth week the human lungs are divided into its five lobes. The expression of growth factors and other signal molecules must be well coordinated. At the edge of growing lung buds cell proliferation needs to be enhanced and at the branching points cell division needs to be inhibited (7, 9, 10).

Pseudoglandular stage

During the pseudoglandular stage, the branching of airways and blood vessels continues, and the development of conducting airways and primitive acinus structures is completed by the end of this stage (Fig. 1). Differentiation of respiratory epithelial cells occurs at this stage, and basal cells, ciliated cells, and goblet cells can be identified in the main bronchi. (8, 9).

Canalicular stage

The acinar structures, which comprise the respiratory bronchioles, alveolar ducts, and primitive alveoli, are formed during the canalicular stage and the blood-air interface begins to develop (Fig. 1). Epithelial cells differentiate to cuboidal type II and subsequently type I cells, which contribute to the formation of the future blood-air barrier (9).

Saccular stage

The saccular stage is critical for extrauterine survival. During this stage the airways distal to the terminal bronchioles are elongated and widened and the gas-exchanging surface area increases as the airway walls thin due to the continuing maturation of type II cells into thin type I cells (Fig. 1) (11). Alveolarization is prepared by deposition of elastic fibers where secondary septa will form to create alveoli (9).

Alveolar stage

During the alveolar stage the secondary alveolar septa consisting of connective tissue and a double capillary layer are formed. The immature double capillary layer becomes a capillary monolayer through apoptosis. The saccules subdivide into smaller units, the alveoli (Fig. 1). At birth the human lung contains >50 million alveoli. Since the adult human lung contains >300 million alveoli, alveolarization occurs postnally as well as prenatally (11, 12). The process is possibly completed by 2-3 years of age in humans (9).

While the human infant is usually born after the initiation of alveolar development, the mouse is born in the saccular stage. The alveolar stage in the mouse starts around postnatal day (PN)4, thus the alveolarization in the mouse is only a postnatal event (Table 1) (13).

Table 1. Developmental stages of the lung in human and mouse.

Stage	**Human (weeks) Term 40 weeks**	**Mouse (days) Term 19 days**
Embryonic	3-7	E9-11.5
Pseudoglandular	5-17	E11.5-16.5
Canalicular	16-26	E16-18
Saccular	24-38	E17.5-PN5
Alveolar	36-2 years	PN4-28

E – embryonic day; PN – postnatal day

Surfactant

Surfactant is a complex mixture of about 90% phospholipids and 10% proteins (6). The major lipid constituent is saturated phosphatidylcholine which accounts for about 50% of surfactant content (10). There are four different surfactant proteins (SP), SP-A, SP-B, SP-C, and SP-D. The alveolar type II cell is the only type of pulmonary cell that produces all the surfactant components. In addition to type II cells, Clara cells may also synthesize and

release SP-A, SP-B, and SP-D, while SP-C is thought to be released by alveolar type II cells only (6). The surfactant is synthesized and stored in the lamellar bodies in type II cells, and secreted by exocytosis to the alveolus (6). The production of surfactant starts around gestational week 24-25 during the canalicular stage in the human lung (Fig. 1) (10). Its primary function is to reduce surface tension and thus prevent the alveoli from collapsing during expiration. Respiratory distress syndrome (RDS, also called hyaline membrane disease), is a disease in premature infants caused by developmental insufficiency of surfactant production and structural immaturity of the lungs (14).

Immune system

The immune system is divided into the innate immune system and the adaptive immune system. The innate responses are those the body mounts without requiring previous contact with the microbe. This first line of defense is characterized by its lack of specificity. It recognizes e.g. lipopolysaccharide (LPS) and glycoproteins from microbes with pattern recognition receptors (PRRs). The adaptive responses, including B and T lymphocytes and the production of antibodies, are highly specific (15).

Inflammation

Inflammation is a complex biological response the body mounts when it comes into contact with harmful stimuli such as pathogens, damaged cells, toxins, or other irritants. It is the organisms own protective attempt to remove the injurious stimuli and initiate the healing process. Inflammation can be either acute or chronic. The initial response to harmful stimuli is achieved by the increased movement of protein, water, and leukocytes from the blood to the injury site, resulting in the cardinal signs of inflammation: swelling, redness, pain, heat, and loss of function. Chronic inflammation is characterized by a continuous infiltration of mononuclear inflammatory cells, tissue destruction, and attempts at healing the tissue by e.g. angiogenesis and fibrosis (15). Pre-exposure to a low dose of endotoxin may markedly reduce the inflammatory response to subsequent endotoxin exposure, indicating an immunosuppressed state called tolerance (16).

Inflammatory cells

The major cells of the immune system are various types of leukocytes. Neutrophils, eosinophils, and basophils belong to a group called polymorphonuclear granulocytes, having multilobed nuclei and abundant membrane-surrounded granules. The two other

classes of leukocytes are monocytes and lymphocytes. All the different classes differentiate from the myeloid precursor cell upon different stimuli (15).

Neutrophils

Neutrophils, the most abundant kind of leukocytes, are the first responders to infection. Production of neutrophils is stimulated by cytokines such as granulocyte colony stimulating factor (G-CSF), produced by many cell types in response to infection. G-CSF acts on neutrophil precursors that proliferate and mature into neutrophils. The cell surface receptors of neutrophils make it possible for them to detect chemical gradients of chemoattractants, e.g. complement component C5a, CXCL8 (interleukin (IL)-8), CCL3 (macrophage inflammatory protein (MIP)-1α), and CCL4 (MIP-1β), and migrate towards the site of inflammation. Neutrophils remove harmful stimuli by phagocytosis (17).

Macrophages

Production of monocytes is stimulated by cytokines such as monocyte colony stimulating factor (M-CSF). Monocytes differentiate into tissue macrophages when they leave the bloodstream. Macrophages can phagocytose harmful agents such as microorganisms. Alveolar macrophages contribute to keeping the lung free from injurious stimuli (15).

Cytokines

Cytokines are extracellular messenger proteins that mediate and regulate the immune response. There are both pro- and anti-inflammatory cytokines. An imbalance between pro- and anti-inflammatory cytokines is considered to be an important factor in the development of lung injury (18, 19). IL-8, tumor necrosis factor (TNF)-α, IL-1, and IL-6 belong to the group of pro-inflammatory cytokines, and they are important mediators in the initiation and persistence of the inflammatory response. Upon exposure to hypoxia, hyperoxia, microorganisms, endotoxin, or other bacterial cell wall components, pro-inflammatory cytokines are synthesized by alveolar macrophages, airway epithelial cells, fibroblasts, and type II pneumocytes in order to promote inflammation. IL-4, IL-10, IL-12, IL-13, and IL-1 receptor antagonist (IL-1Ra) belong to the group of anti-inflammatory cytokines (20).

Interleukin-1

There are three members of the IL-1 family: IL-1α, IL-1β, and IL-1Ra. IL-1 play a central role in the regulation of immune and inflammatory responses. IL-1α and IL-1β are structurally related, with similar three dimensional structures of the β sheets. They act

through the same cell-surface receptors and share biological activities, but are the product of separate genes (21). There are two different antagonists to IL-1α and IL-1β, IL-1Ra and IL-1 receptor type II (IL-1RII). IL-1Ra acts as a specific inhibitor, competing with IL-1α and IL-1β to bind to its cell surface receptor (IL-1RI) without triggering any signal. The second antagonist, IL-1RII, acts as a decoy receptor. IL-1α and IL-1β bind to the receptor but no signal is transduced (21).

There are two different IL-1 receptors, IL-1RI and IL-1RII. As mentioned before, no signal is transduced upon binding to IL-1RII. IL-1RI is expressed on e.g. endothelial cells, epithelial cells, and T-lymphocytes (21). It mediates a very complex pathway, involving multiple kinases and transcription factors. Upon binding to IL-1RI several different complexes are formed, leading to the liberation of NFκB, which can then activate the transcription of various genes in the nucleus (22).

Interleukin-1β

IL-1β is a pro-inflammatory cytokine involved in the initiation and persistence of inflammation. IL-1β is synthesized as a 31 kDa precursor, proIL-1β, which essentially lacks biological activity (21). ProIL-1β is produced by monocytes, macrophages, neutrophils, epithelial cells, endothelial cells, and fibroblasts. IL-1β-converting enzyme (ICE or caspase-1) or proteases such as matrix metalloprotease (MMP)-9 (gelatinase B) and granzyme A must cleave proIL-1β to obtain the mature 17 kDa form of IL-1β with biological activity. ICE, an intracellular protease, is a member of the cysteine protease family (21, 23).

Chemokines

Chemokines are a cytokine family of small (8-14 kDa) structurally related molecules with chemotactic properties. Members of the chemokine family are divided into four groups depending on the spacing of their first two cysteine residues in the amino-terminal region, CXC (α), CC (β), C (γ) and CX_3C (δ). CC and CXC are the two major groups with several different members (24-26). Chemokines are produced as pro-peptides and are cleaved during secretion to produce an active mature protein that functions by activating chemokine receptors. The receptors are all members of the 7-transmembrane spanning (7-TMS), G-protein-coupled receptor family (27).

CXC chemokines

The two N-terminal cysteines of CXC chemokines are separated by one amino acid, represented in the name by "X". CXC chemokines can be further divided into ELR or non-ELR chemokines depending on whether a three amino acid motif, ELR (glutamate-leucine-arginine), is present between the N-terminus and the first cysteine (27). Members expressing this motif (ELR+) e.g. CXCL1 (growth related oncogene-α (Gro-α, KC)) and CXCL5 (epithelial-derived neutrophil activating-78 (ENA-78)), are generally chemotactic for neutrophils but also have angiogenic properties (24). ELR– chemokines attract monocytes and lymphocytes, but normally have poor chemotactic ability for neutrophils (25).

CC chemokines

The CC chemokines have two adjacent cysteines at their amino terminus. They are active on a broad spectrum of cells, e.g. monocytes, lymphocytes, natural killer (NK) cells, and dendritic cells (28). Monocyte chemoattractant protein (MCP)-1 (or CCL2) is a chemoattractant for monocytes and lymphocytes, inducing them to leave the bloodstream and enter the surrounding tissue through upregulation of the expression of integrins required for chemotaxis. MCP-3 (CCL7) is one of the most pluripotent chemokines, acting on many different cell types, e.g. monocytes, lymphocytes, eosinophils, NK cells, and dendritic cells (27).

Antenatal inflammation

Intrauterine infection – chorioamnionitis

Intrauterine infection may take place at various sites. Infection in the fetal membranes is called chorioamnionitis, infection in the umbilical cord is called funisitis, and infection in the amniotic fluid is called amnionitis (29). Most deliveries before 30 weeks are associated with histological chorioamnionitis (29). The most sensitive method of detecting chorioamnionitis is histological examination; clinical signs of chorioamnionitis are only found in a minority of cases (30, 31). Human fetuses exposed to chorioamnionitis may have infiltration of inflammatory cells and increased expression of the pro-inflammatory cytokine CXCL8 in the lung tissue (32). Intrauterine infections may occur quite early in pregnancy and remain undetected for months. For example, amniotic fluid concentration of IL-6 may be elevated already at 15-20 weeks of gestation in pregnancies ending in preterm delivery at 23-30 weeks (33, 34). A fetal inflammatory response is induced by intrauterine infection, leading to elevated levels of pro-inflammatory cytokines, which in turn are associated with premature birth (29, 35). Lahra *et al.* report an inverse

relationship between gestational age and the incidence of histological chorioamnionitis (30).

Experimental studies indicate that fetal exposure to inflammation accelerates the maturation of the fetal lung, thereby protecting the newborn against RDS. Intra-amniotic administration of IL-1 or endotoxin induces surfactant production and improves gas exchange and lung mechanisms in preterm animal models (36-38). Histological chorioamnionitis is also associated with less RDS in preterm neonates (39-42).

In addition to intrauterine infections, extrauterine infections or inflammatory illnesses at other sites, such as periodontitis (43), pneumonia (44), and inflammatory bowel disease (45), are also associated with preterm delivery. A systemic inflammation may be initiated by these conditions (46, 47), leading to preterm labor (48) .

Bronchopulmonary dysplasia

Definition of BPD

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that occurs primarily in preterm infants weighing less than 1000g at birth, born at 23-26 weeks of gestation. BPD is defined as a need of supplemental oxygen for at least 28 days of age, and classified as mild, moderate, or severe depending on the need of supplemental oxygen at 36 weeks postmenstrual age (49). The incidence of BPD is 22% in infants with birth weights of less than 1500g (50). The incidence increases with decreased birth weight, so that 46% of infants with birth weight between 501-750g develop BPD (50). The affected infants are at high risk for mortality and morbidity during the first years of life and many of them have respiratory problems throughout childhood and young adulthood (50-52). BPD was first described by Northway *et al.* in 1967 as a lung injury in preterm infants resulting from oxygen treatment and prolonged mechanical ventilation (53). However, advances in neonatal medicine such as the use of antenatal glucocorticoids, gentler ventilation techniques, and early surfactant treatment have changed the pattern of injury in BPD. The pathology of the new BPD includes extreme lung immaturity with larger simplified alveolar structures, inflammation, and abnormal capillary development (8, 49).

Risk factors for BPD

Gestational age

Advances in neonatal care have led to the survival of smaller and more immature infants. A low gestational age is one of the primarily risk factors for developing BPD. BPD occurs primarily in the very premature infants born in the early saccular stage of lung development. Lung development is rapid between the canalicular and the following saccular stage (54), therefore with every week the fetus stays in the uterus, the chance of survival and a healthy life is increased.

Mechanical ventilation

Mechanical ventilation is harmful to the preterm lung, but it is sometimes necessary for the survival of the very premature infants. Overinflation of the lung, results in leukocyte migration into the lungs, increased permeability, and interstitial and alveolar edema (55-57). Mechanical ventilation can also interfere with alveolar and vascular development in animal models (58, 59). Autopsy studies show that infants who die after >10 days of mechanical ventilation have simple terminal airspaces, a reduced number of alveoli, and therefore a decreased internal lung surface area (60). Different styles of mechanical ventilation can be more or less injurious, but just intubating the infant may increase the risk of developing BPD (57). The duration of ventilation is also an important factor. To avoid ventilator-induced lung injury and thus decrease the risk of BPD, many centers use nasal continuous positive airway pressure (CPAP) (61, 62).

Hyperoxia

Exposure to high levels of oxygen can cause severe inflammation. Hyperoxia is a strong inducer of the production and release of inflammatory mediators in animal models (63). Exposure to hyperoxia in animal models of premature baboons (58), neonatal mice (64, 65) and rats (66, 67) results in a progressive lung disease that resembles BPD.

Inflammation

Preterm infants suffering from BPD have higher and persisting numbers of inflammatory cells (neutrophils and macrophages) and higher levels of pro-inflammatory cytokines, including IL-1β and neutrophil chemokines such as IL-8, in their bronchoalveolar lavage (BAL) fluid than control infants (20, 68-71). Baier and coworkers have shown that the concentrations of CCL2 (MCP-1), CCL4 (MIP-1β), CCL7 (MCP-2), and CCL8 (MCP-3) were increased in tracheal aspirates (TA) from infants who developed BPD (28). Increased levels of CXCL-8 (IL-8), CCL2 (MCP-1), and CCL3 (MIP-1α) in TA during acute lung

injury in ventilated very low birth weight infants have been shown to correlate with adverse outcome (72, 73).

Assessing inflammation by TA and gastric fluid

TA is traditionally used to assess inflammation in newborn infants. For TA to be obtained from premature infants, they need to be intubated. With the increased use of nasal CPAP, intubation is often no longer needed. Gastric fluid samples can be obtained by a non-invasive procedure when the nasogastric catheter is routinely placed for enteral feeding of very premature infants and some fluid is retrieved to ensure correct placement of the tube in the stomach. Since the fetus swallows amniotic fluid and lung fluid secreted from the lungs during fetal life, gastric fluid at birth contains components of amniotic fluid and lung secretions, in addition to possible secretions from the stomach or the esophagus (74). Since gastric aspirates contain lung secretions, gastric aspirates can be used to predict the possibility of premature infants' developing RDS via a mircobubble stability test (75).

Patent ductus arteriosus

Healthy infants born at 30 or more gestation weeks will normally have closed their ductus by the fourth day after birth. On the other hand, infants born at less than 30 weeks of gestation have a 65% incidence of patent ductus arteriosus (PDA) (76). If the ductus does not close, it can be medically closed or surgically ligated.

Retinopathy of prematurity

Extremely premature infants are at a high risk of developing retinopathy of prematurity (ROP). Retinal vascularization is completed at around 34 weeks of gestation. The more premature the infant is, the less the retina is vascularized at birth and the incidence of ROP increases (77). Loss of vision occurs in the most severe form of ROP (78).

Animal models of BPD

The animal models most closely comparable to the development of BPD in humans are the lamb or baboon, which can be ventilated for weeks to months (54). Albertine and coworkers (59) have developed a lamb model in which prematurely born lambs are mechanically ventilated for three to four weeks. In contrast to humans, lambs are born with fully alveolarized lungs. Therefore the lambs in this model are delivered at about

84% of gestation, at which time their degree of lung maturity corresponds to that of the human infant at 24-26 weeks of gestation. The ventilated animals show an altered inflation pattern, impaired alveolar formation with large alveoli, increased muscularization of bronchioles, and inflammation in their lungs. These are changes similar to those seen in the lungs of preterm infants with BPD (59). Coalson and coworkers have developed a 125-day gestation baboon model (term is 185 days) with lung development between the canalicular and saccular stages to study BPD (58). These baboons require early exogenous surfactant administration for survival. They develop clinical, radiographic, and histological features consistent with BPD.

Mouse model of BPD

In the present work (Papers I-II), a transgenic mouse model in which mature human (h)IL-1β is expressed in the lung epithelium in an externally regulated manner is used (79). Production of hIL-1β is induced in the lungs of these mice at selected time points by administration of doxycycline. Doxycycline is a tetracycline antibiotic commonly used to treat a variety of infections. Doxycycline can be administered in drinking water, laboratory chow, or as intraperitoneal (i.p.) injections to mice. In our mouse model, the rat (r)CCSP promoter drives the expression of the reverse tetracycline transactivator (rtTA) protein (80), and the tetracycline operator-cytomegalovirus minimal promoter ($(tetO)_7$CMV) drives the expression of hIL-1β (79). The rtTA protein binds to the $(tetO)_7$ sequence in order to induce hIL-1β expression when doxycycline is present (81). The rCCSP promoter is active in type II pneumocytes as well as in epithelial cells within the trachea, bronchi, and larger bronchioles (80, 82, 83).

Using this model the effects of hIL-1β on the adult murine lung have been studied. Doxycycline was administered to the mice from 3 weeks of age until sacrifice at the age of 8 weeks. Production of hIL-1β was sufficient to induce a phenotype that recapitulates many of the features of chronic obstructive pulmonary disease (COPD) (79); it caused pulmonary inflammation characterized by neutrophil and macrophage infiltration, distal airspace enlargement (consistent with emphysema), disruption of elastin fibers in alveolar septa and fibrosis in airway walls, thickening of conducting airways, enhanced mucin production as well as lymphocytic aggregates in the airways (79).

To study the antenatal and postnatal effects of hIL-1β on lung development, doxycycline was administered from the beginning of gestation until sacrifice of the mice at PN7 (84). Antenatal and postnatal production of hIL-1β resulted in impaired postnatal growth,

disrupted alveolar septation, abnormal capillary development, lung inflammation, and increased mortality. These symptoms both clinically and histologically resemble BPD (84).

AIMS OF THE THESIS

The aims of this thesis were:

- To study the influence of maternal inflammation on the development of hIL-1β-induced lung injury in the newborn mouse.

- To study how production of hIL-1β in the fetal mouse lung at different developmental stages affects lung morphogenesis, inflammation, survival, and postnatal growth of infant mice.

- To study whether gastric aspirate samples can be used to assess the exposure of newborn premature infants to inflammation and whether the levels of inflammatory proteins in gastric aspirates are associated with the development of BPD.

MATERIALS AND METHODS

Conditional expression of hIL-1β – Paper I-II

To generate bitransgenic rCCSP-rtTA-$(tetO)_7$CMV-hIL-1β mice expressing mature hIL-1β in the lung epithelium, we mated homozygous rCCSPrtTA$^{+/+}$ activator transgenic dams (80) that express the rtTA under the control of the rCCSP promoter with $(tetO)_7$CMV-hIL-1β$^{+/-}$ males in which tetracycline-responsive promoter $(tetO)_7$CMV drives the expression of hIL-1β (79) (Fig. 2). Pulmonary epithelial expression of rtTA alone causes emphysema in mice (85). Therefore all control groups consists of rCCSP-rtTA$^{+/-}$ transgenic mice. The mice are in FVB/N background.

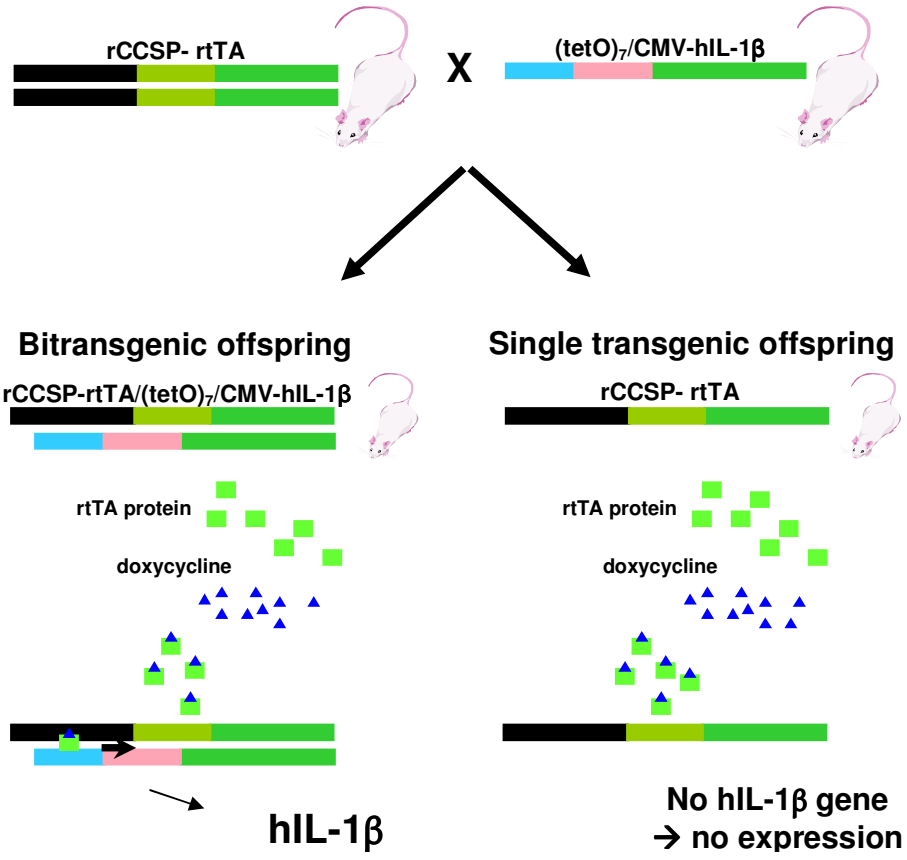

Figure 2. Bitransgenic offspring was produced by mating a homozygous rCCSP-rtTA$^{+/+}$ dam with a heterozygous $(tetO)_7$CMV-hIL-1β$^{+/-}$ male. In the bitransgenic offspring, rtTA binds to the $(tetO)_7$CMV promoter in the presence of doxycycline, and expression of hIL-1β is induced.

Paper I

In this study bitransgenic offspring were also generated by mating bitransgenic rCCSP-rtTA-$(tetO)_7$CMV-hIL-1β dams with wild-type males. One fourth of the offspring become bitransgenic and one fourth become single transgenic rCCSP-rtTA$^{+/-}$ mice (used as controls). The offspring with no transgene and those with only the $(tetO)_7$CMV-hIL-1β transgene were not used in this study. The CCSP promoter becomes active in the fetus on embryonic day (E)14 and thereafter remains active throughout fetal and postnatal life (83, 84). This means that hIL-1β production can be induced by doxycycline in the lungs of bitransgenic dams at any time, whereas hIL-1β production in bitransgenic fetuses is only inducible after E14, as previously shown (84). This property of the transgenic system makes it possible to study how maternal hIL-1β production preceding fetal hIL-1β production modifies the response of the fetus and newborn to its own hIL-1β.

All mice (Paper I-II) were genotyped by PCR analysis of genomic tail DNA using primers specific for transgene constructs; see Table 2.

Table 2. Primers used for genotyping by PCR.

Transgene construct	Primer sequence (5'-3')	Paper
rCCSP-rtTA	Forward: ACT GCC CAT TGC CCA AAC AC	**I, II**
	Reverse: AAA ATC TTG CCA GCT TTC CCC	
$(tetO)_7$CMV-hIL-1β	Forward: CCA TCC ACG CTG TTT TGA CC	**I, II**
	Reverse: ACG GGC ATG TTT TCT GCT TG	

Doxycycline administration

Paper I

Doxycycline (Sigma, St. Louis, MO) was administered in drinking water at a concentration of 0.5 mg/ml from E0 or E15 to pregnant mice, and was continued until E14, PN0, or PN7 as indicated (Fig. 3).

Paper II

Doxycycline was administered in drinking water (0.5 mg/ml) to pregnant dams at E15-16.5 (36h), at E17.5-PN0 (36h), or from E0 until sacrifice of the pups on PN7 (Fig. 3). Postnatal administration of doxycycline to nursing dams in water (1 mg/ml, double dose compared to standard doxycycline administration) and in laboratory chow (1.25 mg/g) did not result in comparable levels of hIL-1β in the lungs of bitransgenic pups. Therefore doxycycline was administered as i.p. injections (0.15 mg/pup/injection, Merckle GmbH,

Blaubeuren, Germany) at PN0, 0.5, and 1 to newborn pups in order to obtain solely postnatal expression and production of hIL-1β in the infant bitransgenic offspring (Fig. 3).

The light-sensitive doxycycline solution was protected from light by covering cage bottles with aluminum foil, and the solution was changed three times per week.

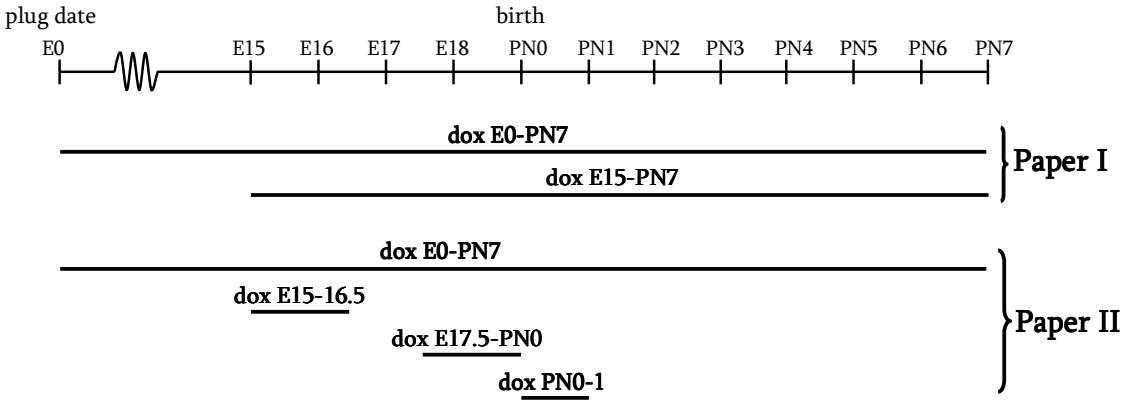

Figure 3. Schedule over the different doxycycline treatments in Paper I and II.

Animal care - Paper I-II

The mice were housed in pathogen-free conditions and all experiments were conducted in accordance with the Animal Research Ethics Committee guidelines at University of Gothenburg. All animals were given access to water and laboratory chow *ad libitum*. Vaginal plugs were observed and the plug date was counted as E0. The date of birth was counted as PN0. Before sample collection from infant mice, the animals were anesthetized by i.p. injection of a mixture of ketamine, xylazine, and acepromazine, and exsanguinated by transection of abdominal vessels.

Maternal tissue - Paper I

Blood samples from pregnant dams at E14 were collected, using EDTA as anticoagulant, by heart punction after anesthesia with an i.p. injection of a mixture of ketamine, xylazine, and acepromazine. Blood smears were prepared and the remaining blood was centrifuged at 1,000 g for 10 minutes to separate the plasma and the blood cell fraction. The lungs, uteri, placentas, and fetal membranes were also collected.

Fetal tissue - Paper II

For antenatal sample collection the pregnant dams were anesthetized with a mixture of ketamine, xylazine, and acepromazine and exsanguinated by transection of abdominal vessels. The uterus with the fetuses was removed and placed on ice until sample collection from the fetuses.

RNA isolation and quantitative RT-PCR - Paper I-II

Total RNA from fetal lung tissue (fetal membranes, maternal blood cells, maternal liver, maternal uterus, and placentas in Paper I) was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and treated with RNase-free DNase (DNA-free, Ambion, Austin, TX). One microgram of total RNA was reverse transcribed (Omniscript, Qiagen, Hilden, Germany) into cDNA. For quantification of mRNA levels, PCR was performed on cDNA equivalent of 20 ng of cDNA, using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA), Mx3000P real-time PCR instrument (Stratagene) and gene-specific, intron-spanning primers (see Table 3). Specificity of PCR was confirmed by gel analysis during reaction optimization and melting curve analysis of each amplification product. Relative quantification of starting amounts of mRNA was performed with Mx3000P instrument software from amplification curves using standard curves obtained from dilution series of cDNA. The results were normalized to β-actin mRNA levels.

Protein measurement with ELISA - Paper I-III

Fetal lung tissue (and placenta and maternal uterus in Paper I) was homogenized in PBS containing protease inhibitor (Complete protease inhibitor, Roche Diagnostics, Basel, Switzerland) and centrifuged at 10,000 × g for 10 minutes to remove cell debris. Supernatant was used for analysis. Total protein concentration was measured using the bicinchoninic acid (BCA) method according to the manufacturer's instructions (Sigma). Concentration of hIL-1β was measured using DuoSet enzyme-linked immunosorbent assay (ELISA) development kit (Table 4).

The concentration of hIL-1β in maternal plasma, maternal uterus, and placenta homogenates in Paper I was measured using Quantikine® HS hIL-1β ELISA development kit (Table 4). DuoSet ELISA development kits were used in Paper II to quantify mouse chemokines CXCL1 and CCL2 (Table 4). In Paper III the concentration of cytokines and chemokines in gastric aspirates were measured using ELISA kits in Table 4.

Table 3. Primers used for quantification of mRNA levels with RT-PCR.

Gene	Primer sequence (5'-3')	Paper
24p3	Forward: ACA ACT GAA TGG GTG GTG AGT GTG	**I**
	Reverse: AGA AGA GGC TCC AGA TGC TCC TTG	
Areg	Forward: TGC TGT TGC TGC TGG TCT TAG GCT	**II**
	Reverse: GAA GGC ATT TCG CTT ATG TGT GAA	
β-actin	Forward: TCC GTA AAG ACC TCT ATG CCA ACA	**I, II**
	Reverse: CTC AGG AGG AGC AAT GAT CTT GAT	
CCL2	Forward: GCT CTC TCT TCC TCC ACC ACC AT	**I, II**
	Reverse: GCT CTC CAG CCT ACT CAT TGG GAT	
CCL7	Forward: TCT GCC ACG CTT CTG TGC CT	**I**
	Reverse: GCT CTT GAG ATT CCT CTT GGG GAT	
CXCL1	Forward: AAA CCG AAG TCA TAG CCA CAC TCA	**I, II**
	Reverse: CTT GGG GAC ACC TTT TAG CAT CTT	
CXCL5	Forward: GCT GGC ATT TCT GTT GCT GTT CA	**I**
	Reverse: ATG ACT TCC ACC GTA GGG CAC TGT	
CXCR2	Forward: CCT CAG ACT TTT GGC TTC CTC GT	**I**
	Reverse: CGC AGT GTG AAC CCG TAG CAG A	
MMP-9	Forward: TTC GCA GAC CAA GAG GGT TTT C	**I**
	Reverse: AAG ATG TCG TGT GAG TTC CAG GGC	
MMP-12	Forward: CTG TCT TTG ACC CAC TTC GCC A	**I**
	Reverse: TCC TGC CTC ACA TCA TAC CTC CAG T	
hIL-1β	Forward: CCA TCC ACG CTG TTT TGA CC	**I**
	Reverse: ACC AAG CTT TTT TGC TGT GAG TCC	
mIL-1β	Forward: AGC CCA TCC TCT GTG ACT CA	**I**
	Reverse: TGT CGT TGC TTG GTT CTC CT	
IL-1RI	Forward: TGG AGG GAC AGT TTG GAT ACA AG	**I**
	Reverse: ATC AGC CTC CTG CTT TTC TTT AC	
IL-1RII	Forward: GAT AAC CTG CTG GTG TGT GA	**I**
	Reverse: TCT GTC CAT TGA GGT GGA GA	
IL-1Ra	Forward: ATA GTG TGT TCT TGG GCA TCC A	**I**
	Reverse: TGT CTT CTT CTT TGT TCT TGC TCA G	
S100A8	Forward: GAG CAA CCT CAT TGA TGT CTA	**I**
	Reverse: TGC ATT GTC ACT ATT GAT GTC CA	
S100A9	Forward: GCC AAC AAA GCA CCT TCT CAG AT	**I**
	Reverse: GCC ATC AGC ATC ATA CAC TCC TCA A	
SAA3	Forward: TGC TCG GGG GAA CTA TGA TGC T	**I**
	Reverse: CCA CTC GTT GGC AAA CTG GTC A	
TLR2	Forward: CCG AAA CCT CAG ACA AAG CGT CA	**I**
	Reverse: TCA CAC ACC CCA GAA GCA TCA CAT	
TLR4	Forward: GCA AAG TCC CTG ATG ACA TTC CTT	**I**
	Reverse: CCA CAG CCA CCA GAT TCT CTA AA	
Ym1	Forward: GCT CAT TGT GGG ATT TCC AGC A	**I, II**
	Reverse: CCT CAG TGG CTC CTT CAT TCA GAA	
Ym2	Forward: TTG GAG GAT GGA AGT TTG GAC CT	**I, II**
	Reverse: TGA CGG TTC TGA GGA GTA GAG ACC A	

Table 4. ELISA kits used for protein measurement.

Protein	Assay standard range	Supplier	Paper
CCL2	3.9-250 pg/ml	R&D Systems *	**II**
CCSP	1-100 ng/ml	Biovendor †	**III**
CXCL1	15.6-1000 pg/ml	R&D Systems	**II**
Gro-α	31.3-2000.0 pg/ml	R&D Systems	**III**
ENA-78	15.6-1000.0 pg/ml	R&D Systems	**III**
IL-8	31.3-2000.0 pg/ml	R&D Systems	**III**
hIL-1β	3.9-250.0 pg/ml	R&D Systems	**I-III**
HS hIL-1β	0.125-8.000 pg/ml	R&D Systems	**I**

* R&D Systems, Abingdon, UK

† Biovendor, Modrice, Czech Repulic

Histology - Paper I-II

Fetal lungs (and maternal lungs in Paper I) were inflation-fixed. The chest cavity was opened and the trachea exposed. PBS-buffered 4% paraformaldehyde was instilled through a blunt cannula in the trachea and the lungs were inflated at a pressure of 25 cm H_2O. After overnight fixation at +4°C, the tissue was rinsed in PBS and graded ethanol, dehydrated and processed through conventional paraffin embedding. Five-micrometer tissue sections were deparaffinized and rehydrated and then stained with hematoxylin and eosin or Alcian blue/periodic acid Schiff (PAS, pH 2.5) (Schiff's reagent; Merck, Darmstadt, Germany) (86) and counterstained with Mayer's hematoxylin.

Immunohistochemistry - Paper I-II

For immunohistochemistry the tissue sections were deparaffinized, rehydrated, and antigen retrieved using citrate buffer (pH 6). Methanol and hydrogen peroxidase was used to block endogenous peroxidase; the sections were then incubated with normal rabbit serum to block non-specific binding. Staining for neutrophils and macrophages was performed using monoclonal rat anti-mouse neutrophils, clone 7/4 (1:50, Serotec, Oxford, UK) and monoclonal rat anti-mouse Mac3, clone M3/84 (1:50, BD Biosciences Pharmingen, San Diego, CA) antibodies, respectively. Biotinylated secondary antibodies, rabbit anti-rat (1:50, Vector Laboratories, Burlingame, CA) and avidin-biotin peroxidase (Vectastain Elite ABC, Vector Laboratories) with peroxidase substrate NovaRed were used according to manufacturer's instructions (Vector Laboratories). Sections were slightly counterstained with Mayer's hematoxylin.

In Paper I, placentas, fetal membranes, and uteri were also collected from dams and placed in PBS-buffered 4% paraformaldehyde overnight at +4°C for fixation. The samples were then treated and stained as above (hematoxylin and eosin, neutrophils, and macrophages).

Detection of proliferating cells - Paper II

Five-micrometer lung tissue sections were deparaffinized, rehydrated, and antigen retrieved using citrate buffer (pH 6). Methanol and hydrogen peroxidase was used to block endogenous peroxidase. The sections were then incubated with normal goat serum to block non-specific binding. Polyclonal rabbit anti-human Ki-67 antibodies (1:500, Novocastra Laboratories Ltd, Newcastle-upon-Tyne, UK) that cross-react with murine Ki-67 were used to detect proliferating cells. Biotinylated secondary antibodies, anti-rabbit (1:200, Vector Laboratories) and avidin-biotin peroxidase (Vectastain Elite ABC, Vector Laboratories) with peroxidase substrate 3.3-diamniobenzidine (DAB, Vector Laboratories) were used according to manufacturer's instructions (Vector Laboratories). Sections were slightly counterstained with nuclear fast red.

In situ detection of apoptotic cells - Paper II

The 3' strand breaks occurring within the DNA during apoptosis can be detected with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL). Five-micrometer tissue sections were stained with the TUNEL method as previously described (87). Briefly, DNA 3'-end labeling was performed with a labeling mixture containing TdT buffer, $CoCl_2$, TdT recombinant transferase, digoxigenin-dideoxy-UTP (ddUTP), and dideoxyadenosine-triphosphat (ddATP) (Roche Diagnostics). Alkaline phosphates-conjugated antidigoxigenin antibody (Roche Diagnostics) and 5-Bromo-4-chromo-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT, Vector Laboratories) as substrate were used to detect cells with DNA fragmentation.

Cell counts - Paper I-II

The numbers of neutrophils, macrophages, and TUNEL-positive cells were counted with a x40 lens, and the number of Ki-67-positive cells were counted with a x100 lens in 10 random high-power fields in each section (≥four animals per group), and average numbers of positive cells per square millimeter were calculated.

The percentage of goblet cells in the epithelium of 12–20 airways in at least four mice from each group was assessed at PN7 on Alcian blue/PAS-stained lung tissue sections. The distribution of airways having less than 10%, 11–40%, 41–80%, or more than 81% PAS-positive cells in the epithelium was then calculated. The trachea and the primary bronchi were excluded from this analysis.

Paper I

The number of white cells in maternal blood was counted using a hemacytometer. Differential leukocyte counts were performed from blood smears stained with Wright's Geimsa stain.

Morphometric analysis - Paper I-II

Quantification of chord length

Quantification of distal airspace size at PN7 (and maternal lung in Paper I; and at PN12 in Paper II) was performed from hematoxylin and eosin-stained lung tissue sections, using the mean chord length as a measure of alveolar size (79). A minimum of 8 representative, non-overlapping fields from lungs of 4-9 mice of each genotype and treatment group were acquired in 8-bit grayscale, using a 20x lens, at a final magnification of 1.89 pixels per micrometer using a Nikon Eclipse E800 microscope and DXM1200 digital camera (Nikon, Tokyo, Japan). Areas of bronchiolar airways and blood vessels were excluded from the analysis. Chord length analysis was performed using the public domain program NIH Image with a chord length macro (available from the U.S. National Institutes of Health at http://rsb.info.nih.gov/nih-image). The images were binarized and a grid with horizontal and vertical straight lines was applied; the lengths of lines overlying alveolar space were then averaged as the mean chord length.

Quantification of alveolar wall thickness

The same binarized images used to quantify chord length were used to assess the thickness of alveolar septa (88). Straight lines (60-80 per field) were drawn at 90^{o} angles across the narrowest segments of septa of distal airspaces. The mean length of the lines crossing the septa was determined using the NIH Image software ImageJ (available at http://rsb.info.nih.gov/nih-image).

Patients and study design - Paper III

The study was approved by the Regional Ethics Board for Human Studies at the University of Gothenburg. Premature infants born at the Sahlgrenska University Hospital whose gestational age was less than 29 weeks were included. Gastric aspirate samples were retrieved within 1 hour after birth from 56 infants. Five infants died before the age of 28 days and were therefore excluded from the study. Clinical data including birth weight, gestational age, gender, prenatal steroids, time of rupture of membranes (ROM), surfactant therapy, requirement of supplemental oxygen, CPAP or ventilator therapy, presence of clinical chorioamnionitis, PDA, medical or surgical treatment of PDA, and ROP were entered to the database from the patients' charts and the maternal records.

Clinical chorioamnionitis was defined as the presence of maternal fever, elevated C-reactive protein (CRP), and supporting clinical evidence such as uterine tenderness, fetal tachycardia, or purulent vaginal discharge. BPD was defined as oxygen requirement at 28 days of age and classified as mild, moderate, or severe according to the NICHD definition (49).

Statistics - Paper I-II

Calculations were made using the statistical software GraphPad Prism™ software (GraphPad Software Inc., San Diego, CA). Measurement values are expressed as mean ± SEM. As appropriate, groups were compared with Student's two-tailed unpaired t-test or with nonparametric Mann-Whitney U-test. Neonatal mortality data were analyzed using Kaplan-Meier survival analysis and logrank (Mantel-Cox) test. Reported p-values are two-sided and $p \leq 0.05$ was considered statistically significant.

Paper III

Calculations were made using the statistical software SPSS (version 18.0). Data are presented as median [interquartile range] or as mean±SEM, as indicated. Group comparisons of continuous variables were tested with the Mann-Whitney-U test and categorical variables were tested with Fisher's exact test. Multiple linear regression was used to evaluate relationships between cytokine levels and gestational age adjusting for mode of delivery and chorioamnionitis. Logistic regression was used to test whether BPD was associated with cytokine levels or ROP after adjustment for gestational age. Relationships between cytokine levels and BPD severity was tested with Jonckheere-Terpstra, a non-parametric test for ordered groups. Reported p-values are two-sided and considered statistically significant when $p \leq 0.05$.

RESULTS

Paper I

Inflammation in hIL-1β-expressing dams

Pulmonary expression of hIL-1β in the lungs of pregnant dams during the first 14 days of gestation did not cause airspace enlargement; however, the bitransgenic dams had a pulmonary inflammation with increased numbers of neutrophils and macrophages in the lungs. The bitransgenic dams also had a mild systemic inflammation characterized by a higher white blood cell count, an altered differential count, and increased mRNA expression of several genes associated with inflammation (24p3, MMP-9, S100A8, S100A9, and serum amyloid A (SAA)3) (Paper I; Fig. 1A-D).

Inflammation in uterus, placenta and fetal membranes

Expression and production of hIL-1β was found in the uteri of pregnant hIL-1β-producing dams, but not in the placentas or fetal membranes. The number of neutrophils in the uterus and placenta was higher in bitransgenic dams than in those of control dams (Paper I; Fig. 2A-G), the number of macrophages was not affected (Paper I; Fig. 2H).

Maternal hIL-1β expression affected growth and survival in hIL-1β-expressing pups

In all maternal groups, pups expressing hIL-1β had lower body weight at PN7 than their control littermates. Interestingly, maternal hIL-1β production preceding the fetal hIL-1β production resulted in better growth of bitransgenic pups compared with the other groups of bitransgenic pups (Paper I; Fig. 3A). The survival of bitransgenic pups of hIL-1β-producing dams was better when doxycycline was administered from the beginning of gestation (76%), than that of bitransgenic pups of control dams (52%) (** $p \leq 0.01$, Fig. 4). When doxycycline was administered from E15 there was no difference in survival between the two groups of bitransgenic pups (36% vs. 48%) (Fig. 4). However, the survival of bitransgenic pups born to bitransgenic dams was better when doxycycline was administered from E0 than when it was administered from E15 (*** $p \leq 0.001$, Fig. 4).

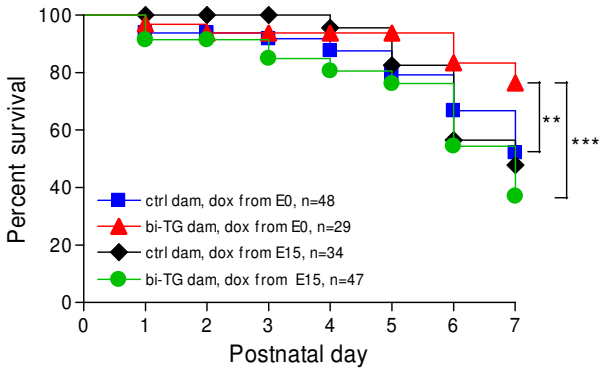

*Figure 4. Survival of bitransgenic pups. Bitransgenic pups born to bitransgenic dams with doxycycline from E0 had significantly better survival than those born to control dams and than those born to bitransgenic dams with doxycycline from E15. ** p≤0.01, *** p≤0.001.*

Maternal hIL-1β expression affected postnatal lung morphogenesis and modulated inflammation

The lungs of bitransgenic pups of both groups of control dams and of bitransgenic dams given doxycycline from E15 had enlarged distal airspaces, impaired septation, and thickened alveolar walls (Fig. 5E, G-H), compared with controls (Fig. 5A-D). In contrast, airspaces were smaller, more septa were present, and alveolar walls were thinner in the lungs of bitransgenic pups of bitransgenic dams given doxycycline from E0 (Fig. 5F), compared with the other bitransgenic pups (Fig. 5E, G-H). These results were confirmed by measurement of alveolar chord length and alveolar wall thickness (Paper I; Fig. 4I-J).

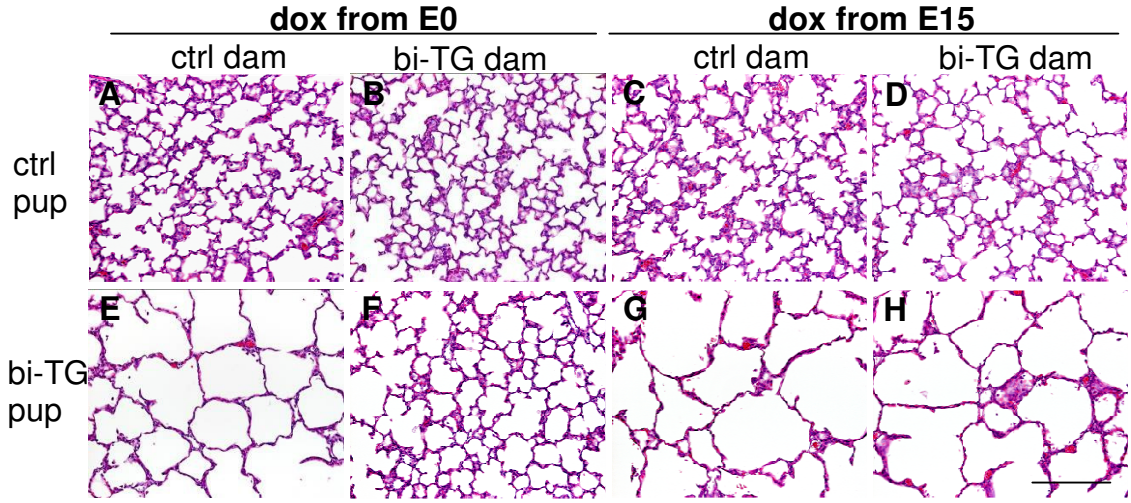

Figure 5. Lung histology at PN7 (hematoxylin and eosin), (A-D) control (ctrl) pups, (E-H) bitransgenic (bi-TG) pups. Scale bar, 200 μm.

Inflammation in the alveolar regions was assessed by immunostaining of lung sections with specific antibodies against neutrophils and macrophages. Fetal hIL-1β expression induced infiltration of neutrophils into the lungs of bitransgenic pups of both control dams and of bitransgenic dams given doxycycline from E15, compared with control pups. No increased infiltration of neutrophils was seen in hIL-1β-expressing pups of bitransgenic dams given doxycycline from E0 (Paper I; Fig. 5A-I). Fetal hIL-1β production resulted in infiltration of the lungs with macrophages in all groups of bitransgenic offspring; however, maternal hIL-1β production preceding the fetal hIL-1β production suppressed the number of macrophages in the alveolar regions of bitransgenic pups (Paper I; Fig. 6A-I).

Maternal hIL-1β production suppressed hIL-1β-induced airway remodeling and goblet cell hyperplasia

Fetal expression of hIL-1β resulted in an accumulation of inflammatory cells in and around the airways, thicker airways, and goblet cell hyperplasia in bitransgenic offspring of control dams and of bitransgenic dam given doxycycline from E15, whereas few inflammatory cells and PAS-positive goblet cells were seen in the airways of bitransgenic pups of bitransgenic dams given doxycycline from E0 (Paper I; Fig. 7A-I). Human IL-1β expression in infant mice resulted in increased expression of chitinase-like lectins Ym1 and Ym2 compared with control pups, but the increase was blunted in bitransgenic pups of bitransgenic dams given doxycycline from E0 (Paper I; Fig. 7J-K).

Maternal hIL-1β production modified the expression of inflammatory genes

Expression of hIL-1β in the lungs of infant mice induced the expression of several inflammatory genes, such as CC chemokines CCL2 and CCL7, CXC chemokines CXCL1 and CXCL5, chemokine receptor CXCR2, and acute-phase protein SAA3. However, maternal hIL-1β production preceding the fetuses' own hIL-1β production inhibited the expression of these inflammatory genes in response to hIL-1β in the lungs of infant mice (Paper I; Fig. 8A-F). The expression of toll-like receptor (TLR)2 and TLR4 was also studied. Human IL-1β production in the fetal lung induced the expression of TLR2; this effect was absent in bitransgenic offspring of hIL-1β-producing dams given doxycycline from E0 (Paper I; Fig. 8G). The expression of TLR4 was elevated in all groups of bitransgenic offspring; hIL-1β producing pups of control dams had higher levels than those of bitransgenic dams (Paper I; Fig. 8H).

Paper II

Production of hIL-1β in the bitransgenic fetal and newborn lung

Doxycycline treatment at E15-16.5 resulted in a peak of hIL-1β production during E16-18, i.e. during the late canalicular-early saccular stage, whereas doxycycline treatment at E17.5-PN0 resulted in maximal hIL-1β production during PN1-3, i.e. during the saccular stage. Doxycycline treatment at E0-PN7 induced hIL-1β production from E14, when the CCSP promoter becomes active, to PN7, i.e. from the pseudoglandular to the alveolar stage. Doxycycline administered as i.p. injections (0.15 mg/injection) at PN0, 0.5, and 1, resulted in hIL-1β production from PN3-12, i.e. from the late saccular-alveolar stage. The peak concentrations of hIL-1β in the lungs were similar in the two 36h treatment groups, around 90 ng hIL-1β/mg protein. The level of hIL-1β resulting from prolonged doxycycline treatment (E0-PN7) reached a plateau at around 140 ng hIL-1β/mg protein at E16 and remained approximately at this level until PN4. Doxycycline injections at PN0-1 resulted in a maximal level of around 210 ng hIL-1β/mg protein at PN6 (Fig. 6).

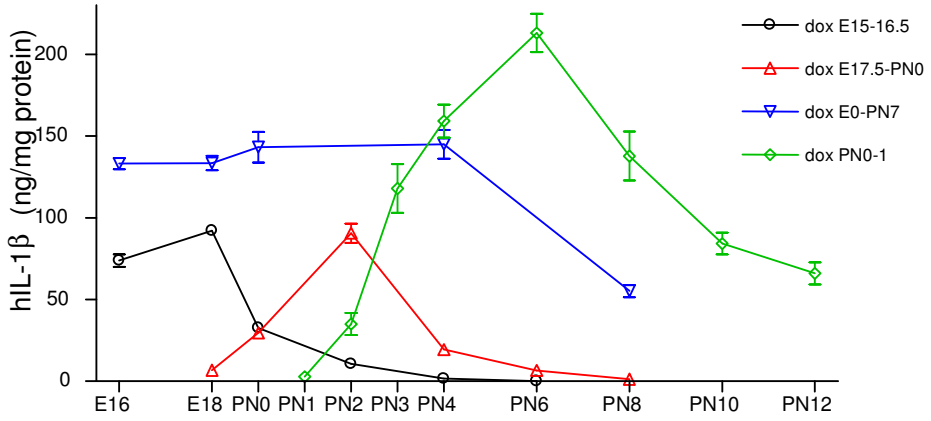

Figure 6. Production of hIL-1β in the lungs of fetuses and newborn bitransgenic mice. n≥4 in each group.

The timing of hIL-1β production affects postnatal growth and survival

Doxycycline treatment at E15-16.5 did not affect the postnatal growth of bitransgenic pups compared with control pups (Paper II; Fig. 2A). In contrast, postnatal growth was poorer in bitransgenic offspring when doxycycline was administered at E17.5-PN0, E0-PN7, or PN0-1, compared with controls as well as with bitransgenic pups given doxycycline at E15-16.5 (Paper II; Fig. 2A). Except for a few deaths in the immediate postnatal period, all control pups in the different treatment groups as well as all the

bitransgenic pups given doxycycline at E15-16.5 or at PN0-1 survived until PN7. When doxycycline was administered at E17.5-PN0 or at E0-PN7 the survival of bitransgenic offspring was significantly reduced to 50% and 52%, respectively (Paper II; Fig. 2B).

Production of hIL-1β during the saccular stage disrupts lung development

Alveolar septation was ongoing in all four groups of control pups as well as in the bitransgenic pups given doxycycline at E15-16.5 or at PN0-1 (Fig. 7A-E, H). Human IL-1β production following doxycycline at E17.5-PN0 or at E0-PN7 resulted in disrupted alveolar development (Fig. 7F-G) and consequently a significantly increased alveolar chord length (Paper II; Fig. 3I). However, hIL-1β production in the late saccular to alveolar stage (doxycycline at PN0-1) was sufficient to negatively affect alveolar wall thinning. Bitransgenic pups given doxycycline at E17.5-PN0 or at E0-PN7 had greater alveolar wall thickness than those of their controls and than those bitransgenic pups given doxycycline at E15-16.5 or at PN0-1 (Paper II; Fig. 3J). Production of hIL-1β in the alveolar stage caused a slight increase in alveolar chord length at PN12.

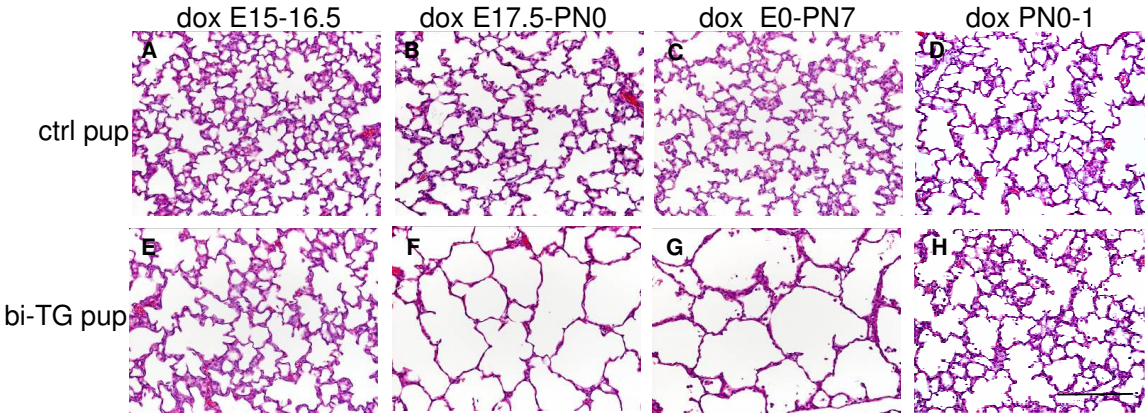

Figure 7. Lung histology at PN7 (hematoxylin and eosin), (A-D) ctrl pups, (E-H) bi-TG pups. Scale bar, 200 μm.

Infiltration of neutrophils and macrophages to the lungs of hIL-1β producing pups

Doxycycline treatment at E17.5-PN0, E0-PN7, or PN0-1 caused massive infiltration of neutrophils and macrophages into the lungs of hIL-1β producing offspring. Production of hIL-1β in the late canalicular-early saccular stage of lung development (doxycycline at E15-16.5) was not sufficient to cause an inflammation in the lungs of bitransgenic pups (Paper II; Fig. 4-5). The expression and production of CCL2 and the expression of CXCL1

paralleled the numbers of inflammatory cells (Paper II; Fig. 6A-C). Production of hIL-1β in the canalicular stage caused a slight increase in the CXCL1 production; the increase was significantly lower than the increase in the other groups with bitransgenic mice (Paper II; Fig. 6D).

The timing of hIL-1β production influenced airway remodeling

The airways of bitransgenic offspring with hIL-1β production in the saccular stage (doxycycline at E17.5-PN0) or from the pseudoglandular to the alveolar stage (doxycycline at E0-PN7) had thicker airways with an accumulation of inflammatory cells in and around the airways, and more goblet cell hyperplasia, than control mice as well as than the other two groups of bitransgenic pups given doxycycline at E15-16.5 or at PN0-1 (Paper II; Fig. 7A-I). The expression of chitinase-like lectins, Ym1 and Ym2, and amphiregulin (Areg) was upregulated in infant bitransgenic mice given doxycycline at E17.5-PN0 or at E0-PN7, whereas the expression was blunted in bitransgenic offspring given doxycycline at PN0-1. Doxycycline treatment at E15-16.5 did not change the expression of Ym1, Ym2, and Areg (Paper II; Fig. 7J-L).

Apoptosis and proliferation in the lungs of infant mice

Bitransgenic pups given doxycycline at E17.5-PN0, at E0-PN7 or at PN0-1 had more apoptotic cells in the distal lung than their controls (Paper II; Fig. 7A). However, the number of apoptotic cells in bitransgenic pups given doxycycline at PN0-1 was significantly smaller than the number of apoptotic cells in bitransgenic pups given doxycycline at E0-PN7. No significant differences were seen in the numbers of proliferating cells between control and bitransgenic pups in any treatment group (Paper II; Fig. 7B).

Paper III

There were 51 infants enrolled in this study, 44 (86%) of these infants had BPD, defined as requirement of supplementary oxygen at 28 days of age. Of the 44 infants with BPD, 24 (54%) had mild BPD, defined as no oxygen requirement at 36 weeks of corrected age, 13 (30%) had moderate BPD, defined as oxygen requirement of less than 30% at 36 weeks of postmenstrual age, and 7 (16%) had severe BPD, defined as higher oxygen requirement than 30% at 36 weeks of postmenstrual age or requirement of ventilator or CPAP support.

Maternal characteristics of infants with vs. without BPD

The maternal characteristics (ROM, clinical chorioamnionitis, maternal antepartum antibiotic therapy, and antenatal steroids) were not significantly different among those with infants developing BPD and those without BPD. Similarly, no differences were found when comparing infants who had no or mild BPD with those who had moderate or severe BPD (Paper III; Table I). The incidence of caesarean section was higher among the infants with no BPD than among those with BPD (Paper III; Table I).

Infant characteristics at 28 days of age

As expected, the gestational age of infants developing BPD was lower than that of infants not developing BPD (Paper III; Table II). The maximal fraction of inspired oxygen (FiO_2) during the first three days of life tended to be higher, but not significant, in infants with BPD than those without BPD. The difference in oxygen requirement between the two groups became significant by day 7 of life (Paper III; Table II). There was no difference in the number of days with ventilator, but treatment with nasal CPAP was more prolonged in infants developing BPD than in infants without BPD (Paper III; Table II).

Infant characteristics at 36 weeks of age

The gestational age and birth weight of infants with moderate or severe BPD were lower than those of infants with no or mild BPD (Paper III; Table II). The maximal FiO_2 during the first three days of life, the FiO_2 on day 7 of life, intubation rate, the number of days on ventilator, the number of days on CPAP, surfactant treatment, and the incidence of sepsis were higher in infants with moderate or severe BPD than in infants with no or mild BPD (Paper III; Table II). The probability of receiving postnatal steroids was higher in the group of infants developing moderate or severe BPD than in those developing no or mild BPD, not surprisingly, since postnatal steroids were given only to facilitate extubation of infants who were respirator-dependent at a postnatal age of at least 7-10 days (Paper III; Table II).

PDA and ROP

There was no difference in incidence of PDA or treatment of PDA between those infants with moderate or severe BPD and those with no or mild BPD. However, a strong association between PDA ligation and the development of moderate or severe BPD was found (Paper III; Table II). After the adjustment for gestational age, the association between BPD and surgery for PDA was no longer significant. ROP was also associated with moderate or severe BPD at 36 weeks postmenstrual age (Paper III; Table II).

Clinical chorioamnionitis and mode of delivery

The gastric fluid levels of IL-8, Gro-α, ENA-78 and IL-1β were higher in infants exposed to clinical chorioamnionitis than in those whose mothers did not have clinical chorioamnionitis (Paper III; Table III), as well as in the infants who were delivered vaginally compared with those born by caesarean section (Paper III; Table IV). In addition, the infants delivered vaginally had lower gestational age than those born by caesarean section (Paper III; Table IV).

Cytokines in gastric aspirate fluid and the development BPD

Unadjusted comparisons of cytokine concentrations showed that the levels of IL-8, Gro-α, ENA-78, and IL-1β in gastric fluid at birth were higher in infants with moderate or severe BPD. A nonparametric test for ordered differences among classes showed that there was a significant order among the groups of infants with no BPD, mild BPD, and moderate or severe BPD in regard to the levels of inflammatory cytokines (Paper III; Fig. 1). After adjustment for gestational age, the difference between the groups was no longer significant.

DISCUSSION

Maternal inflammation and expression of hIL-1β in the uterus

Paper I shows that maternal hIL-1β production preceding fetal hIL-1β production protects infant mice from hIL-1β-induced lung injury. Bitransgenic pups born to bitransgenic dams exposed to prior maternal hIL-1β production have fewer neutrophils and macrophages in their lungs, better alveolar septation and alveolar wall thinning, less goblet cell hyperplasia, better growth, and higher survival rates than bitransgenic pups born to control dams.

The expression of hIL-1β in the present transgenic system is driven by the rCCSP promoter. Besides being highly expressed in the lung, CCSP, also known as uteroglobin, is expressed at low levels in the pregnant and pseudopregnant uterine epithelium of rats and mice (89-91). Uteroglobin is the founding member of a superfamily of proteins called the *Secretoglobin* superfamily (92). This study shows, for the first time uterine expression of a transgene driven by the CCSP promoter. The levels of expression and production were extremely low compared with pulmonary levels. However, this very low concentration of hIL-1β was sufficient to cause infiltration of the uterine endometrium and the decidua basalis of the placenta with neutrophils in bitransgenic pregnant dams. Importantly, no neutrophils or macrophages were seen in the fetal membranes, yolk sac or amnion of offspring to either control or bitransgenic dams. This shows that though the tissues are adjacent, uterine hIL-1β production does not induce an inflammation in the fetal membranes.

The peripheral white cell count and the expression of the inflammatory genes 24p3, MMP-9, S100A8, and S100A9 was elevated in the blood cells of bitransgenic dams. These data confirm the presence of a systemic inflammatory response to hIL-1β in bitransgenic dams. It has been proposed that MMPs play a key role in remodeling of the lung in various diseases due to their ability to cleave structural proteins such as collagens and elastin (93). The lipocalin 24p3, secreted by macrophages, neutrophils, and various other cell types, has been implicated in the regulation of inflammatory responses, the control of cell growth and development, tissue involution, and apoptosis (94). Expression of neutrophil gelatinase-associated lipocalin (NGAL), a human homologue of 24p3, has been observed to be induced in epithelial cells during inflammation and, more specifically, by IL-1β (95). S100A8 and S100A9 have pro-inflammatory properties and they are chemotactic for neutrophils and monocytes (96, 97).

Preterm delivery can be induced by inflammatory cytokines, e.g. IL-1β and TNF-α, in experimental animal models (98, 99). In Paper I, hIL-1β production did not cause preterm labor. Perhaps the inflammation was not severe enough and the amount of inflammatory cytokines in the pregnant mouse was too low. The lack of preterm labor may also be partially explained by the relatively high resistance to inflammation-induced preterm labor that the FVB/N strain has, compared with other strains of mice (100).

Fetal lung development is affected by maternal hIL-1β production

Bitransgenic pups (born to control dams) had thicker airways and goblet cell hyperplasia and increased expression of Ym1 and Ym2 than control pups. Maternal hIL-1β production preceding fetal hIL-1β production suppressed the expression of Ym1 and Ym2 and inhibited the airway remodeling and goblet cell hyperplasia. Thick airway epithelia and goblet cell hyperplasia are common features of BPD (101). Chitinase-like proteins, with chitin-binding activity (102), Ym1 and Ym2 are upregulated in allergic airway inflammation and may play a role in the development of asthma (103-106). YKL-40, a human chitinase-like protein homologue, is elevated in patients with COPD (107) and in patients with asthma, and the amount in a degree correlating to the severity of the disease (108). Possibly, the blunted expression of Ym1 and Ym2 played a part in preventing hIL-1β-induced lung injury in infant bitransgenic mice.

Maternal hIL-1β production induces fetal tolerance to hIL-1β

In the present mouse model, hIL-1β production causes a massive infiltration of the lungs with neutrophils and macrophages (84). However, when maternal hIL-1β production starts before the fetuses produce hIL-1β, the infiltration of inflammatory cells into the lungs is strongly decreased. The mRNA expression of mIL-1β, several neutrophil- and monocyte-attractant chemokines, SAA3, TLR2, and TLR4 was down-regulated in the lungs of hIL-1β–expressing newborns exposed to prior maternal inflammation compared with hIL-1β–expressing pups of control dams. After the initiation of a systemic inflammation, humans often have a period of silencing of pro-inflammatory genes. This silencing or tolerance may persist for days or even weeks (109). IL-1 has been shown to be a potential mediator in a tolerance process in rats (110). After intratracheal pre-treatment with IL-1 a tolerance to oxidative lung injury was developed. A variety of stimuli have been shown to be able to induce a state of tolerance, e.g. endotoxin, peptidoglycan, and TNF (16). Tolerance to hIL-1β, or silencing of pro-inflammatory genes, may be a central

mechanism in Paper I. The tolerance of infant mice towards hIL-1β was absent if hIL-1β production was initiated at the same time in both the dam and the fetuses.

IL-1Ra and IL-1RII are known antagonists to IL-1β (21). To test if the tolerance to hIL-1β of bitransgenic pups of bitransgenic dams given doxycycline from E0 was due to increased expression of IL-1Ra and IL-1RII, the expression was measured with quantitative RT-PCR. Interestingly, we found that the expression of IL-1RII was inhibited in bitransgenic offspring of bitransgenic dams given doxycycline from E0 compared with the other groups with bitransgenic offspring. Human IL-1β expression in infant mice caused increased expression of IL-1Ra in all groups with bitransgenic offspring. Thus, the blunted inflammatory response in bitransgenic offspring of hIL-1β-producing dams given doxycycline from E0 was not due to upregulation of IL-1Ra or IL-1RII.

Production of hIL-1β in the saccular stage affects lung development

In the present mouse model, hIL-1β production from the pseudoglandular to the alveolar stage in the lungs results in disrupted alveolar development (84). In Paper II we showed that doxycycline administration at E17.5-PN0, causing production of hIL-1β solely in the mid-saccular stage, was sufficient to cause a similar lung injury in bitransgenic pups as hIL-1β expression throughout gestation until PN7. Production of hIL-1β in the late saccular-alveolar stage only caused a slight increase in alveolar size and wall thickness. These results are consistent with clinical findings showing that the risk for BPD is highest in children born at 23-27 weeks of gestation, during the early saccular stage of lung development, whereas it is low in children born after 32 weeks of gestation, i.e. in the late saccular-alveolar stage (49, 54).

Areg, a member of the epidermal growth factor (EGF) family, has been shown to upregulate mucin gene expression in epithelial cells (111). In Paper II, the expression of Areg, Ym1, and Ym2 was strongly increased in bitransgenic pups with hIL-1β production in the mid-saccular or in the pseudoglandular-alveolar stage. Thus, the expression of these proteins paralleled the severity of airway remodeling and goblet cell hyperplasia.

Timing of hIL-1β production affects inflammation

Increased concentrations of the chemokines CCL2 and IL-8 (human functional homologue of mouse CXCL1) in TA and increased number of neutrophils and macrophages in BALF in preterm newborns are associated with the development of BPD (20, 72, 112). The

expression and production of CCL2 and CXCL1 paralleled the number of neutrophils and macrophages in the lungs of bitransgenic mice in Paper II. Interestingly, the magnitude of inflammation is not directly related to the severity of lung disease. The infant mice with hIL-1β expression in the late saccular to the alveolar stage of lung development had a substantial pulmonary inflammation but lung development appeared almost normal and the survival was 100%. Similarly, Moss *et al.* (113) used a fetal sheep model to show that a single proinflammatory exposure to the fetus via the amniotic fluid alters lung function and structure. Lung growth was altered depending on the timing of endotoxin exposure. The results suggest that the stage of lung development is a more important factor than the magnitude of inflammation when it comes to development of hIL-1β-induced lung injury.

Doxycycline and MMP-9 activity

Since postnatal oral administration of doxycycline to lactating dams resulted in very low levels of hIL-1β production in bitransgenic pups, doxycycline was instead given by i.p. at PN0-1. Increased activity of MMP-9 has been reported in the lungs of human infants and of baboons developing BPD (93, 114). In the present mouse model, hIL-1β production has been shown to increase the production of MMP-9 in the lungs of newborn mice (115). Since doxycycline is a non-selective MMP inhibitor, the higher dose of doxycycline given to the mice treated at PN0-1 may have resulted in lower MMP-9 activity in the lungs of these mice compared to the other treatment groups. A study by Lukkarinen *et al.* (115) showed that hIL-1β-induced lung injury is enhanced in infant mice with null MMP-9 loci compared with wild-type mice. These results suggest that MMP-9 activity in the inflamed newborn lung protects the lung against inflammatory injury. Thus, inhibition of MMP activity by doxycycline does not explain why the lung disease in the mice treated with doxycycline at PN0-1 was milder than in the groups treated at E0-PN7 or at E17.5-PN0.

Gastric fluid cytokines and the development of BPD

The concentrations of inflammatory mediators in gastric aspirate fluid of very immature infants shortly after birth were associated with BPD. There was significant increasing order among the three groups of patients (no BPD, mild BPD, and moderate or severe BPD) in the levels of IL-8, Gro-α, ENA-78, and IL-1β in gastric fluid at birth. The results were no longer significant after adjustment for gestational age, the major risk factor for BPD. Previous studies have shown that IL-1β, IL-6, and IL-8 concentrations in TA are higher in infants who developed BPD than in infants who did not have BPD (70, 116-119). The presence of IL-1β in tracheal aspirate samples has also been shown to predict

prolonged need of ventilation and oxygen supplementation (117). However, these results were not adjusted for gestational age.

Using gastric aspirate fluid to assess inflammation in the infant at birth has potential advantages. Gastric fluid can be obtained from most infants, avoiding the selection bias that TA sampling involves. Infants not requiring intubation are excluded from studies based on TA samples, resulting in exclusion of the healthiest patients. In our study, only 24% of the infants were intubated during the first hour of life, since it is a general policy in our unit to use nasal CPAP as a first-line treatment in preterm infants requiring respiratory support. A neutrophil influx into the airways occurs immediately after intubation (57, 120). Gastric aspirate levels of inflammatory markers are unlikely to be influenced by intubation to the same extent as TA levels.

Gastric fluid cytokines and chorioamnionitis

As expected, IL-8, Gro-α, ENA-78, and IL-1β concentrations in gastric fluid were strongly increased in preterm infants who were exposed to clinical chorioamnionitis. Increased concentrations of these cytokines in amniotic fluid are associated with microbial invasion of the amniotic cavity (121-124). The incidence of clinical chorioamnionitis tended to be higher among the mothers whose infants developed BPD compared to those with no BPD. There are studies suggesting that chorioamnionitis (39, 125) and elevated concentrations of inflammatory cytokines in amniotic fluid (70, 116), in TA (71), or in cord blood (126) are linked with the development of BPD. However, Andrews *et al.* (42) found no connection between antenatal inflammation and BPD, consistent with other studies (127-130), whereas Van Marter *et al.* (131) found that chorioamnionitis decreased the risk of BPD. These correlations are not consistent across different reports, probably due to the nature, duration, and magnitude of the various inflammatory exposures, varying gestational ages, and use of antenatal and postnatal therapies, such as maternal glucocorticoids, surfactant and oxygen administration, and ventilator treatment.

A study by Miralles *et al.* (132) showed that the presence of microbes in gastric fluid at birth strongly correlates with the occurrence of chorioamnionitis. Detection of microbes by analysis of bacterial 16s ribosomal RNA in gastric fluid is also associated with the development of severe BPD (74). These studies support the idea that gastric fluid may provide an alternative to TA to identify antenatal inflammation.

CCSP levels and the development of BPD

The level of CCSP tended to be lower in gastric aspirates from infants who developed BPD than in infants who did not. Since CCSP is produced in the airways by nonciliated secretory cells, CCSP in gastric aspirates originates from swallowed lung secretion. An earlier study has shown that the levels of CCSP in TA on the first day of life are lower in infants who develop BPD than in infants who do not (133), suggesting that low CCSP production predicts the development of BPD.

PDA ligation is associated with the development of BPD

In our study no association between BPD and PDA was found. However, the development of moderate or severe BPD was strongly associated with PDA ligation. BPD and PDA have previously been shown to be associated (no data on PDA ligation was shown) (129). Clyman *et al.* (134) have shown that infants who had surgery of the ductus within 24 hours of birth regardless of the presence or absence of symptoms of PDA have a higher risk of developing BPD compared with infants who underwent surgery only if they had symptomatic PDA. An association between surgical ligation of the ductus and BPD has also been demonstrated in a study where the infants were treated with prophylactic indomethacin (135). Vasodilator prostaglandins appear to be the dominant vasodilator that opposes ductus constriction (76). Ibuprofen is a prostaglandin inhibitor used to close the ductus medically. In the present study, ductus ligation was reserved for infants who failed to close their ductus after the ductus had been treated with ibuprofen without closure.

CONCLUSIONS

Paper I shows that maternal hIL-1β production preceding fetal hIL-1β production protects the newborn against hIL-1β-induced lung inflammation and injury. This protective effect is absent when hIL-1β production starts simultaneously in dam and fetus.

Paper II shows that production of hIL-1β in the saccular stage, but not in the late canalicular-early saccular or late saccular-alveolar stage, is sufficient to cause an inflammatory BPD-like illness in infant mice.

Paper III shows that gastric aspirate can be used instead of more invasive methods to assess the exposure of newborn premature infants to inflammation and to assess the impact of perinatal inflammation on neonatal outcomes.

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REFERENCES

1. Vander A, Sherman J, Luciano D 2001 Respiration In: Human Physiology, The Mechanisms of Body Function. McGraw-Hill, pp 463-504.
2. Krinke GJ 2004 Normative Histology of Organs. In: Hedrich H. The laboratory Mouse. Elsevier, Oxford, UK, pp 133-165.
3. Massaro GD, Singh G, Mason R, Plopper CG, Malkinson AM, Gail DB 1994 Biology of the Clara cell. Am J Physiol 266:L101-106.
4. Berthiaume Y, Voisin G, Dagenais A 2006 The alveolar type I cells: the new knight of the alveolus? J Physiol 572:609-610.
5. Chen J, Chen Z, Chintagari NR, Bhaskaran M, Jin N, Narasaraju T, Liu L 2006 Alveolar type I cells protect rat lung epithelium from oxidative injury. J Physiol 572:625-638.
6. Fehrenbach H 2001 Alveolar epithelial type II cell: defender of the alveolus revisited. Respir Res 2:33-46.
7. Kotecha S 2000 Lung growth for beginners. Paediatr Respir Rev 1:308-313.
8. Coalson JJ 2003 Pathology of new bronchopulmonary dysplasia. Semin Neonatol 8:73-81.
9. Roth-Kleiner M, Post M 2003 Genetic control of lung development. Biol Neonate 84:83-88.
10. Jobe AH 2002 Lung development and maturation. In Fanaroff AA, Martin RJ (eds) Neonatal - Perinatal Medicine. Disease of the Fetus and Infant. Mosby Inc., St. Louis, MO, pp 973-991.
11. Kotecha S 2000 Lung growth: implications for the newborn infant. Arch Dis Child Fetal Neonatal Ed 82:F69-74.
12. Burri PH 2006 Structural aspects of postnatal lung development - alveolar formation and growth. Biol Neonate 89:313-322.
13. Ten Have-Opbroek AA 1991 Lung development in the mouse embryo. Exp Lung Res 17:111-130.
14. Rodriguez RJ, Martin RJ, Fanaroff AA 2002 Respiratory distress syndrome and its management. In: Fanaroff, A.A. and Martin, R.J. Neonatal-perinatal medicine: Diseases of the fetus and infant. Mosby Inc, St Louis, pp 1001-1011.
15. Mölne J, Wold A 2007 Inflammation. Liber AB, Stockholm.
16. West MA, Heagy W 2002 Endotoxin tolerance: a review. Crit Care Med 30:S64-73.
17. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L 2000 Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 80:617-653.
18. Keane MP, Strieter RM 2002 The importance of balanced pro-inflammatory and anti-inflammatory mechanisms in diffuse lung disease. Respir Res 3:5.
19. Kakkera DK, Siddiq MM, Parton LA 2005 Interleukin-1 balance in the lungs of preterm infants who develop bronchopulmonary dysplasia. Biol Neonate 87:82-90.
20. Speer CP 2006 Pulmonary inflammation and bronchopulmonary dysplasia. J Perinatol 26 Suppl 1:S57-62; discussion S63-54.

21. Dinarello CA 1996 Biologic basis for interleukin-1 in disease. Blood 87:2095-2147.
22. Qin J, Jiang Z, Qian Y, Casanova JL, Li X 2004 IRAK4 kinase activity is redundant for interleukin-1 (IL-1) receptor-associated kinase phosphorylation and IL-1 responsiveness. J Biol Chem 279:26748-26753.
23. Schonbeck U, Mach F, Libby P 1998 Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. J Immunol 161:3340-3346.
24. Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, Cassatella MA 2000 The neutrophil as a cellular source of chemokines. Immunol Rev 177:195-203.
25. Laing KJ, Secombes CJ 2004 Chemokines. Dev Comp Immunol 28:443-460.
26. Zlotnik A, Yoshie O 2000 Chemokines: a new classification system and their role in immunity. Immunity 12:121-127.
27. Rollins BJ 1997 Chemokines. Blood 90:909-928.
28. Baier RJ, Majid A, Parupia H, Loggins J, Kruger TE 2004 CC chemokine concentrations increase in respiratory distress syndrome and correlate with development of bronchopulmonary dysplasia. Pediatr Pulmonol 37:137-148.
29. Goldenberg RL, Hauth JC, Andrews WW 2000 Intrauterine infection and preterm delivery. N Engl J Med 342:1500-1507.
30. Lahra MM, Jeffery HE 2004 A fetal response to chorioamnionitis is associated with early survival after preterm birth. Am J Obstet Gynecol 190:147-151.
31. Thomas W, Speer CP 2010 Chorioamnionitis: Important Risk Factor or Innocent Bystander for Neonatal Outcome? Neonatology 99:177-187.
32. Schmidt B, Cao L, Mackensen-Haen S, Kendziorra H, Klingel K, Speer CP 2001 Chorioamnionitis and inflammation of the fetal lung. Am J Obstet Gynecol 185:173-177.
33. Ghidini A, Jenkins CB, Spong CY, Pezzullo JC, Salafia CM, Eglinton GS 1997 Elevated amniotic fluid interleukin-6 levels during the early second trimester are associated with greater risk of subsequent preterm delivery. Am J Reprod Immunol 37:227-231.
34. Wenstrom KD, Andrews WW, Hauth JC, Goldenberg RL, DuBard MB, Cliver SP 1998 Elevated second-trimester amniotic fluid interleukin-6 levels predict preterm delivery. Am J Obstet Gynecol 178:546-550.
35. Andrews WW, Hauth JC, Goldenberg RL, Gomez R, Romero R, Cassell GH 1995 Amniotic fluid interleukin-6: correlation with upper genital tract microbial colonization and gestational age in women delivered after spontaneous labor versus indicated delivery. Am J Obstet Gynecol 173:606-612.
36. Bry K, Lappalainen U, Hallman M 1997 Intraamniotic interleukin-1 accelerates surfactant protein synthesis in fetal rabbits and improves lung stability after premature birth. J Clin Invest 99:2992-2999.
37. Jobe AH, Newnham JP, Willet KE, Sly P, Ervin MG, Bachurski C, Possmayer F, Hallman M, Ikegami M 2000 Effects of antenatal endotoxin and glucocorticoids on the lungs of preterm lambs. Am J Obstet Gynecol 182:401-408.

38. Willet KE, Kramer BW, Kallapur SG, Ikegami M, Newnham JP, Moss TJ, Sly PD, Jobe AH 2002 Intra-amniotic injection of IL-1 induces inflammation and maturation in fetal sheep lung. Am J Physiol Lung Cell Mol Physiol 282:L411-420.
39. Watterberg KL, Demers LM, Scott SM, Murphy S 1996 Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. Pediatrics 97:210-215.
40. Hannaford K, Todd DA, Jeffery H, John E, Blyth K, Gilbert GL 1999 Role of ureaplasma urealyticum in lung disease of prematurity. Arch Dis Child Fetal Neonatal Ed 81:F162-167.
41. Shimoya K, Taniguchi T, Matsuzaki N, Moriyama A, Murata Y, Kitajima H, Fujimura M, Nakayama M 2000 Chorioamnionitis decreased incidence of respiratory distress syndrome by elevating fetal interleukin-6 serum concentration. Hum Reprod 15:2234-2240.
42. Andrews WW, Goldenberg RL, Faye-Petersen O, Cliver S, Goepfert AR, Hauth JC 2006 The Alabama Preterm Birth study: polymorphonuclear and mononuclear cell placental infiltrations, other markers of inflammation, and outcomes in 23- to 32-week preterm newborn infants. Am J Obstet Gynecol 195:803-808.
43. Goldenberg RL, Culhane JF 2006 Preterm birth and periodontal disease. N Engl J Med 355:1925-1927.
44. Goodnight WH, Soper DE 2005 Pneumonia in pregnancy. Crit Care Med 33:S390-397.
45. Dominitz JA, Young JC, Boyko EJ 2002 Outcomes of infants born to mothers with inflammatory bowel disease: a population-based cohort study. Am J Gastroenterol 97:641-648.
46. Antunes G, Evans SA, Lordan JL, Frew AJ 2002 Systemic cytokine levels in community-acquired pneumonia and their association with disease severity. Eur Respir J 20:990-995.
47. Pussinen PJ, Tuomisto K, Jousilahti P, Havulinna AS, Sundvall J, Salomaa V 2007 Endotoxemia, immune response to periodontal pathogens, and systemic inflammation associate with incident cardiovascular disease events. Arterioscler Thromb Vasc Biol 27:1433-1439.
48. El-Shazly S, Makhseed M, Azizieh F, Raghupathy R 2004 Increased expression of pro-inflammatory cytokines in placentas of women undergoing spontaneous preterm delivery or premature rupture of membranes. Am J Reprod Immunol 52:45-52.
49. Jobe AH, Bancalari E 2001 Bronchopulmonary dysplasia. Am J Respir Crit Care Med 163:1723-1729.
50. Fanaroff AA, Stoll BJ, Wright LL, Carlo WA, Ehrenkranz RA, Stark AR, Bauer CR, Donovan EF, Korones SB, Laptook AR, Lemons JA, Oh W, Papile LA, Shankaran S, Stevenson DK, Tyson JE, Poole WK 2007 Trends in neonatal morbidity and mortality for very low birthweight infants. Am J Obstet Gynecol 196:147 e141-148.

51. Vrijlandt EJ, Boezen HM, Gerritsen J, Stremmelaar EF, Duiverman EJ 2007 Respiratory health in prematurely born preschool children with and without bronchopulmonary dysplasia. J Pediatr 150:256-261.
52. Doyle LW, Anderson PJ 2009 Long-term outcomes of bronchopulmonary dysplasia. Semin Fetal Neonatal Med 14:391-395.
53. Northway WH, Jr., Rosan RC, Porter DY 1967 Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med 276:357-368.
54. Coalson JJ 2006 Pathology of bronchopulmonary dysplasia. Semin Perinatol 30:179-184.
55. Jobe AH, Ikegami M 1998 Mechanisms initiating lung injury in the preterm. Early Hum Dev 53:81-94.
56. Dreyfuss D, Saumon G 1998 Ventilator-induced lung injury: lessons from experimental studies. Am J Respir Crit Care Med 157:294-323.
57. Kallapur SG, Jobe AH 2006 Contribution of inflammation to lung injury and development. Arch Dis Child Fetal Neonatal Ed 91:F132-135.
58. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA 1999 Neonatal chronic lung disease in extremely immature baboons. Am J Respir Crit Care Med 160:1333-1346.
59. Albertine KH, Jones GP, Starcher BC, Bohnsack JF, Davis PL, Cho SC, Carlton DP, Bland RD 1999 Chronic lung injury in preterm lambs. Disordered respiratory tract development. Am J Respir Crit Care Med 159:945-958.
60. Hislop AA, Wigglesworth JS, Desai R, Aber V 1987 The effects of preterm delivery and mechanical ventilation on human lung growth. Early Hum Dev 15:147-164.
61. Polin RA, Sahni R 2002 Newer experience with CPAP. Semin Neonatol 7:379-389.
62. Verder H, Bohlin K, Kamper J, Lindwall R, Jonsson B 2009 Nasal CPAP and surfactant for treatment of respiratory distress syndrome and prevention of bronchopulmonary dysplasia. Acta Paediatr 98:1400-1408.
63. Wagenaar GT, ter Horst SA, van Gastelen MA, Leijser LM, Mauad T, van der Velden PA, de Heer E, Hiemstra PS, Poorthuis BJ, Walther FJ 2004 Gene expression profile and histopathology of experimental bronchopulmonary dysplasia induced by prolonged oxidative stress. Free Radic Biol Med 36:782-801.
64. Bonikos DS, Bensch KG, Ludwin SK, Northway WH, Jr. 1975 Oxygen toxicity in the newborn. The effect of prolonged 100 per cent O2 exposure on the lungs of newborn mice. Lab Invest 32:619-635.
65. Warner BB, Stuart LA, Papes RA, Wispe JR 1998 Functional and pathological effects of prolonged hyperoxia in neonatal mice. Am J Physiol 275:L110-117.
66. Roberts RJ, Weesner KM, Bucher JR 1983 Oxygen-induced alterations in lung vascular development in the newborn rat. Pediatr Res 17:368-375.
67. Han RN, Buch S, Tseu I, Young J, Christie NA, Frndova H, Lye SJ, Post M, Tanswell AK 1996 Changes in structure, mechanics, and insulin-like growth factor-related gene expression in the lungs of newborn rats exposed to air or 60% oxygen. Pediatr Res 39:921-929.

68. Merritt TA, Cochrane CG, Holcomb K, Bohl B, Hallman M, Strayer D, Edwards DKr, Gluck L 1983 Elastase and alpha 1-proteinase inhibitor activity in tracheal aspirates during respiratory distress syndrome. Role of inflammation in the pathogenesis of bronchopulmonary dysplasia. J Clin Invest 72:656-666.
69. Munshi UK, Niu JO, Siddiq MM, Parton LA 1997 Elevation of interleukin-8 and interleukin-6 precedes the influx of neutrophils in tracheal aspirates from preterm infants who develop bronchopulmonary dysplasia. Pediatr Pulmonol 24:331-336.
70. Yoon BH, Romero R, Jun JK, Park KH, Park JD, Ghezzi F, Kim BI 1997 Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. Am J Obstet Gynecol 177:825-830.
71. Rindfleisch MS, Hasday JD, Taciak V, Broderick K, Viscardi RM 1996 Potential role of interleukin-1 in the development of bronchopulmonary dysplasia. J Interferon Cytokine Res 16:365-373.
72. Baier RJ, Loggins J, Kruger TE 2001 Monocyte chemoattractant protein-1 and interleukin-8 are increased in bronchopulmonary dysplasia: relation to isolation of Ureaplasma urealyticum. J Investig Med 49:362-369.
73. Murch SH, Costeloe K, Klein NJ, MacDonald TT 1996 Early production of macrophage inflammatory protein-1 alpha occurs in respiratory distress syndrome and is associated with poor outcome. Pediatr Res 40:490-497.
74. Oue S, Hiroi M, Ogawa S, Hira S, Hasegawa M, Yamaoka S, Yasui M, Tamai H, Ogihara T 2009 Association of gastric fluid microbes at birth with severe bronchopulmonary dysplasia. Arch Dis Child Fetal Neonatal Ed 94:F17-22.
75. Verder H, Ebbesen F, Linderholm B, Robertson B, Eschen C, Arroe M, Lange A, Grytter C, Bohlin K, Bertelsen A 2003 Prediction of respiratory distress syndrome by the microbubble stability test on gastric aspirates in newborns of less than 32 weeks' gestation. Acta Paediatr 92:728-733.
76. Clyman RI 2006 Mechanisms regulating the ductus arteriosus. Biol Neonate 89:330-335.
77. Heidary G, Vanderveen D, Smith LE 2009 Retinopathy of prematurity: current concepts in molecular pathogenesis. Semin Ophthalmol 24:77-81.
78. 2005 The International Classification of Retinopathy of Prematurity revisited. Arch Ophthalmol 123:991-999.
79. Lappalainen U, Whitsett JA, Wert SE, Tichelaar JW, Bry K 2005 Interleukin-1beta causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung. Am J Respir Cell Mol Biol 32:311-318.
80. Tichelaar JW, Lu W, Whitsett JA 2000 Conditional expression of fibroblast growth factor-7 in the developing and mature lung. J Biol Chem 275:11858-11864.
81. Kistner A, Gossen M, Zimmermann F, Jerecic J, Ullmer C, Lubbert H, Bujard H 1996 Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. Proc Natl Acad Sci U S A 93:10933-10938.
82. Glasser SW, Korfhagen TR, Wert SE, Whitsett JA 1994 Transgenic models for study of pulmonary development and disease. Am J Physiol 267:L489-497.

83. Perl AK, Wert SE, Loudy DE, Shan Z, Blair PA, Whitsett JA 2005 Conditional recombination reveals distinct subsets of epithelial cells in trachea, bronchi, and alveoli. Am J Respir Cell Mol Biol 33:455-462.
84. Bry K, Whitsett JA, Lappalainen U 2007 IL-1beta disrupts postnatal lung morphogenesis in the mouse. Am J Respir Cell Mol Biol 36:32-42.
85. Sisson TH, Hansen JM, Shah M, Hanson KE, Du M, Ling T, Simon RH, Christensen PJ 2006 Expression of the reverse tetracycline-transactivator gene causes emphysema-like changes in mice. Am J Respir Cell Mol Biol 34:552-560.
86. Prophet EB, Mills B, Arrington JB, Sobin LH 1992 Armed Forces Institute of Pathology - Laboratory Methods in Histotechnology. American Registry of Pathology, Washington, D.C.
87. Lukkarinen HP, Laine J, Kaapa PO 2003 Lung epithelial cells undergo apoptosis in neonatal respiratory distress syndrome. Pediatr Res 53:254-259.
88. Ashour K, Shan L, Lee JH, Schlicher W, Wada K, Wada E, Sunday ME 2006 Bombesin inhibits alveolarization and promotes pulmonary fibrosis in newborn mice. Am J Respir Crit Care Med 173:1377-1385.
89. Hagen G, Wolf M, Katyal SL, Singh G, Beato M, Suske G 1990 Tissue-specific expression, hormonal regulation and 5'-flanking gene region of the rat Clara cell 10 kDa protein: comparison to rabbit uteroglobin. Nucleic Acids Res 18:2939-2946.
90. Ray MK, Wang G, Barrish J, Finegold MJ, DeMayo FJ 1996 Immunohistochemical localization of mouse Clara cell 10-KD protein using antibodies raised against the recombinant protein. J Histochem Cytochem 44:919-927.
91. Mukherjee AB, Zhang Z, Chilton BS 2007 Uteroglobin: a steroid-inducible immunomodulatory protein that founded the Secretoglobin superfamily. Endocr Rev 28:707-725.
92. Klug J, Beier HM, Bernard A, Chilton BS, Fleming TP, Lehrer RI, Miele L, Pattabiraman N, Singh G 2000 Uteroglobin/Clara cell 10-kDa family of proteins: nomenclature committee report. Ann N Y Acad Sci 923:348-354.
93. Tambunting F, Beharry KD, Hartleroad J, Waltzman J, Stavitsky Y, Modanlou HD 2005 Increased lung matrix metalloproteinase-9 levels in extremely premature baboons with bronchopulmonary dysplasia. Pediatr Pulmonol 39:5-14.
94. Tong Z, Wu X, Ovcharenko D, Zhu J, Chen CS, Kehrer JP 2005 Neutrophil gelatinase-associated lipocalin as a survival factor. Biochem J 391:441-448.
95. Cowland JB, Sorensen OE, Sehested M, Borregaard N 2003 Neutrophil gelatinase-associated lipocalin is up-regulated in human epithelial cells by IL-1 beta, but not by TNF-alpha. J Immunol 171:6630-6639.
96. Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA 2003 Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. J Immunol 170:3233-3242.
97. Vandal K, Rouleau P, Boivin A, Ryckman C, Talbot M, Tessier PA 2003 Blockade of S100A8 and S100A9 suppresses neutrophil migration in response to lipopolysaccharide. J Immunol 171:2602-2609.

98. Bry K, Hallman M 1993 Transforming growth factor-beta 2 prevents preterm delivery induced by interleukin-1 alpha and tumor necrosis factor-alpha in the rabbit. Am J Obstet Gynecol 168:1318-1322.
99. Sadowsky DW, Adams KM, Gravett MG, Witkin SS, Novy MJ 2006 Preterm labor is induced by intraamniotic infusions of interleukin-1beta and tumor necrosis factor-alpha but not by interleukin-6 or interleukin-8 in a nonhuman primate model. Am J Obstet Gynecol 195:1578-1589.
100. Salminen A, Paananen R, Vuolteenaho R, Metsola J, Ojaniemi M, Autio-Harmainen H, Hallman M 2008 Maternal endotoxin-induced preterm birth in mice: fetal responses in toll-like receptors, collectins, and cytokines. Pediatr Res 63:280-286.
101. Bancalari E, Gonzalez A 2000 Clinical course and lung function abnormalities during development of chronic lung disease. In Bland RD, Coalson JJ (eds) Chronic lung disease in early infancy. Marcel Dekker, Inc., New York, pp 41-64.
102. Shuhui L, Mok YK, Wong WS 2009 Role of mammalian chitinases in asthma. Int Arch Allergy Immunol 149:369-377.
103. Webb DC, McKenzie AN, Foster PS 2001 Expression of the Ym2 lectin-binding protein is dependent on interleukin (IL)-4 and IL-13 signal transduction: identification of a novel allergy-associated protein. J Biol Chem 276:41969-41976.
104. Iwashita H, Morita S, Sagiya Y, Nakanishi A 2006 Role of eosinophil chemotactic factor by T lymphocytes on airway hyperresponsiveness in a murine model of allergic asthma. Am J Respir Cell Mol Biol 35:103-109.
105. Ober C, Tan Z, Sun Y, Possick JD, Pan L, Nicolae R, Radford S, Parry RR, Heinzmann A, Deichmann KA, Lester LA, Gern JE, Lemanske RF, Jr., Nicolae DL, Elias JA, Chupp GL 2008 Effect of variation in CHI3L1 on serum YKL-40 level, risk of asthma, and lung function. N Engl J Med 358:1682-1691.
106. Cai Y, Kumar RK, Zhou J, Foster PS, Webb DC 2009 Ym1/2 promotes Th2 cytokine expression by inhibiting 12/15(S)-lipoxygenase: identification of a novel pathway for regulating allergic inflammation. J Immunol 182:5393-5399.
107. Letuve S, Kozhich A, Arouche N, Grandsaigne M, Reed J, Dombret MC, Kiener PA, Aubier M, Coyle AJ, Pretolani M 2008 YKL-40 is elevated in patients with chronic obstructive pulmonary disease and activates alveolar macrophages. J Immunol 181:5167-5173.
108. Chupp GL, Lee CG, Jarjour N, Shim YM, Holm CT, He S, Dziura JD, Reed J, Coyle AJ, Kiener P, Cullen M, Grandsaigne M, Dombret MC, Aubier M, Pretolani M, Elias JA 2007 A chitinase-like protein in the lung and circulation of patients with severe asthma. N Engl J Med 357:2016-2027.
109. McCall CE, Yoza BK 2007 Gene silencing in severe systemic inflammation. Am J Respir Crit Care Med 175:763-767.
110. Repine JE 1994 Interleukin-1-mediated acute lung injury and tolerance to oxidative injury. Environ Health Perspect 102 Suppl 10:75-78.
111. Okumura S, Sagara H, Fukuda T, Saito H, Okayama Y 2005 FcepsilonRI-mediated amphiregulin production by human mast cells increases mucin gene expression in epithelial cells. J Allergy Clin Immunol 115:272-279.

112. Kotecha S, Chan B, Azam N, Silverman M, Shaw RJ 1995 Increase in interleukin-8 and soluble intercellular adhesion molecule-1 in bronchoalveolar lavage fluid from premature infants who develop chronic lung disease. Arch Dis Child Fetal Neonatal Ed 72:F90-96.
113. Moss TJ, Newnham JP, Willett KE, Kramer BW, Jobe AH, Ikegami M 2002 Early gestational intra-amniotic endotoxin: lung function, surfactant, and morphometry. Am J Respir Crit Care Med 165:805-811.
114. Ekekezie, II, Thibeault DW, Simon SD, Norberg M, Merrill JD, Ballard RA, Ballard PL, Truog WE 2004 Low levels of tissue inhibitors of metalloproteinases with a high matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio are present in tracheal aspirate fluids of infants who develop chronic lung disease. Pediatrics 113:1709-1714.
115. Lukkarinen H, Hogmalm A, Lappalainen U, Bry K 2009 Matrix metalloproteinase-9 deficiency worsens lung injury in a model of bronchopulmonary dysplasia. Am J Respir Cell Mol Biol 41:59-68.
116. Ghezzi F, Gomez R, Romero R, Yoon BH, Edwin SS, David C, Janisse J, Mazor M 1998 Elevated interleukin-8 concentrations in amniotic fluid of mothers whose neonates subsequently develop bronchopulmonary dysplasia. Eur J Obstet Gynecol Reprod Biol 78:5-10.
117. Cayabyab RG, Jones CA, Kwong KY, Hendershott C, Lecart C, Minoo P, Ramanathan R 2003 Interleukin-1beta in the bronchoalveolar lavage fluid of premature neonates: a marker for maternal chorioamnionitis and predictor of adverse neonatal outcome. J Matern Fetal Neonatal Med 14:205-211.
118. Choi CW, Kim BI, Kim HS, Park JD, Choi JH, Son DW 2006 Increase of interleukin-6 in tracheal aspirate at birth: a predictor of subsequent bronchopulmonary dysplasia in preterm infants. Acta Paediatr 95:38-43.
119. De Dooy J, Ieven M, Stevens W, De Clerck L, Mahieu L 2007 High levels of CXCL8 in tracheal aspirate samples taken at birth are associated with adverse respiratory outcome only in preterm infants younger than 28 weeks gestation. Pediatr Pulmonol 42:193-203.
120. Thomson MA, Yoder BA, Winter VT, Giavedoni L, Chang LY, Coalson JJ 2006 Delayed extubation to nasal continuous positive airway pressure in the immature baboon model of bronchopulmonary dysplasia: lung clinical and pathological findings. Pediatrics 118:2038-2050.
121. Romero R, Ceska M, Avila C, Mazor M, Behnke E, Lindley I 1991 Neutrophil attractant/activating peptide-1/interleukin-8 in term and preterm parturition. Am J Obstet Gynecol 165:813-820.
122. Romero R, Mazor M, Brandt F, Sepulveda W, Avila C, Cotton DB, Dinarello CA 1992 Interleukin-1 alpha and interleukin-1 beta in preterm and term human parturition. Am J Reprod Immunol 27:117-123.
123. Cohen J, Ghezzi F, Romero R, Ghidini A, Mazor M, Tolosa JE, Goncalves LF, Gomez R 1996 GRO alpha in the fetomaternal and amniotic fluid compartments during pregnancy and parturition. Am J Reprod Immunol 35:23-29.

124. Keelan JA, Yang J, Romero RJ, Chaiworapongsa T, Marvin KW, Sato TA, Mitchell MD 2004 Epithelial cell-derived neutrophil-activating peptide-78 is present in fetal membranes and amniotic fluid at increased concentrations with intra-amniotic infection and preterm delivery. Biol Reprod 70:253-259.
125. Viscardi RM, Muhumuza CK, Rodriguez A, Fairchild KD, Sun CC, Gross GW, Campbell AB, Wilson PD, Hester L, Hasday JD 2004 Inflammatory markers in intrauterine and fetal blood and cerebrospinal fluid compartments are associated with adverse pulmonary and neurologic outcomes in preterm infants. Pediatr Res 55:1009-1017.
126. An H, Nishimaki S, Ohyama M, Haruki A, Naruto T, Kobayashi N, Sugai T, Kobayashi Y, Mori M, Seki K, Yokota S 2004 Interleukin-6, interleukin-8, and soluble tumor necrosis factor receptor-I in the cord blood as predictors of chronic lung disease in premature infants. Am J Obstet Gynecol 191:1649-1654.
127. Redline RW, Wilson-Costello D, Hack M 2002 Placental and other perinatal risk factors for chronic lung disease in very low birth weight infants. Pediatr Res 52:713-719.
128. Kent A, Dahlstrom JE 2004 Chorioamnionitis/funisitis and the development of bronchopulmonary dysplasia. J Paediatr Child Health 40:356-359.
129. Akram Khan M, Kuzma-O'Reilly B, Brodsky NL, Bhandari V 2006 Site-specific characteristics of infants developing bronchopulmonary dysplasia. J Perinatol 26:428-435.
130. Kaukola T, Tuimala J, Herva R, Kingsmore S, Hallman M 2009 Cord immunoproteins as predictors of respiratory outcome in preterm infants. Am J Obstet Gynecol 200:100 e101-108.
131. Van Marter LJ, Dammann O, Allred EN, Leviton A, Pagano M, Moore M, Martin C 2002 Chorioamnionitis, mechanical ventilation, and postnatal sepsis as modulators of chronic lung disease in preterm infants. J Pediatr 140:171-176.
132. Miralles R, Hodge R, McParland PC, Field DJ, Bell SC, Taylor DJ, Grant WD, Kotecha S 2005 Relationship between antenatal inflammation and antenatal infection identified by detection of microbial genes by polymerase chain reaction. Pediatr Res 57:570-577.
133. Ramsay PL, DeMayo FJ, Hegemier SE, Wearden ME, Smith CV, Welty SE 2001 Clara cell secretory protein oxidation and expression in premature infants who develop bronchopulmonary dysplasia. Am J Respir Crit Care Med 164:155-161.
134. Clyman R, Cassady G, Kirklin JK, Collins M, Philips JB, 3rd 2009 The role of patent ductus arteriosus ligation in bronchopulmonary dysplasia: reexamining a randomized controlled trial. J Pediatr 154:873-876.
135. Chorne N, Leonard C, Piecuch R, Clyman RI 2007 Patent ductus arteriosus and its treatment as risk factors for neonatal and neurodevelopmental morbidity. Pediatrics 119:1165-1174.