Mechanisms of Lung Injury in a Mouse Model of Bronchopulmonary Dysplasia

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2009

ISBN 978-91-628-7969-3
Internet-id: http://hdl.handle.net/2077/21084

Printed by Chalmers Reproservice, Gothenburg, Sweden, 2009

ABSTRACT

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that affects preterm infants. Increased levels of inflammatory mediators in the amniotic fluid and in the lungs of preterm infants are associated with the development of BPD. It has been shown that infant transgenic mice that express interleukin (IL)-1β in the lung epithelium from approximately embryonal day 14 (pseudoglandular stage of lung development) develop a pulmonary injury that resembles BPD, supporting the idea that inflammation plays an important role in the pathogenesis of BPD. The mechanisms by which inflammation causes lung injury have not been identified.

The aim of this thesis was to define mechanisms by which perinatal inflammatory lung injury develops by using transgenic mice that express IL-1β in the lung epithelium in an inducible manner.

The β6 integrin subunit has previously been shown to be involved in the progression of pulmonary diseases in adult mice. To investigate the involvement of the β6 integrin subunit in IL-1β-induced lung disease in the neonate, lung development of IL-1β-expressing mice lacking the β6 integrin subunit were compared with that of IL-1β-expressing mice with wild-type β6 loci. Absence of the β6 integrin subunit alleviated the IL-1β-induced lung injury, as demonstrated by smaller alveoli, thinner alveolar walls, and a milder lung inflammation than IL-1β-expressing mice with wild-type β6 integrin loci. The results suggest that the β6 integrin subunit plays a role in the development of neonatal lung disease.

Increased levels of matrix metalloproteinase (MMP)-9 and an imbalance between proteases and antiproteases in the lungs of infants and animals developing BPD have led to the hypothesis that MMP-9 may be involved in the pathogenesis of the disease. No differences in lung histology were detected between mice with wild-type MMP-9 loci and mice with null MMP-9 loci, implying a non-essential role of MMP-9 during lung development. However, IL-1β caused a more severe alveolar hypoplasia in mice deficient in MMP-9 than in MMP-9 wild-type mice, suggesting that MMP-9 may have a protective role during inflammatory lung injury.

A short-term exposure of IL-1 has been shown to accelerate development of the surfactant system in fetal rabbits and lambs. Using transgenic mice where the expression of IL-1β is restricted to the distal lung epithelium, the effects on lung development and function of chronic prenatal IL-1β production were studied. Distal lung expression of IL-1β disrupted acinar bud formation prior to birth and decreased the expression of the important surfactant proteins SP-B and SP-C. The 100% mortality observed among the IL-1β-expressing mice was probably due to the inflammation-induced structural changes and to deficient surfactant function. The results suggest that an early and continuous inflammatory stimulus in the distal lung epithelium causes severe lung injury and disrupts surfactant production.

LIST OF PAPERS

The thesis is based on the following papers:

I. Beta6 integrin subunit deficiency alleviates lung injury in a mouse model of bronchopulmonary dysplasia.
Hogmalm A, Sheppard D, Lappalainen U, Bry K.
Am J Respir Cell Mol Biol In press. E-publ. Aug 28, 2009.

II. Matrix metalloproteinase-9 deficiency worsens lung injury in a model of bronchopulmonary dysplasia.
Lukkarinen H, Hogmalm A, Lappalainen U, Bry K.
Am J Respir Cell Mol Biol 2009;41(1):59-68.

III. Expression of IL-1β in the distal lung epithelium disrupts lung development in fetal mice.
Hogmalm A, Lappalainen U, Bry K.
Manuscript

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LIST OF ABBREVIATIONS

AB	alcian blue
ALI	acute lung injury
BAL	bronchoalveolar lavage
BPD	bronchopulmonary dysplasia
CCSP	Clara cell secretory protein
CMV	cytomegalovirus
CXCR2	CXC chemokine receptor 2
E	embryonal day
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbet assay
hIL-1β	human interleukin-1β
HPF	high power field
hSP-C	human surfactant protein-C
ICAM	intercellular adhesion molecule
IL	interleukin
KC	keratinocyte-derived chemokine
MCP	monocyte chemoattractant protein
MIP	macrophage-inflammatory protein
MMP	matrix metalloproteinase
PAS	periodic acid Schiff
PBS	phosphate buffered saline
PECAM	platelet endothelial cell adhesion molecule
PN	postnatal day
RDS	respiratory distress syndrome
rCCSP	rat Clara cell secretory protein
RT-PCR	real-time polymerase chain reaction
rtTA	reverse tetracycline transactivator
SAA	serum amyloid A
SP	surfactant protein
tetO	tetracycline operator
TIMP	tissue inhibitor of metalloproteinase
TGF	transforming growth factor
TUNEL	TdT-mediated dUTP nick-end labeling
TTF	thyroid transcription factor
VEGF	vascular endothelial growth factor

INTRODUCTION

Structure of the lung

The main function of the lung is to transport oxygen from the air into the bloodstream and to release carbon dioxide from the bloodstream to the atmosphere. The gas exchange is performed by epithelial cells within the thin alveolar-capillary membrane in the peripheral lung (Marieb, 2001). The lungs of humans, as well as mice, are composed of five lobes. In humans, the left lung is divided into two lobes and the right lung into three, whereas the left lung of the mouse forms a single lobe and the right lung is subdivided into four lobes (superior, middle, postcaval, and inferior) (Braun *et al.*, 2004).

The respiratory system consists of conductive and respiratory zones. The conductive zone contains the trachea, the bronchi, the bronchioles, and the terminal bronchioles. The airway epithelium contains ciliated cells, basal cells, and mucus-producing goblet cells. The bronchiolar epithelium contains non-ciliated Clara cells that secrete Clara cells secretory protein (CCSP). The principal roles of the conductive zone are to transfer air towards the site of gas exchange, to humidify and warm the inhaled air, and to remove irritants such as dust and bacteria to protect the airway from injury. The respiratory zone contains the respiratory bronchioles, the alveolar ducts and alveoli, which are the actual sites of gas exchange (Marieb, 2001).

The alveolar epithelium consists mainly of thin squamous alveolar type I cells, which together with the closely situated capillary endothelial cells form the thin alveolar-capillary membrane that is responsible for gas exchange. The alveolar epithelium also consists of cuboidal type II epithelial cells that secrete surfactant (Marieb, 2001). When the alveolar epithelium is exposed to toxic agents that lead to destruction of the type I epithelial cells, type II epithelial cells can proliferate and may act as precursor cells for type I cells (Adamson and Bowden, 1974; Mendelson, 2000). In addition to specialized epithelial cells, the alveoli contains alveolar macrophages that internalize and destroy foreign material, such as infectious microorganims, and clear the alveoli from damaged or old epithelial cells (Marieb, 2001).

Lung development

During the development of the lung, branching morphogenesis occurs to increase the lung surface area to provide sufficient respiratory function after birth. The development of the mammalian lung can be divided into five stages; the embryonic, the pseudoglandular, the canalicular, the saccular, and the alveolar stage (Table 1) (Burri, 2006; Maeda *et al.*, 2007).

Table 1. Timing of the developmental stages in human and mouse

Stage	**Human (weeks)** Term 40 weeks	**Mouse (days)** Term 19 days
Embryonic	3 – 7	9 – 12
Pseudoglandular	5 – 17	12 – 16
Canalicular	16 – 26	16 – 17
Saccular	24 – 38	17 – PN 5
Alveolar	36 – (1-2 years)	PN 4 – 28

PN = postnatal day

The embryonic stage

The embryonic stage of lung development begins with the formation of an outgrowth from the primitive foregut endodermal epithelium. The outgrowth separates from the primitive esophagus to form the tracheal rudiment and gives rise to two primary bronchial buds, which branch into the surrounding mesenchyme to divide the lung into the five lobes. The pulmonary vessels arise from the aortic arches and the left atrium and grow into the mesenchyme along the developing airways (Jobe, 2002; Maeda *et al.*, 2007).

The pseudoglandular stage

During the pseudoglandular stage, the branching of airways and vascular system continues and forms the bronchial tree with conductive airways, terminal bronchioles, and primitive acinar structures. Differentiation of epithelial cells results in the appearance of ciliated cells, goblet cells and basal cells in the main bronchi (Jobe, 2002). The expression of surfactant proteins during the pseudoglandular stage (Khoor *et al.*, 1993; Khoor *et al.*, 1994; Wert *et al.*, 1993) indicates epithelial cell differentiation in the primitive acinar structures during this stage of lung development. The vascular system develops along the bronchial and bronchiolar tubules (Jobe, 2002).

The canalicular stage

The respiratory structures undergo further subdivision and widening to form clusters of acinar tubules and buds during the canalicular stage. The epithelial differentiation is concentrated in the peripheral part of the respiratory tree, and this is accompanied by the growth and development of intra-acinar double capillary network in close relation with the epithelium (Jobe, 2002). During this stage, differentiated bronchiolar Clara cells start to synthesize CCSP (Singh *et al.*, 1988; Zhou *et al.*, 1996a), and the cuboidal cells differentiate into specialized type II epithelial cells and subsequently into type I epithelial cells that subsequently will form the alveolar epithelium (Jobe, 2002).

The saccular stage

During the saccular stage, the segments distal to the terminal bronchioles dilate and expand, resulting in formation of alveolar saccules and ducts, and in reduction of interstitial tissue. The peripheral epithelial cells continue to differentiate, and the capillaries become more closely associated with the type I epithelial cells. Elastin is deposited in areas where the future alveolar septa will form to create alveoli from the terminal alveolar saccules (Burri, 2006; Jobe, 2002).

The alveolar stage

During the alveolar stage, the saccules subdivide into the smaller alveoli. The formed alveolar walls (secondary septa) contain a double capillary and connective tissue. During alveolarization, a single capillary layer is formed to enable efficient gas exchange after birth (Burri, 2006).

In the human lung, alveoli begin to form during the weeks preceding birth but the large majority of alveoli are formed postnatally (Burri, 2006). Alveolarization continues into early childhood (1-2 years). At birth, 50-150 million alveoli exist in the human lung, whereas the adult lung contains 300 million alveoli (Burri, 2006; Jobe, 2002). Since the mouse is born

during the saccular stage, its alveolarization takes place postnatally beginning around postnatal day (PN) 4, but as in humans the alveoli continue to develop beyond the neonatal period.

Surfactant

The thin layer of liquid that lines the alveolar epithelium contains surfactant, the primary functions of which are to lower the surface tension, thus preventing the alveoli from collapsing at the end of expiration, and to enhance compliance. Lung surfactant is a complex of phospholipids (80-90%) and proteins (~10%) synthesized and secreted primarily by alveolar type II cells. Disaturated phospatidylcholine accounts for ~50% of surfactant content and allows packing of the surface film that reduces surface tension to nearly zero. There are four different surfactant proteins, the hydrophobic surfactant protein (SP)-B and SP-C and the hydrophilic SP-A and SP-D (Jobe, 2002). The expression of these proteins is developmentally regulated (Mendelson, 2000).

SP-B

SP-B is required for the formation of lamellar bodies in type II cells and for the spreading of lipids in the surface film to enhance the stability of the film, to enhancing proper surfactant function (Clark *et al.*, 1995; Jobe, 2002). SP-B knock-out mice develop severe neonatal lung disease and die immediately after birth (Clark *et al.*, 1995). Similarly, hereditary SP-B deficiency is lethal in human neonates (Clark and Clark, 2005). Since absence of SP-B inhibits the processing of SP-C (Vorbroker *et al.*, 1995), decreased production of SP-B in premature infants may also decrease SP-C.

SP-C

SP-C contributes to lowering of surface tension but its presence is not essential for surfactant function as SP-C-deficient mice are born without signs of respiratory distress (Glasser *et al.*, 2001; Glasser *et al.*, 2003). In the aging mouse, however, lack of SP-C causes interstitial lung disease and emphysema (large alveoli) associated with inflammation (Glasser *et al.*, 2003). SP-C may be involved in the recycling of surfactant since SP-C increases the reuptake of surfactant phospholipids into type II epithelial cells *in vitro* (Horowitz *et al.*, 1996). SP-C may also participate in innate host defense (Augusto *et al.*, 2003; Glasser *et al.*, 2008).

SP-A and SP-D

Mice deficient in SP-A or SP-C appear to have normal lung function and do not develop symptoms of neonatal respiratory disease (Botas *et al.*, 1998; Korfhagen *et al.*, 1996). However, lack of SP-D induces an accumulation of surfactant in the lumen of alveoli, suggesting a role of SP-D in surfactant homeostasis (Botas *et al.*, 1998). SP-A and SP-D have important roles in host defense of the lung. They bind microorganisms and facilitate the uptake of infectious pathogens by alveolar macrophages (Jobe, 2002). Mice lacking either SP-A or SP-D are more susceptible to bacterial and viral infections than wild-type mice (LeVine *et al.*, 1999a; 1999b; 2000; 2004).

Premature birth and RDS

The incidence of preterm delivery, i.e. birth at gestational age of less than 37 weeks, is approximately 13% in the United States (Martin *et al.*, 2007), whereas only 5-6% of births in Sweden are preterm (Ringborg *et al.*, 2006).

Due to lung immaturity, preterm infants are at a risk of developing respiratory distress syndrome (RDS, or hyaline-membrane disease). Fanaroff *et al*. and Lemons *et al*. reported that 44% and 50%, respectively, of preterm infants with a birth weight of <1500 g (VLBW) develop RDS (Fanaroff *et al.*, 2007; Lemons *et al.*, 2001). According to a Swedish study, 37% of infants born before 32 weeks' gestational age develop RDS (Lundqvist *et al.*, 2009). The incidence of RDS increases with lower birth weight and lower gestational age (Fanaroff *et al.*, 2007; Lemons *et al.*, 2001). The lungs of infants who die from RDS display diffuse atelectasis and very few dilated alveoli and have membranes of fibrotic material and cellular debris from the injured epithelium that line the airspaces (Rodriguez *et al.*, 2002). RDS is a risk factor for the development of chronic lung disease of the neonate, also called bronchopulmonary dysplasia (BPD).

Bronchopulmonary dysplasia

Definition and incidence

BPD is a chronic pulmonary disease that affects premature infants. For infants born before 32 weeks gestational age, BPD is defined as the need for supplemental oxygen for at least 28 days and the severity (mild, moderate or severe) of BPD is determined by the need for oxygen at 36 weeks' postmenstrual age or at discharge (Jobe and Bancalari, 2001). Previously, BPD was defined either as oxygen requirement for at least 28 days or as oxygen requirement at 36 weeks' postmenstrual age. BPD occurs primarily in premature infants weighing less than 1000 g at birth and born at 24-26 weeks of gestation, i.e. during the canalicular/saccular stage when neither the alveolar nor the distal vascular development is completed (Coalson, 2006). These infants have clinical signs of respiratory disease, such as tachypnea and retractions (Jobe and Bancalari, 2001). The most important risk factor for BPD is lung immaturity, as the incidence of BPD is negatively correlated to birth weight and gestational age (Fanaroff *et al.*, 2007; Lemons *et al.*, 2001). The incidence of BPD, defined as requirement of supplemental oxygen at 36 weeks' postmenstrual age, is ~50% in infants with birth weight 501-750 g, ~34% in infants with birth weight 751-1000 g, ~15% in infants with birth weight 1001-1250 g, and ~7% in infants with birth weight 1251-1500 g (Fanaroff *et al*., 2007; Lemons *et al*., 2001).

Pathology

BPD was first described by Northway and co-workers in 1967 as a lung injury caused by mechanical ventilation and oxygen support when treating preterm infants for RDS (Northway *et al.*, 1967). This "old" BPD was characterized by fewer alveoli, fibrosis, severe airway epithelial hyperplasia, and areas of overinflation or atelectasis. Since then, improved mechanical ventilatory strategies, the use of antenatal glucocorticosteriods, and exogenous surfactant treatment have resulted in the survival of more immature and smaller preterm infants (Coalson, 2006). Thus, the lung injury seen in infants with BPD has changed, and the "new" BPD pathology is displayed as extreme lung immaturity and is characterized by large alveoli, impaired vascular development, and an inflammatory response. In contrast to the

"old" BPD, "new" BPD has less pronounced airway hyperplasia and fibroproliferation, and does not show areas of severe overinflation (Coalson, 2003; 2006).

Long-term outcomes for BPD infants

Infants with BPD are more likely to need prolonged hospitalization and readmission in the first years of life than preterm infants without BPD (Vrijlandt *et al.*, 2007). Some infants with BPD continue to show function abnormalities into childhood and even early adulthood (Doyle *et al.*, 2006; Wong *et al.*, 2008; Vrijlandt *et al.*, 2007). Due to their immaturity at birth, infants with BPD are at risk of poor neurodevelopmental outcome (Ehrenkranz *et al.*, 2005; Schmidt *et al.*, 2003).

Inflammation

The immune system comprises the innate immune system and the adapted immune system. Innate immunity provides the first line of defense that is not specific to a particular pathogen. However, the innate immune system has the ability to recognize a given class of molecules. Toll-like receptors (TLRs) for example recognizes lipopolysaccharide (LPS) of Gram-negative bacteria, triggering an inflammatory response. Neutrophils and macrophages which can remove pathogens and damaged cells by phagocytosis are important cells of the innate immune system. The adaptive immune response, which includes T and B lymphocytes, is specific to a particular antigen challenge and includes self- and non-self recognition and immunologic memory (Goldsby *et al.*, 2003).

In the lung, the respiratory epithelium lined by mucus traps microorganisms, and ciliated epithelial cells remove the mucus from the lung. The resident alveolar macrophages represent, together with the respiratory epithelium, the first line of defense in the lung, and macrophages are important in the clearance of foreign material by phagocytosis (Marieb, 2001). When activated, alveolar macrophages secrete cytokines and chemokines that attract neutrophils and monocytes from the circulation (Maus *et al.*, 2002). Cytokines are peptides and proteins that modulate the activity of cells under normal and pathological conditions. Many cytokines are either pro- or anti-inflammatory. However, the same cytokine can have both pro- and anti-inflammatory properties. A chemokine is a cytokine that mediates chemoattraction.

As previously mentioned, the collectins SP-A and SP-D can bind bacteria to enhance their susceptibility to phagocytosis (Jobe, 2002). In addition to leukocytes, bronchiolar epithelial cells and alveolar type II cells are capable of secreting chemoattractants (Standiford, *et al.*, 1998; van der Velden *et al.*, 1998) to promote the infiltration of inflammatory cells to the lung (Fehrenbach, 2001; O'Brien *et al.*, 1998). Neutrophils are often the first inflammatory cells to infiltrate the site of inflammatory response (Kaplanski *et al.*, 2003). It has been suggested that they are important for the subsequent increase in lung monocytes/macrophages (Janardhan *et al.*, 2006).

Inflammation and BPD

Most preterm deliveries that occur before 30 weeks of gestation are associated with antenatal infection, e.g. chorioamnionitis (Goldenberg *et al.*, 2000). Yoon *et al*. demonstrated that amniotic fluid of mothers whose infants later developed BPD had elevated levels of several pro-inflammatory mediators, including interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor α (Yoon *et al.*, 1997). Elevated levels of IL-6 in cord plasma are associated with BPD

after preterm delivery (Yoon *et al.*, 1999). Preterm infants with early tracheal colonization are at higher risk of developing BPD (Young *et al.*, 2005).

Ogden *et al*. demonstrated that neutrophils infiltrate the lungs of preterm infants with RDS soon after birth (Ogden *et al.*, 1983). The neutrophil count in bronchoalveolar lavage (BAL) fluid declines rapidly by the end of the first week of life in infants who recover from RDS. In contrast, in infants who develop BPD, the levels of neutrophils are even higher than in RDS infants and remain elevated for several weeks (Ogden *et al.*, 1983). Increased numbers of alveolar macrophages soon after birth are also associated with the development of RDS and BPD, and infants who develop BPD have higher numbers of macrophages than infants who recover from RDS (Merritt *et al.*, 1983). In addition, the development of BPD is associated with increased levels of pro-inflammatory and chemotactic factors, such as IL-1β, IL-6, IL-8, CC chemokines and transforming growth factor (TGF)-β1 in the lungs of preterm infants (Baier *et al.*, 2004; Kakkera *et al.*, 2005; Kotecha *et al.*, 1996a; Kotecha *et al.*, 1996b; Lecart *et al.*, 2000; Munshi *et al.*, 1997; Tullus *et al.*, 1996). Infants developing BPD have increased levels of intercellular adhesion molecule (ICAM)-1 and E-selectin, which enable circulating inflammatory cells to transmigrate into the tissue (Kim *et al.*, 2004; Ramsay *et al.*, 1998). During the inflammatory process, activated inflammatory cells and resident pulmonary cells contribute to an increased production of proteinases that may play an important role in the development of injury. Infants who develop BPD have an imbalance between proteases and antiproteases (Speer, 2006). Mechanical ventilation and supplemental oxygen given to preterm infants can cause an inflammatory response and interrupt the alveolar and vascular development of the lung (Coalson *et al.*, 1999; Deng *et al.*, 2000; Jobe *et al.*, 2002; Warner *et al.*, 1998; Yoder *et al.*, 2000), and may possibly thus accelerate the development of injury and BPD in the already inflamed lung.

Interleukin-1β

IL-1β is a highly inflammatory cytokine that affects nearly every cell type and is an important part of the inflammatory response. IL-1β is, together with IL-1α and IL-1 receptor antagonist (IL-1Ra), a member of the IL-1 gene family. IL-1α and IL-1β are structurally related, and both are synthesized as precursors and act through the same cellular receptors (Dinarello, 1997; Krakauer and Oppenheim, 1998).

Synthesis of IL-1β

IL-1β is produced by monocytes, macrophages, and to a lesser extent by neutrophils, epithelial cells, endothelial cells, and fibroblasts. It is synthesized within the cell as a precursor that at first remains within the cytoplasm, although a small amount of pro-IL-1β can be secreted. The intracellular 31 kDa pro-IL-1β is processed into the mature 17 kDa form primarily by the IL-1 converting enzyme (ICE or caspase-1) (Krakauer and Oppenheim, 1998). Precursor IL-1β can also be cleaved by elastase, chymotropsin (a mast cell chymase), granzyme A, and matrix metalloproteinases (MMPs), e.g. MMP-9 (Dinarello, 2002; Dinarello, 1997).

IL-1β receptors and IL-1β-signaling

IL-1β can bind two kind of receptors, IL-1 receptor type I (IL-1RI) and IL-1 receptor type II (IL-1RII). IL-1RI is expressed on e.g. endothelial cells, epithelial cells, fibroblasts, and T lymphocytes. Binding of IL-1β to IL-1RI results in signal transduction. In contrast, binding of

IL-1β to IL-1RII does not give rise to an intracellular signal. IL-1β can also bind a soluble form of IL-1RII. This bond is nearly irreversible and prevents IL-1β actions. Another IL-1β-antagonist is IL-1Ra that competes with IL-1β to bind to IL-1RI, thereby preventing IL-1β from inducing a biological response (Dinarello, 1997; Krakauer and Oppenheim, 1998). However, a significant inhibition of IL-1β's biological effects is only achieved when a large proportion (70-80%) of the binding of IL-1β is blocked (Dayer, 2002).

Effects of IL-1β

IL-1β is a proinflammatory cytokine that is involved in the initiation and persistence of inflammatory response. This cytokine affects a large variety of cells and contributes to many responses in the human body. For example, the production of several pro-inflammatory cytokines and chemokines, e.g. IL-1β itself, IL-6, IL-8, tumor necrosis factor α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) and of complement compotent 5a (C5a) is induced by IL-1β in endothelial cells, monocytes/macrophages, and fibroblasts, thus increasing the attraction and activity of neutrophils, monocytes, and macrophages (Krakauer and Oppenheim, 1998). By inducing the expression of the cell adhesion molecules ICAM-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin on vascular endothelial cells, IL-1β participates in the recruitment of leukocytes to the tissue (Dinarello, 2002; Krakauer and Oppenheim, 1998).

IL-1β-expressing mouse as a model of BPD

Elevated levels of IL-1β in the amniotic fluid and in the lungs of preterm infants are associated with the development of BPD (Kakkera *et al.*, 2005; Kotecha *et al.*, 1996; Yoon *et al.*, 1997). Bry *et al*. recently demonstrated that perinatal expression of human IL-1β (hIL-1β) in the lung epithelium causes a pulmonary disease resembling BPD in infant mice (Bry *et al.*, 2007). These transgenic mice express the hIL-1β transgene from approximately embryonal day (E) 14 in the proximal lung epithelium, and to some extent in the distal lung epithelium (Perl *et al.*, 2002). Postnatally, IL-1β-expressing mice display pulmonary inflammation with neutrophils and macrophages, large alveoli, thick alveolar walls, and abnormal capillary structure in the alveolar walls (Bry *et al.*, 2007). In addition, mice expressing IL-1β have poor postnatal growth and high mortality. Since the mice were not subjected to other potential causes of injury, such as mechanical ventilation, oxygen therapy, or preterm delivery, the study demonstrated that IL-1β-induced inflammation by itself can promote the development of a BPD-like illness. This model provides an opportunity to identify mechanisms of inflammatory lung injury in the neonate.

The αvβ6 integrin

Integrins are heterodimeric transmembrane glycoproteins that are involved in cellular growth, migration, differentiation, and survival by mediating interactions between cells and between cells and the extracellular matrix (ECM) (Hynes, 2002; Sheppard, 2003). Integrins are expressed in many different cell types and organs. One integrin is often capable of binding different ligands, and one specific ligand can often bind to different integrin receptors. Integrin ligation can affect the expression of genes encoding MMPs, cytokines, inhibitors of cell cycle progression, and factors involved in apoptosis (Sheppard, 1998). Each integrin is composed of one α-subunit and one β-subunit. To date, 18 human α-subunits and 8 β-subunits have been identified. These subunits form 24 different heterodimers (Sheppard, 2003). Some

integrins are involved in inflammation, such as β2- and α4-integrins, which mediate leukocyte adhesion by binding adhesion molecules on endothelial cells, as well as the αvβ6 integrin.

Expression and function of the αvβ6 integrin

The β6 integrin subunit was first identified in primary cultures of airway epithelial cells (Sheppard *et al.*, 1990). This subunit binds exclusively the αv subunit, forming the αvβ6 integrin (Busk *et al.*, 1992). The expression of the αvβ6 integrin has been detected predominantly in epithelial cells (Breuss *et al.*, 1993; Breuss *et al.*, 1995). Breuss *et al.* showed that this integrin is expressed basally or at cell–cell borders in the human fetal lung (~18 weeks' gestational age) in epithelial cells of the branching distal airways, while it is generally absent from proximal airways (Breuss *et al.*, 1995). In the newborn monkey lung, αvβ6 integrin is strongly expressed in alveolar epithelial cells and in cells lining the respiratory bronchioles, but is rarely detected in airways. In healthy adult primates, the expression of the αvβ6 integrin is undetectable except in the uterus and kidney. Pulmonary expression of the β6 integrin subunit is markedly upregulated in response to injury and inflammation. Injury of the airway epithelium induces the expression of several integrin subunits, including the αv subunit and the β6 subunit, suggesting that the αvβ6 integrin participates in epithelial repair (Dosanjh *et al.*, 2004; Pilewski *et al.*, 1997). In skin wounds, the expression of the αvβ6 integrin is highly induced in keratinocytes soon after injury (Breuss *et al.*, 1995; Haapasalmi *et al.*, 1996), and its expression is important for keratinocyte migration *in vitro* (Huang *et al.*, 1998a), suggesting a possible role of the αvβ6 integrin in migration of epithelial cells to traumatized areas. However, the αvβ6 integrin is not essential to the healing of epidermal wounds (Huang *et al.*, 1998b). The expression of αvβ6 integrin is also induced in alveolar type II epithelial cells after acute lung injury (ALI) (Breuss *et al.*, 1995), and in the respiratory epithelium of smokers (Weinacker *et al.*, 1995), and in adult patients with allergic alveolitis, chronic obstructive pulmonary disease (COPD) (Breuss *et al.*, 1995), or cystic fibrosis (Pilewski *et al.*, 1997).

The αvβ6 integrin mediates attachment of epithelial cells to the ECM by binding fibronectin (Busk *et al.*, 1992; Weinacker *et al.*, 1994), vitronectin (Huang *et al.*, 1998a) or tenascin-C (Prieto *et al.*, 1993; Yokosaki *et al.*, 1996) and mediates the spreading and proliferation of epithelial cells (Agrez *et al.*, 1994). The αvβ6 integrin is an *in vivo* activator of the profibrotic cytokine TGF-β1 (Munger *et al.*, 1999), which is thought to be involved in BPD (Kotecha *et al.*, 1996a; Lecart *et al.*, 2000; Vicencio *et al.*, 2004), inflammation (Sheppard, 2006), and adult pulmonary diseases (Munger *et al.*, 1999; Pittet *et al.*, 2001).

Knock-out mice lacking the β6 integrin subunit

To identify *in vivo* functions of the αvβ6 integrin, the study of knock-out mice lacking the β6 integrin subunit (Huang *et al.*, 1996) has been a useful approach, as the β6 subunit pairs exclusively with the αv subunit. The αvβ6 integrin is not essential to normal embryonic development, since β6 knock-out mice homozygous for the null mutation are born in expected Mendelian ratios (Huang *et al.*, 1996). Mice lacking the β6 integrin reproduce normally and appear healthy until adulthood, except for juvenile baldness on forehead, neck and thighs. However, adult β6-deficient mice develop spontaneous lung inflammation (Huang *et al.*, 1996; Huang *et al.*, 1998b) and age-related MMP-12-dependent emphysema (Morris *et al.*, 2003). Surprisingly, β6-deficient mice are protected from bleomycin-induced fibrosis, a pulmonary disease often preceded by inflammation (Munger *et al.*, 1999). Absence of the β6 integrin subunit also protects adult mice from bleomycin-induced ALI (Pittet *et al.*, 2001).

Since spontaneous lung inflammation in β6-deficient mice precedes the bleomycin-induced injury in these studies, it is suggested that the spontaneous inflammation may have rendered the lungs resistant to subsequent injury (Pittet *et al.*, 2001). It was recently demonstrated that absence of the αvβ6 integrin protects adult mice from IL-1β-induced ALI by completely inhibiting IL-1β-mediated protein permeability across alveolar epithelial cells (Ganter *et al.*, 2008). The role of the αvβ6 integrin in pulmonary diseases of the fetus or newborn has not previously been investigated.

Matrix metalloproteinase-9

MMPs, a family of zinc-dependent proteinases, are divided into several subclasses according to substrate specificity and structural characteristics. The group of gelatinases includes MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Gelatinases degrade ECM proteins, contribute to tissue remodeling, and modulate cell migration, vascularization, and the activity of inflammatory factors (Atkinson and Senior, 2003; Chakrabarti and Patel, 2005).

Expression of MMP-9

MMP-9 is expressed during lung development, but it is not essential to normal lung development since the lungs of MMP-9-deficient mice appear normal (Betsuyaku *et al.*, 2000; Chakrabarti and Patel, 2005). The adult lung normally does not contain MMP-9, but inflammation induces transcription of MMP-9 and attracts neutrophils that secrete pro-MMP-9. Resident cells such as bronchiolar epithelial cells, Clara cells, alveolar type II cells, fibroblasts, and endothelial cells produce MMP-9 when stimulated. MMP-9 is also produced by leukocytes, most notably neutrophils, which produce MMP-9 and secrete MMP-9 from granules in an inducible manner (Atkinson and Senior, 2003; Chakrabarti and Patel, 2005).

MMP-9 is released as a proenzyme, requiring activation by cleavage of the prodomain by e.g. stromelysin-1 (MMP-3) or MMP-2. The activity of MMP-9 is controlled by the expression of tissue inhibitor of metalloproteinase (TIMP)-1, which can bind and inactivate both active MMP-9 and pro-MMP-9 (Atkinson and Senior, 2003; Chakrabarti and Patel, 2005).

MMP-9 function

MMP-9 degrades gelatin, collagen, elastin, vitronectin and fibonectin, as well as cytokines and growth factors (Atkinson and Senior, 2003; Chakrabarti and Patel, 2005). It also activates pro-IL-1β (Schönbeck *et al.*, 1998) and latent TGF-β (Yu and Stamenkovic, 2000). MMP-9 changes the structure of the neutrophil chemoattractant IL-8, thus increasing its chemotactic activity (Van den Steen *et al.*, 2000), and by degrading α1-antitrypsin MMP-9 protects the activity of neutrophil elastase (Liu *et al.*, 2000). On the other hand, MMP-9 decreases the attraction of inflammatory cells by inactivating keratinocyte-derived chemokine (KC) (Van den Steen *et al.*, 2000).

MMP-9 in lung injury

MMP-9-deficient adult mice are protected from IL-13-induced emphysema and are partially protected from edema in bleomycin-induced ALI despite high numbers of inflammatory cells in BAL fluid (Lanone *et al.*, 2002; Warner *et al.*, 2001), suggesting that MMP-9 is involved in adult pulmonary disease.

Increased levels of MMP-9 and an imbalance between MMP-9 and TIMP-1 in preterm infants have been associated with the development of BPD (Ekekezie *et al.*, 2004; Fukunaga *et al.*, 2009; Schulz *et al.*, 2004). In addition, elevated expression of MMP-9 and higher ratios of MMP-9 to TIMP-1 have been observed in a baboon model of BPD (Tambunting *et al.*, 2005). Decreased expression of MMP-9 has been associated with arrested alveolarization in hyperoxia-exposed newborn rat lungs (Hosford *et al.*, 2004), whereas increased production of MMP-9 were induced by hyperoxia in another study (Cederqvist *et al.*, 2006). Recently, MMP-9-deficient newborn mice have been shown to be protected from hyperoxia-induced lung injury (Chetty *et al.*, 2008). Blocking the activity of MMP (Ekekezie *et al.*, 2004), or more specifically, of MMP-9 (Chetty *et al.*, 2008) has been suggested as a potential therapeutic approach to prevent BPD. On the other hand, Albaiceta *et al*. demonstrated that lack of MMP-9 worsens ventilator-induced lung injury (Albaiceta *et al.*, 2008). Thus, the role of MMP-9 in BPD is poorly defined.

AIMS OF THE THESIS

The general purpose of this thesis was to identify mechanisms involved in inflammation-induced lung injury using a transgenic mouse model of neonatal lung disease. In these mice, hIL-1β is expressed in the lung epithelium in an inducible manner.

The specific aims were:

- To investigate the role of the β6 integrin subunit in IL-1β-induced lung injury in newborn mice.

- To investigate the role of MMP-9 in IL-1β-induced lung injury in newborn mice.

- To study how expression of IL-1β in the distal lung epithelium affects pulmonary development and the expression of surfactant proteins in the lungs of fetal mice.

MATERIALS AND METHODS

The tetracycline-dependent transgenic mouse model (Papers I-III)

The tetracycline-inducible system (Gossen and Bujard, 1992; Gossen *et al.*, 1995; Kistner *et al.*, 1996) consists of two transgenic mouse lines: an activator line and an operator line. The activator line expresses the reverse tetracycline transactivator (rtTA) transgene that is driven by either the rat CCSP (rCCSP) promoter or the human SP-C (hSP-C) promoter. Both of these promoters express rtTA in the lung epithelium. The rCCSP promoter directs the expression of rtTA primarily to bronchiolar cells but also to some alveolar cells, whereas the hSP-C promoter results in the expression of rtTA primarily by type II epithelial cells (Akeson *et al.*, 2003; Perl *et al.*, 2002; Perl *et al.*, 2009). In the present studies, the operator line contains the $(tetO)_7$ tetracycline operator, the inactive cytomegalovirus (CMV) minimal promoter, and the target transgene, mature hIL-1β (Lappalainen *et al.*, 2005). Mice bearing the rCCSP-rtTA or hSP-C-rtTA transgene continuously express rtTA from ~E14 or ~E11, respectively (Bry *et al.*, 2007; Perl *et al.*, 2002). In the presence of doxycycline, rtTA binds the $(tetO)_7$ element, thus activating the CMV promoter that induces the expression of the target transgene. To be able to express the target transgene, the mouse needs to have both transgenic constructs (these mice are referred to as bitransgenic mice) and receive doxycycline. Single-transgenic mice bearing only one of the transgenic constructs (the activator line or the operator line) are not able to express the target transgene.

To produce bitransgenic rCCSP-rtTA/$(tetO)_7$-CMV-hIL-1β offspring and single-transgenic rCCSP-rtTA offspring, mice bearing the rCCSP-rtTA construct were mated with mice bearing the $(tetO)_7$-CMV-hIL-1β construct (Fig. 1) (Papers I, II). To produce bitransgenic hSP-C-rtTA/$(tetO)_7$-CMV-hIL-1β offspring and single-transgenic hSP-C-rtTA offspring, mice bearing the hSP-C-rtTA construct were mated with mice bearing the $(tetO)_7$-CMV-hIL-1β construct (Paper III). Doxycycline administered to pregnant and nursing dams passes transplacentally to the fetuses and via milk to the pups, resulting in expression of IL-1β in bitransgenic offspring but not in single-transgenic offspring (Fig. 1). The expression of IL-1β in bitransgenic mice is induced by doxycycline from ~E11 in the SP-C model (Paper III) and from ~E14 in the CCSP model (Papers I, II).

Since the expression of rtTA, controlled by the rCCSP promoter or the hSP-C promoter, can affect the structure and function of the lung (Sisson *et al.*, 2006), it is necessary to use single-transgenic rCCSP-rtTA and hSP-C-rtTA littermates as controls to specifically study the effects of IL-1β in rCCSP-rtTA/$(tetO)_7$-CMV-hIL-1β and hSP-C-rtTA/$(tetO)_7$-CMV-hIL-1β mice, respectively.

To investigate the role of the β6 integrin subunit in IL-1β-induced lung injury (Paper I), mice lacking both alleles of the β6 integrin subunit ($\beta6^{-/-}$) were mated with transgenic rCCSP-rtTA mice and transgenic $(tetO)_7$-CMV-hIL-1β mice to produce rCCSP-rtTA $\beta6^{-/-}$ mice and $(tetO)_7$-CMV-hIL-1β $\beta6^{-/-}$ mice. The single-transgenic offspring were then mated to produce bitransgenic rCCSP-rtTA/$(tetO)_7$-CMV-hIL-1β $\beta6^{-/-}$ offspring and single-transgenic rCCSP-rtTA $\beta6^{-/-}$ offspring. Abbreviations used for transgenic mice are shown in Table 2.

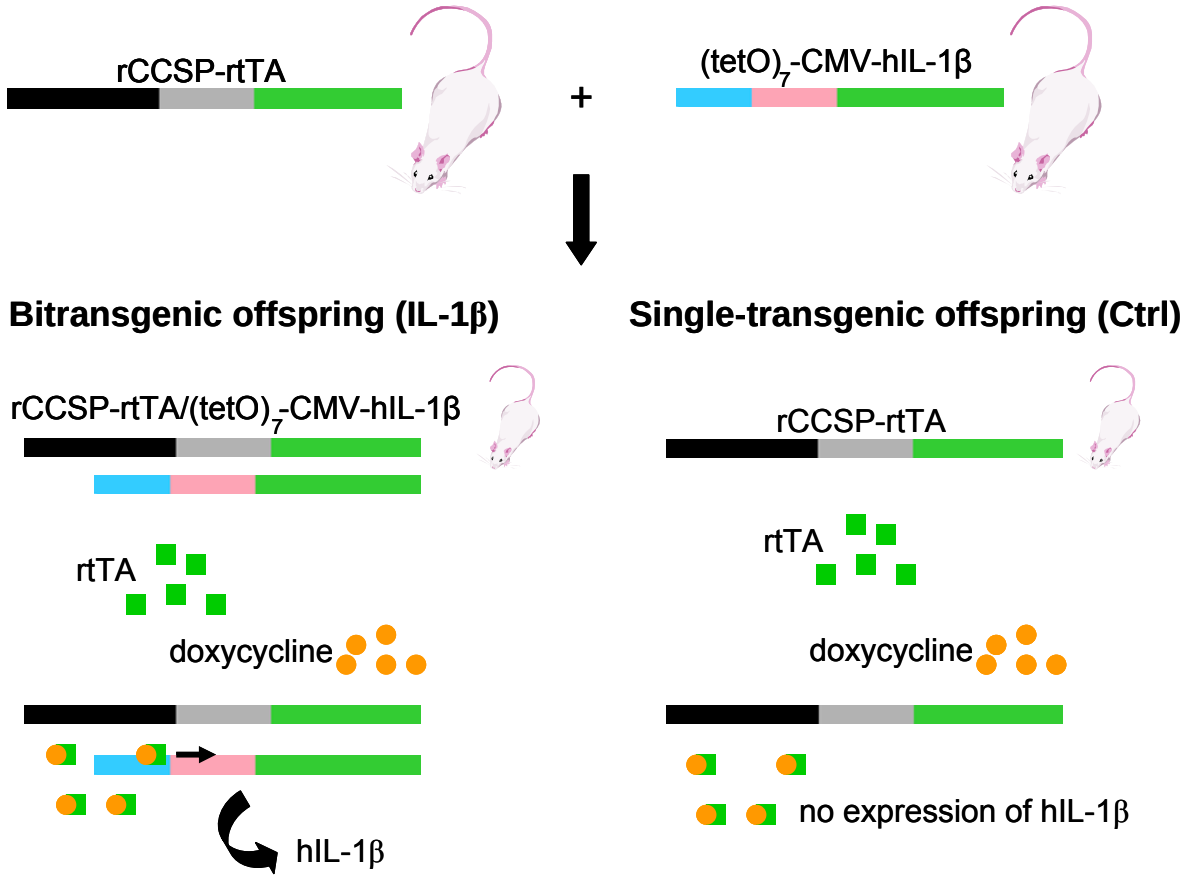

Figure 1. To produce bitransgenic (rCCSP-rtTA/$(tetO)_7$-CMV-hIL-1β) offspring and littermate single-transgenic (rCCSP-rtTA) offspring, mice bearing the rCCSP-rtTA transgenic construct were mated with mice bearing the $(tetO)_7$-CMV-hIL-1β transgenic construct. In the presence of doxycycline, rtTA binds the $(tetO)_7$ element in the bitransgenic offspring and the expression of IL-1β is induced. In the single-transgenic offspring, rtTA is present, but since the mouse does not have the $(tetO)_7$-CMV-hIL-1β transgenic construct, IL-1β cannot be induced. In Paper III, the rtTA expression was similarly controlled by the hSP-C promoter.

Table 2. Abbreviations used for transgenic mice

Abbreviation	Transgene	Paper
control/$\beta6^{+/+}$	rCCSP-rtTA mouse with wild-type integrin β6 loci	I
control/$\beta6^{-/-}$	rCCSP-rtTA mouse with null integrin β6 loci	I
IL-1β/$\beta6^{+/+}$	rCCSP-rtTA/$(tetO)_7$-CMV-hIL-1β mouse with wild-type integrin β6 loci	I
IL-1β/$\beta6^{-/-}$	rCCSP-rtTA/$(tetO)_7$-CMV-hIL-1β mouse with null integrin β6 loci	I
control/MMP-$9^{+/+}$	rCCSP-rtTA mouse with wild-type MMP-9 loci	II
control/MMP-$9^{-/-}$	rCCSP-rtTA mouse with null MMP-9 loci	II
IL-1β/MMP-$9^{+/+}$	rCCSP-rtTA/$(tetO)_7$-CMV-hIL-1β mouse with wild-type MMP-9 loci	II
IL-1β/MMP-$9^{-/-}$	rCCSP-rtTA/$(tetO)_7$-CMV-hIL-1β mouse with null MMP-9 loci	II

To investigate the role of MMP-9 in IL-1β-induced lung injury (Paper II), mice lacking both alleles of MMP-9 (MMP-$9^{-/-}$) were mated with transgenic rCCSP-rtTA mice and transgenic $(tetO)_7$-CMV-hIL-1β mice to produce rCCSP-rtTA MMP-$9^{-/-}$ mice and $(tetO)_7$-CMV-hIL-1β MMP-$9^{-/-}$ mice. The single-transgenic offspring were then mated to produce bitransgenic

rCCSP-rtTA/$(tetO)_7$-CMV-hIL-1β MMP-$9^{-/-}$ offspring and single-transgenic rCCSP-rtTA MMP-$9^{-/-}$ offspring. Abbreviations used for transgenic mice are shown in Table 2.

All mice were in FVB/N background.

Genotype determination (Papers I-III)

DNA was extracted from mouse tails for genotyping by polymerase chain reation (PCR) analysis with subsequent separation on agarose gel containing ethidium bromide and detection with UV light. The specific primers (5' to 3′) for transgenic constructs are given in Table 3.

Table 3. Primers used for genotyping by PCR

Construct/Gene	Primer sequence (5' - 3')	Paper
rCCSP-rtTA	F in rCCSP: ACT GCC CAT TGC CCA AAC AC	I, II
	R in rtTA: AAA ATC TTG CCA GCT TTC CCC	
hSP-C-rtTA	F in hSP-C: GAC ACA TAT AAG ACC CTG GTC A	III
	R in rtTA: AAA ATC TTG CCA GCT TTC CCC	
$(tetO)_7$-CMV-hIL-1β	F in CMV: CCA TCC ACG CTG TTT TGA CC	I, II, III
	R in hIL-1β: ACG GGC ATG TTT TCT GCT TG	
β6 integrin	F: TAG CTT CCA GCC AAG GTG GG	I
	R: TCT GAG GGA CTG GTA TGT GTG TCC	
MMP-9	F: GTG GGA CCA TCA TAA CAT CAC A	II
	R: CTC GCG GCA AGT CTT CAG AGT A	

Administration of doxycycline (Papers I-III)

Doxycycline (0.5 mg/ml, Sigma, St. Louis, MO) was administered in drinking water to pregnant and nursing dams to induce hIL-1β transgene expression in the lungs of bitransgenic fetuses and pups. The administration of doxycycline extended from the beginning of pregnancy until sacrifice of fetuses on E15 (Paper III) or E18.5 (Paper III) or of pups on PN0 (Paper I), PN4 (Paper II) or PN7 (Papers I-III). Since the doxycycline solution is light-sensitive and its activity decreases at room temperature after 72 h (Perl *et al.*, 2002), cage bottles with doxycycline were covered with aluminum foil and the solution was changed three times per week.

Animal care (Papers I-III)

The mice were housed in pathogen-free conditions at Experimental Biomedicine, University of Gothenburg and all animal experiments were approved by the Ethical Committee of Gothenburg. All animals were given access to water and chow *ad libitum*. For sample collection of fetal lungs on E14, E15 or E18.5, fetuses were removed by hysterectomy after anesthesia by intraperitoneal injection of a mixture of ketamine, xylazine, and acepromazine to the pregnant dam. For lung sample collection from infant mice on PN0, PN4 or PN7, pups were anesthetized by intraperitoneal injection of a mixture of ketamine, xylazine, and acepromazine, the abdomen was opened, and the animal was exsanguinated by transection of the abdominal aorta. The day of plug was counted as E0 and the day of birth as PN0. After birth, the pups were counted each day to assess survival data until sample collection. Fetuses and pups were weighed at the time of sacrifice.

Protein measurements with ELISA (Papers I-III)

Lung tissue was homogenized in sterile-filtered phosphate buffered saline (PBS) containing protease inhibitor (Complete; Roche Diagnostics, Basel, Switzerland) and centrifuged at 10,000 g at 4ºC for 10 min to remove cell debris prior to analysis of the collected supernatant. Total protein concentration was measured using the bicinchoninic acid method (Sigma). To measure hIL-1β in whole lung homogenates (Papers I-III), enzyme-linked immunosorbent assay (ELISA) DuoSet specific for human IL-1β, with no cross-reactivity with murine IL-1β, was used (R&D Systems, Abingdon, UK).

DuoSet ELISA development kits (R&D Systems) were used to quantify mouse KC (Papers I, II), macrophage-inflammatory protein (MIP)-2 (Paper I), monocyte chemoattractant protein (MCP)-1 (Papers I, II), osteopontin (Paper I), and vascular endothelial growth factor (VEGF; Paper II). Assay standard concentration ranges were 3.9-250 pg/ml (IL-1β, MCP-1), 7.8-1000 pg/ml (VEGF) and 15.6-1000 pg/ml (KC, MIP-2, osteopontin).

Active TGF-β1 was measured according to manufacturer's instructions (R&D Systems) (Paper I). To enable determination of total TGF-β1 levels, acidification of the samples was performed according to manufacturer's instructions (R&D Systems) to transform latent TGF-β1 into the immunoreactive form. After acidification the samples were neutralized prior to ELISA. Assay standard concentration range was 7.8-1000 pg/ml.

Western blot to detect degraded hIL-1β (Paper II)

Since MMPs can degrade mature IL-1β (Ito *et al.*, 1996; Schönbeck *et al.*, 1998), the presence of degraded hIL-1β (17 kDa) in whole lung homogenates was studied in MMP-$9^{+/+}$ and MMP-$9^{-/-}$ mice by western blotting. Lung tissue was homogenized in sterile-filtered PBS containing protease inhibitor (Complete; Roche). The detergent Nonidet P-40 (Roche Diagnostics) was added to the homogenized tissue to liberate membrane-bound proteins before centrifugation at 10,000 g at 4ºC for 10 min. The supernatant was collected for analysis. Equal amounts of total protein were separated by molecular mass by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-12% gradient gel (Invitrogen, Carlsbad, CA). A protein ladder (10-200 kDa; Cell Signaling Technology, Danvers, MA) was also loaded on the gel. After electrophoresis, proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Bioscience, Little Chalfont, UK). The membrane was blocked with 5% milk powder (Cell Signaling Technology) before overnight (4ºC) incubation with the primary anti-human IL-1β rabbit polyclonal antibody (Abcam, Cambridge, UK). Thereafter, the membrane was incubated with anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (Cell Signaling Technology) and anti-biotin HRP-linked antibody (Cell Signaling Technology). Enhanced chemiluminescence was used according to manufacturer's instructions (Lumigen Inc., Southfield, MI) to visualize the proteins, which were detected in a luminescence image analyzer (LAS-100, Fujifilm; Stamford, CT) and quantified with Image Gauge v4.2 software (Fujifilm). After detection of hIL-1β the same membrane was used to detect β-actin as a loading control, and the densiometric values for IL-1β were normalized to β-actin.

Lung histology (Papers I-III)

For fixation of postnatal lung samples, the lung was inflation-fixed by instillation of 4% PBS-buffered paraformaldehyde at a pressure of 25 cm H_2O. The fixative was perfused into the lung via a blunt cannula inserted in the trachea. After fixation the lungs were taken out of the

thoracic cavity and placed in 4% PBS-buffered paraformaldehyde. For sample collection of embryonal lungs, the lungs were transferred to 4% PBS-buffered paraformaldehyde without preceding inflation fixation. After overnight fixation at 4°C, the tissue was dehydrated through a graded series of ethanol and xylene prior to paraffin embedding. Five-micrometer thick tissue sections were used for staining with hematoxylin and eosin, alcian blue/periodic acid Schiff (AB/PAS), and for immunohistochemistry.

Determination of the alveolar chord length (Papers I-III)

Chord length analysis of the distal lung was used as a measure of the size of the airspaces. A minimum of ten representative non-overlapping fields (20x lens) per lung section stained with hematoxylin and eosin were acquired in 8-bit grayscale image using a Nikon Eclipse E800 microscope and a Nikon DXM1200 digital camera. Final magnification was 1.89 pixels per micrometer. Chord length analysis was performed using the public domain program NIH Image (available from the U.S. National Institutes of Health at http://rsb.info.nih.gov/nih-image) to measure the intra-alveolar distance in binarized images of lung tissue at PN4 or PN7. Areas of bronchiolar airways and blood vessels were not included in the analysis. The mean chord length in a single image was calculated using the NIH Image program.

Determination of alveolar wall thickness (Papers I, II)

The same images that were used for measuring the mean chord length were used for measuring the thickness of distal airspace walls at PN4 or PN7 with ImageJ (available from NIH at http://rsb.info.nih.gov/nih-image) (Papers I, II). A minimum of ten non-overlapping fields (images) were analyzed by drawing at least 30 straight lines at 90º angles across the narrowest segment of the wall. The mean length of lines crossing the walls was determined using ImageJ.

Quantification of airspaces in the fetal lung (Paper III)

Airspace area fraction at E18.5 was measured in lung sections stained with hematoxilin and eosin. Using the public domain program ImageJ, the percentage of airspace area of total lung area was determined using binarized lung images. Five representative non-overlapping fields from the lungs of five mice per group were analyzed. The same images were also used to count the number of airspaces.

Detection of mucus-producing cells (Papers I, II)

To visualize mucus production in airway cells, AB/PAS staining was performed as previously described (Cook, 1996) (Papers I, II). Briefly, dehydrated paraffin sections were stained in alcian blue solution (pH 2.5) to detect acid mucins followed by detection of neutral mucins with periodic acid and Schiff's reagent (Merck, Darmstadt, Germany). Nuclei were stained with Mayer's hematoxilin. The number of AB/PAS-positive and AB/PAS-negative cells within the airways was counted and the percentage of positive cells per airway was calculated. At least ten airways were analyzed per section. The distribution of airways having various percentages of AB/PAS-positive cells (<20%, 20-80%, or >80%) was compared between different genotypes (Paper I).

Immunohistochemistry (Papers I-III)

For immunohistochemistry, lung tissue sections were deparaffinized and rehydrated. Citrate buffer (pH 6) (CCSP, Mac3, MMP-9, neutrophil-7/4, pro-SP-C, SP-B) or 0.1% trypsin (platelet endothelial cell adhesion molecule (PECAM)-1) was used as antigen retrieval and methanol with hydrogen peroxide was used to block endogenous peroxidase. Sections were incubated with appropriate serum to block non-specific binding before the use of primary antibodies (Table 4) and biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA). After incubation with a secondary antibody, sections were incubated with avidin-biotin peroxidase (Vectastain Elite ABC; Vector Laboratories) and immunoreactivity was visualized using peroxidase substrate; 3.3-diaminobenzidine (DAB; Vector Laboratories; MMP-9, pro-SP-C, SP-B) or NovaRED (Vector Laboraties; CCSP, Mac3, neutrophil-7/4, PECAM-1). Sections were lightly counterstained with nuclear fast red after incubation with DAB or with Mayer's hematoxylin (nuclear counterstaining) after incubation with NovaRED. Immunostained cells were counted in the distal airspaces and/or distal septal walls in at least ten non-overlapping high power fields (HPFs, 400x or 1000x magnification) from at least four animals per group. Due to the small lung size at E14 (Paper II), neutrophils and macrophages were counted in five HPFs. In Papers I and III, the number of positive cells per square millimeter septa or square millimeter airspace was calculated.

Table 4. Antibodies used for immunohistochemistry

Primary antibody	Source	Dilution	Company	Paper
CCSP	Rabbit polyclonal	1:500	Seven Hills Bioreagents Cincinnati, OH	III
Ki-67	Rabbit polyclonal	1:500	Novocastra Laboratories Ltd. Newcastle-upon-Tyne, UK	I, III
Mac3, clone M3/84	Rat monoclonal	1:50	BD PharMingen, San Diego, CA	I, II, III
MMP-9	Goat polyclonal	1:100	R&D Systems, Abingdon, UK	II
Neutrophils, clone 7/4	Rat monoclonal	1:50	Serotec, Oxford, UK	I, II, III
PECAM-1 (CD31)	Rat monoclonal		BD PharMingen, San Diego, CA	II
Pro-SP-C	Rabbit polyclonal	1:1000	Chemicon International Temecula, CA	III
SP-B	Rabbit polyclonal	1:500	Chemicon International Temecula, CA	III

Detection of proliferating cells with Ki-67 immunostaining (Papers I, III)

Five-micrometer thick lung tissue sections were deparaffinized, rehydrated and treated with citrate buffer (pH 6) and methanol with hydrogen peroxide. After incubation with goat serum, polyclonal rabbit anti-human Ki-67 antibody that cross-reacts with murine Ki-67 (Table 4) was used to detect proliferating cells (Scholzen and Gerdes, 2000), followed by biotinylated goat anti-rabbit secondary antibody (Vector Laboratories). Avidin-biotin peroxidase (Vectastain Elite ABC; Vector Laboratories) and DAB (Vector Laboratories) were used according to manufacturer's instructions, and sections were counterstained with nuclear fast red. The number of Ki-67-positive cells was counted in the distal lung in at least ten non-overlapping HPFs (400x magnification).

Detection of apoptotic cells with TUNEL (Papers I-III)

During apoptosis, 3' strand breaks occur within the DNA. These breaks can be detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL). The template-independent TdT (Roche Diagnostics) catalyzes the addition of nucleotides to the 3'-end of single- and double-stranded DNA. The TUNEL method was performed on 5 μm thick paraffin-embedded lung tissue sections as previously described (Lukkarinen *et al.*, 2003). Briefly, TdT-incorporated digoxigenin-dideoxyuridine-triphospate (dig-ddUTP, Roche) into fragmented DNA was detected with an anti-digoxigenin alkaline phosphates-labeled antibody (Roche Diagnostics). Addition of 5-Bromo-4-chromo-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Vector Laboratories) resulted in a substrate color reaction, regarded as representing DNA fragmentation. TUNEL-positive cells were counted in the distal lung in at least 10 non-overlapping fields.

Quantitative real-time RT-PCR (Papers I-III)

Lung tissue was transferred to RNA-stabilizing solution (RNA-later; Ambion, Austin, TX) immediately after opening of the thorax. Total RNA was isolated using TRIzol reagent according to manufacturer's instructions (Invitrogen) and treated with RNase-free DNase (DNA-free; Ambion). After measuring the RNA concentration (NanoDrop Technologies Inc., Wilmington, DE), one microgram of total RNA was reverse transcribed (Omniscript; Qiagen, Hilden, Germany) into cDNA. Twenty nanograms of cDNA were analyzed by quantitative real-time (RT) PCR on Mx3000P RT-PCR instrument (Stratagene, La Jolla, CA) using Brilliant SYBR Green Q-PCR Master mix (Stratagene) and primers specific for each gene (Table 5). The results were normalized to β-actin mRNA levels.

Gelatin zymography (Paper II)

Zymography was used to evaluate the gelatinolytic activity of MMP-2 and MMP-9. Lung tissue was homogenized in sterile PBS and centrifuged at 10,000 g at 4ºC for 10 min to remove cell debris. The supernatant was used for analysis. Total protein concentration was measured using bicinchoninic acid method (Sigma). Equal amounts of total protein, together with Novex Tris-Glycine sodium dodecylsulphate (SDS) sample buffer (Invitrogen), were loaded on a 10% Novex Zymogram gel containing gelatin (Invitrogen) and processed according to manufacturer's instructions. Proteins were separated according to molecular mass on the Zymogram gel by electrophoresis. To distinguish between the different MMPs, the position of the samples after separation was compared to a ladder containing proteins of known molecular masses, also loaded on the Zymogram gel. The MMPs, including the pro-forms, were activated and allowed to digest the gelatin of the gel. Thereafter, the gels were stained with Simply Blue (Invitrogen) in order to obtain a dark-blue background to visualize the enzymatic activity as clear bands, which were quantified using NIH Image Software.

Statistical analysis (Papers I-III)

Data are presented as mean ± SEM. Groups of normally distributed data were compared with Student's t test followed by Bonferroni correction. Two-way ANOVA was used for analysis of time-dependent MMP-9 mRNA expression (Paper II). Postnatal survival data were analyzed using Kaplan-Meier survival analysis and logrank test (Papers I-III). P values < 0.05 were considered statistically significant. Statistical analysis was assessed using GraphPad Prism™ software (GraphPad Software Inc., San Diego, CA).

Table 5. Primers used for quantitative RT-PCR

Gene	Primer sequence (5' - 3')	Paper
β-actin	F: TCC GTA AAG ACC TCT ATG CCA ACA	I, II, III
	R: CTC AGG AGG AGC AAT GAT CTT GAT	
CCSP	F: GAT ACC CTC CCA CAA GAG ACC AGG ATA	III
	R: GGC AGT GAC AAG GCT TTA GCA GTA GAA	
CXCR2	F: CCT CAG ACT TTT GGC TTC CTC GT	I
	R: CGC AGT GTG AAC CCG TAG CAG A	
hIL-1β	F: CCA TCC ACG CTG TTT TGA CCT C	I, II, III
	R: ACC AAG CTT TTT TGC TGT GAG TCC	
KC	F: AAA CCG AAG TCA TAG CCA CAC TCA	I, II, III
	R: CTT GGG GAC ACC TTT TAG CAT CTT	
MCP-1	F: GCT CTC TCT TCC TCC ACC ACC AT	I, II
	R: GCT CTC CAG CCT ACT CAT TGG GAT	
MCP-3	F: TCT GCC ACG CTT CTG TGC CT	I, III
	R: GCT CTT GAG ATT CCT CTT GGG GAT	
MIP-2	F: CCC CCT GGT TCA GAA AAT CAT C	I
	R: AAC TCT CAG ACA GCG AGG CAC ATC	
MMP-9	F: TTC GCA GAC CAA GAG GGT TTT C	II
	R: AAG ATG TCG TGT GAG TTC CAG GGC	
Osteopontin	F: CGG TGA AAG TGA CTG ATT CTG GCA	I
	R: CGC AAG GAG ATT CTG CTT CTG AGA	
Pendrin	F: GCA GAA CCA GGT CAA ATC CAG A	III
	R: TCT CAG GAA GCA AGT CTA CGC A	
S100A8	F: GAG CAA CCT CAT TGA TGT CTA	I, III
	R: TGC ATT GTC ACT ATT GAT GTC CA	
S100A9	F: GCC AAC AAA GCA CCT TCT CAG AT	I, III
	R: GCC ATC AGC ATC ATA CAC TCC TCA A	
SAA3	F: TGC TCG GGG GAA CTA TGA TGC T	I, III
	R: CCA CTC GTT GGC AAA CTG GTC A	
SP-A	F: GGA GCT TCA GAC TGC ACT CTA CGA GA	III
	R: GAC TGA CTG CCC ATT GGT GGA AA	
SP-B	F: CCA AAC CCC ACA CCT CT GAGA A	III
	R: GCT TGT CCT CTG GAG CAG GCT	
SP-C	F: GAT ACT GGT TCC GAG TCC GAT TCT	III
	R: TTC TAC CGA CCC TGT GGA TGC T	
SP-D	F: AGA GGT TGC CTT CTC CCA CTA TCA	III
	R: GCC CAC ATC TGT CAT ACT CAG GAA	
TIMP-1	F: AAG TCC CAG AAC CGC AGT GAA GA	II
	R: TCC GTC CAC AAA CAG TGA GTG TCA	
TIMP-2	F: CCC TCT GTG ACT TCA TTG TGC CCT	II
	R: TGG TGC CCA TTG ATG CTC TTC TCT	
TTF-1	F: GCT GCC GCC TTA CCA GGA	III
	R: CGT GGG TGT CAG GTG AAT CAT	
VEGF-A	F: CAC CCA CGA CAG AAG GAG AGC A	III
	R: GCA CAC AGG ACG GCT TGA AGA TGT A	
Ym1	F: GCT CAT TGT GGG ATT TCC AGC A	I, III
	R: CCT CAG TGG CTC CTT CAT TCA GAA	
Ym2	F: TTG GAG GAT GGA AGT TTG GAC CT	I, III
	R: TGA CGG TTC TGA GGA GTA GAG ACC A	

F: forward primer, R: reverse primer

RESULTS

Paper I

To study the role of the β6 integrin subunit in IL-1β-induced lung disease in infant mice, the lung development was studied in mice expressing IL-1β with wild-type or null β6 integrin loci.

Similar production of IL-1β in IL-1β/β6$^{+/+}$ and IL-1β/β6$^{-/-}$ mice

Absence of the β6 integrin subunit did not change the mRNA expression or protein production of IL-1β in bitransgenic mice.

Absence of β6 integrin subunit improved growth and survival in IL-1β-expressing mice

IL-1β inhibited the postnatal growth in both β6$^{+/+}$ and β6$^{-/-}$ mice, but to a lower degree in the absence of the β6 integrin subunit despite the lower body weight of control/β6$^{-/-}$ mice compared to control/β6$^{+/+}$ mice at PN7 (Paper I; Fig. 1A). Only ~50% of the IL-1β/β6$^{+/+}$ mice survived until PN7, whereas 98% of the IL-1β/β6$^{-/-}$ mice survived until PN7 (Paper I; Fig. 1B).

Absence of β6 integrin subunit ameliorated alveolar development in IL-1β-expressing mice

Absence of the β6 integrin did not cause detectable changes in alveolar size or alveolar wall thickness at PN7 in control mice (Fig. 2) (Paper I; Fig. 2A-F). Perinatal production of IL-1β in bitransgenic mice disrupted alveolarization in β6$^{+/+}$ and in β6$^{-/-}$ mice, as demonstrated by greater alveolar chord length (a measure of alveolar size) (Fig. 2), and thicker alveolar walls than in littermate controls. Interestingly, IL-1β/β6$^{-/-}$ mice had smaller alveoli and thinner septal walls than IL-1β/β6$^{+/+}$ mice (Fig. 2), indicating better alveolar development during chronic inflammation in infant mice lacking the αvβ6 integrin.

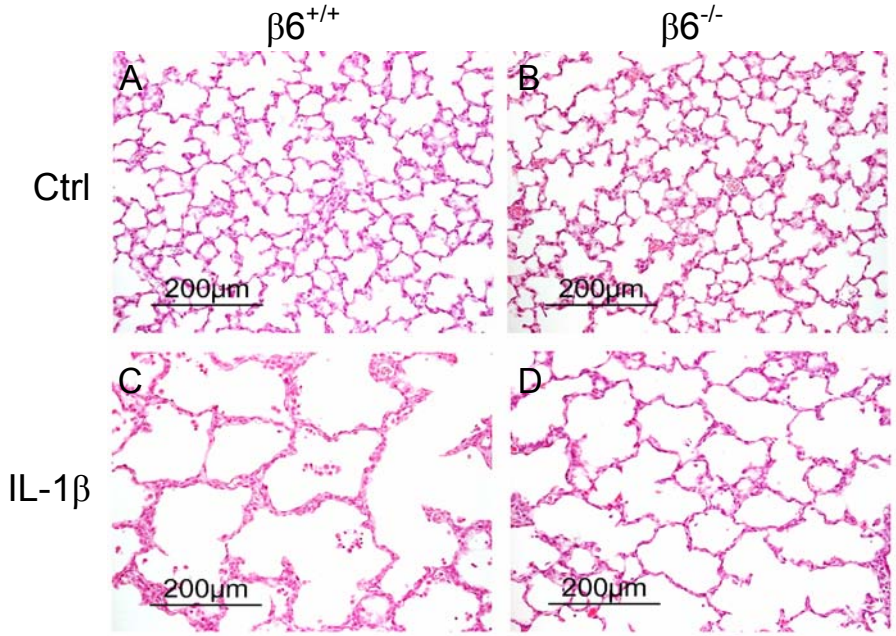

Figure 2. Lung histology at PN7 of control mice (Ctrl) with (A) wild-type β6 integrin subunit loci (β6$^{+/+}$) or (B) null β6 integrin subunit loci (β6$^{-/-}$) and IL-1β-expressing mice (IL-1β) with (C) wild-type β6 integrin subunit loci (β6$^{+/+}$) or (D) null β6 integrin subunit loci (β6$^{-/-}$).

Absence of β6 integrin subunit alleviated the IL-1β-induced inflammation

Expression of IL-1β caused infiltration of the lungs of $\beta6^{+/+}$ and $\beta6^{-/-}$ mice by neutrophils and macrophages (Paper I; Fig. 3A-F). While the IL-1β/$\beta6^{-/-}$ mice had similar numbers of neutrophils within the distal septa as IL-1β/$\beta6^{+/+}$ mice, the number of neutrophils within the alveolar spaces was lower in IL-1β/$\beta6^{-/-}$ mice. The distribution of neutrophils between the alveolar septa and lumen was different in $\beta6^{+/+}$ and $\beta6^{-/-}$ mice: 69% of neutrophils in IL-1β/$\beta6^{+/+}$ mice, but only 39% of neutrophils in IL-1β/$\beta6^{-/-}$ mice, were in the alveolar spaces (Fig. 3). This indicates that absence of the β6 integrin subunit affected the transmigration of neutrophils into the alveolar spaces. In addition, absence of the β6 integrin subunit suppressed the IL-1β-induced increase of macrophages in the alveolar spaces and septa.

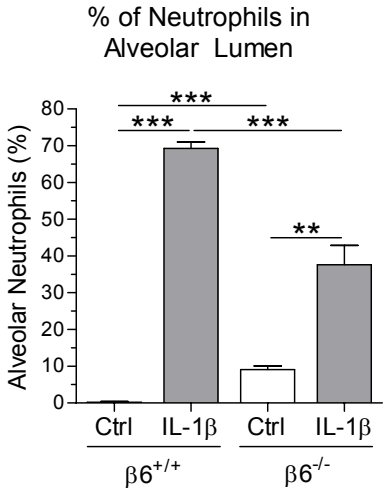

Figure 3. Precentage (%) of total distal neutrophils detected in the alveolar spaces at PN7 in control (Ctrl) and IL-1β-expressing (IL-1β) mice with wild-type ($\beta6^{+/+}$) or null ($\beta6^{-/-}$) β6 integrin loci.

The IL-1β-induced infiltration of inflammatory cells was associated with enhanced expression and production of the neutrophil chemoattractants KC and MIP-2, the monocyte chemoattractants MCP-1 and MCP-3, as well as the chemotactic proteins S100A8, S100A9, serum amyloid A3 (SAA3) and osteopontin in both $\beta6^{+/+}$ and $\beta6^{-/-}$ mice (Paper I; Fig. 4A-D, Fig. 4F-4H, Fig. 5A-E). However, the IL-1β-induced levels of KC, MIP-2, S100A8, S100A9, SAA3 and osteopontin were lower in the absence of the β6 integrin subunit. In contrast, MCP-1 and MCP-3 levels were elevated in the lungs of IL-1β/$\beta6^{-/-}$ mice compared to IL-1β/$\beta6^{+/+}$ mice. The expression of the CXC chemokine receptor CXCR2 was similarly elevated by IL-1β in $\beta6^{+/+}$ and $\beta6^{-/-}$ mice (Paper I; Fig. 4E).

At PN7, control/$\beta6^{-/-}$ mice had an increased number of neutrophils in the alveolar lumen, and elevated expression and production of the neutrophil- and macrophage-chemoattractants KC, MIP-2, MCP-1, MCP-3, S100A8, S100A9, and SAA3 compared with control/$\beta6^{+/+}$ mice (Paper I; Fig. 3A, Fig. 4A-4D, Fig. 4F-4H, Fig. 5A-C). In contrast, the numbers of macrophages in control/$\beta6^{-/-}$ and control/$\beta6^{+/+}$ mice were similar, and the level of osteopontin, a macrophage chemotactic protein, was lower in control/$\beta6^{-/-}$ mice (Paper I; Fig. 3D-E, Fig. 5D-E).

Pittet *et al.* suggested that spontaneous inflammation observed in adult $\beta6^{-/-}$ mice conditions their lungs to resist subsequent injury (Pittet *et al.*, 2001). Since control/$\beta6^{-/-}$ mice had more alveolar neutrophils and greater expression of several chemoattractants at PN7 than control/$\beta6^{+/+}$ mice, we sought to determine if this spontaneous inflammatory response was present at E14, the time at which production of hIL-1β starts in the lungs of bitransgenic mice. Such a spontaneous inflammation might explain the resistance of $\beta6^{-/-}$ mice to IL-1β-induced inflammatory lung injury. At E14, the expression of none of the chemoattractants investigated was higher in control/$\beta6^{-/-}$ than in control/$\beta6^{+/+}$ fetuses (Paper I; Table 1). In addition, the numbers of lung neutrophils and macrophages were similar in control/$\beta6^{-/-}$ and control/$\beta6^{+/+}$ fetuses (Paper I; Table 1). Thus, absence of the β6 integrin subunit did not cause spontaneous inflammation in the lungs of fetal mice.

Absence of β6 integrin subunit alleviated IL-1β-induced goblet cell hyperplasia

In addition to morphological changes in the distal lung of IL-1β-expressing mice, the proximal airways were affected by IL-1β. Expression of IL-1β caused thickening and goblet cell hyperplasia of the airway epithelium and accumulation of large numbers of lymphocytes around the airways of $\beta6^{+/+}$ mice. These pathological changes induced by IL-1β were suppressed in the absence of the β6 integrin subunit (Paper I; Fig. 6A-E). In addition, the IL-1β-induced expression of the chitinase-like lectins Ym1 and Ym2 was lower in $\beta6^{-/-}$ mice than in $\beta6^{+/+}$ mice (Paper I; Fig. 6F-G).

IL-1β enhanced the levels of active TGF-β1

Since the αvβ6 integrin is an *in vivo* activator of TGF-β1, we measured the levels of total and active TGF-β1 in the lungs of infant mice. IL-1β increased the levels of active TGF-β1, but not of total TGF-β1 levels, in $\beta6^{+/+}$ and $\beta6^{-/-}$ mice (Paper I; Fig. 8A-B). However, the levels of active TGF-β1 in whole lung homogenates did not differ between IL-1β/$\beta6^{+/+}$ and IL-1β/$\beta6^{-/-}$ mice.

Apoptosis and proliferation

In control/$\beta6^{+/+}$ mice the presence of apoptotic cells in airspaces and distal septa, as detected by TUNEL staining, decreased from PN0 to PN7 (Paper I; Fig. 7A-C). At birth, IL-1β inhibited apoptosis in $\beta6^{+/+}$ mice but not in $\beta6^{-/-}$ mice. However, control/$\beta6^{-/-}$ mice had a slightly lower number of apoptotic cells than control/$\beta6^{+/+}$ mice. At PN7, IL-1β induced apoptosis in both $\beta6^{+/+}$ and $\beta6^{-/-}$ mice. The numbers of apoptotic cells in the alveolar spaces did not differ between IL-1β/$\beta6^{+/+}$ and IL-1β/$\beta6^{-/-}$ mice, but the alveolar septa of IL-1β/$\beta6^{-/-}$ mice contained fewer apoptotic cells than those of IL-1β/$\beta6^{+/+}$ mice.

The number of proliferating cells in the distal lung increased from birth to PN7 in control/$\beta6^{+/+}$ mice, but the degree of proliferation did not vary significantly among any of the genotypes investigated at either PN0 or PN7 (Paper I; Fig. 7D).

Paper II

IL-1β induced MMP-9 expression in the newborn lung

In control/MMP-$9^{+/+}$ mice, the expression of MMP-9 increased immediately after birth and remained elevated to PN5, but decreased thereafter (Paper II; Fig. 1A). IL-1β enhanced the age-dependent increase in MMP-9 expression several-fold and remained at high levels at PN7–9. IL-1β also increased pro-MMP-9 and MMP-9 activity. In contrast, levels of pro-MMP-2 and mature MMP-2 were not altered by the expression of IL-1β. As expected, MMP-$9^{-/-}$ mice lacked MMP-9 activity.

To study the possible involvement of MMP-9 in IL-1β-induced lung injury, lung development and presence of inflammation were studied in IL-1β-expressing newborn mice with null or wild-type MMP-9 loci.

Absence of MMP-9 did not affect the production of IL-1β

The levels of hIL-1β detected by ELISA and the levels of degraded hIL-1β (17 kDa) detected by Western blot were similar in IL-1β/MMP-$9^{+/+}$ and IL-1β/MMP-$9^{-/-}$ mice.

IL-1β decreased postnatal growth and survival

Expression of IL-1β decreased postnatal growth and survival to a similar extent in MMP-$9^{+/+}$ and MMP-$9^{-/-}$ mice (Paper II; Fig. 4A-B).

Absence of MMP-9 worsened IL-1β-induced alveolar hypoplasia

Alveolar development appeared normal in the absence of MMP-9, judging by alveolar size and septal wall thickness at PN4 and PN7 in control mice (Fig. 4) (Paper II; Fig. 3A-C). Expression of IL-1β disrupted alveolarization in both MMP-$9^{+/+}$ and MMP-$9^{-/-}$ pups, as demonstrated by larger alveoli than in littermate controls (Fig. 4). Surprisingly, the IL-1β-induced lack of septation was worse in the MMP-9-deficient mice (Fig. 4). Even though IL-1β/MMP-$9^{-/-}$ pups had abnormally thick septal walls in the beginning of the alveolar stage at PN4, septal thinning subsequently occurred and septal thickness reached control levels by PN7.

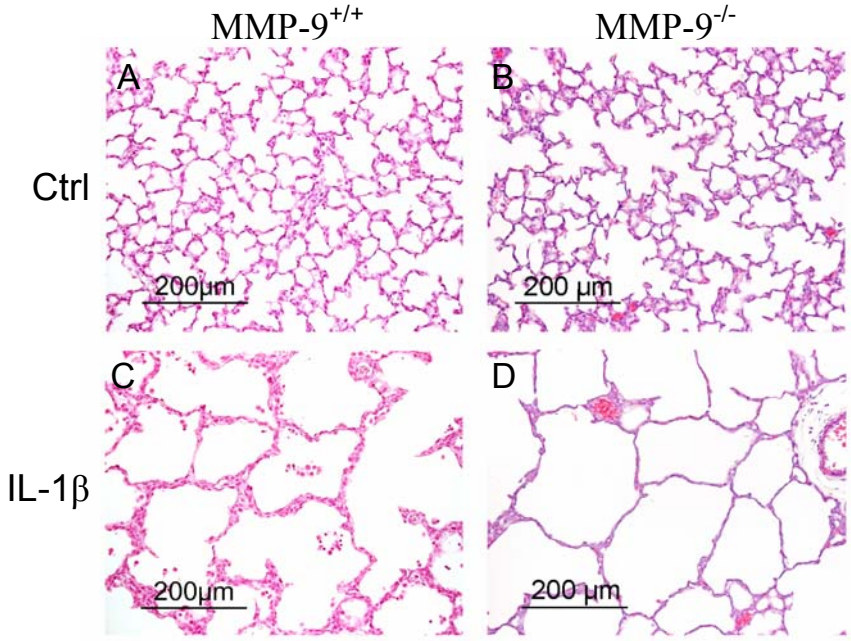

Figure 4. Lung histology at PN7 of control mice (Ctrl) with (A) wild-type MMP-9 loci (MMP-$9^{+/+}$) or (B) null MMP-9 loci (MMP-$9^{-/-}$) and IL-1β-expressing mice (IL-1β) with (C) wild-type MMP-9 loci (MMP-$9^{+/+}$) or (D) null MMP-9 loci (MMP-$9^{-/-}$).

Altered numbers of inflammatory cells in the lungs of IL-1β/MMP-$9^{-/-}$ mice

IL-1β expression increased the infiltration of neutrophils and macrophages into the lungs at PN4 to a similar extent in MMP-$9^{+/+}$ and MMP-$9^{-/-}$ mice (Paper II; Fig. 6B, Fig. 7B). The number of neutrophils had decreased by PN7 in both IL-1β-expressing groups but was still higher than in littermate controls. However, IL-1β/MMP-$9^{-/-}$ mice had fewer neutrophils at PN7 than IL-1β/MMP-$9^{+/+}$ mice. At PN7, more macrophages had infiltrated the lungs of IL-1β/MMP-$9^{-/-}$ mice than those of IL-1β/MMP-$9^{+/+}$ mice. The altered IL-1β-induced

accumulation of neutrophils and macrophages in MMP-9-deficient mice was not associated with changes in the production of the neutrophil chemoattractant KC or the monocyte chemokine MCP-1 (Paper II; Table 1). IL-1β/MMP-$9^{-/-}$ mice had a larger proportion of macrophages that contained phagocytozed material than IL-1β/MMP-$9^{+/+}$ mice (Paper II; Fig. 7C-D).

Increased apoptosis in IL-1β/MMP-$9^{-/-}$ mice

The number of apoptotic cells was similar in control/MMP-$9^{+/+}$ and control/MMP-$9^{-/-}$ mice. The IL-1β-induced apoptosis was strongly increased by MMP-9 deficiency at PN4 and PN7 (Paper II; Fig. 5A-B).

Vascularization

Despite similar levels of VEGF production at PN4 in all groups of mice, IL-1β altered the structure of capillaries in the alveolar septa (Paper II; Fig. 8). In control MMP-$9^{-/-}$ and MMP-$9^{+/+}$ mice, the vascular endothelium, identified by PECAM staining, was in close proximity to the alveolar spaces and formed outsproutings towards secondary crests. IL-1β expression caused abnormal appearance of the capillaries. In IL-1β-expressing MMP$9^{+/+}$ and MMP$9^{-/-}$ mice, the capillaries had fewer branching points and outsproutings towards secondary crests than in littermate controls.

Paper III

To examine how expression of IL-1β, mainly in type II epithelial cells, affects pulmonary development and surfactant function, mice expressing the hIL-1β transgene in the distal lung epithelium under the control of the hSP-C promoter were studied.

IL-1β resulted in 100% mortality postnatally

Expression of IL-1β did not affect the body weight of fetuses at E15 or E18.5 (Paper III; Fig. 1B). Mice expressing IL-1β had a mortality of 94% (32 out of 34) at PN0, and none of the IL-1β-expressing pups survived beyond PN1 (Paper III; Fig. 1A). In the absence of doxycycline, all bitransgenic pups survived until sacrifice at PN7 and gained weight as expected. Nine per cent (3 pups of 34) of control mice died at birth or during the first day of life.

IL-1β caused prenatal lung inflammation

IL-1β induced an inflammatory response with elevated numbers of neutrophils and macrophages in the distal lung parenchyma and airspaces at E18.5 and increased the expression of several neutrophil- and monocyte-chemoattractant proteins, namely KC, MCP-3, S100A8, S100A9, and SAA3, at E15 and E18.5 (Paper III; Fig. 3A-C).

IL-1β production resulted in prenatal arrest in acinar bud formation

The lungs of IL-1β-expressing fetuses demonstrated arrested lung morphogenesis with a lower number of distal airspaces and a decreased area of distal air sacs as a percentage of total distal lung area compared to control mice (Fig. 5) (Paper III; Fig. 2A-D). The decreased mRNA expression of VEGF in IL-1β-expressing fetuses suggests that vascular development

may have been inhibited in the present model (Paper III; Fig. 5). IL-1β did not cause detectable changes in apoptosis or proliferation in the lungs at E18.5 (Paper III; Fig. 7A-B).

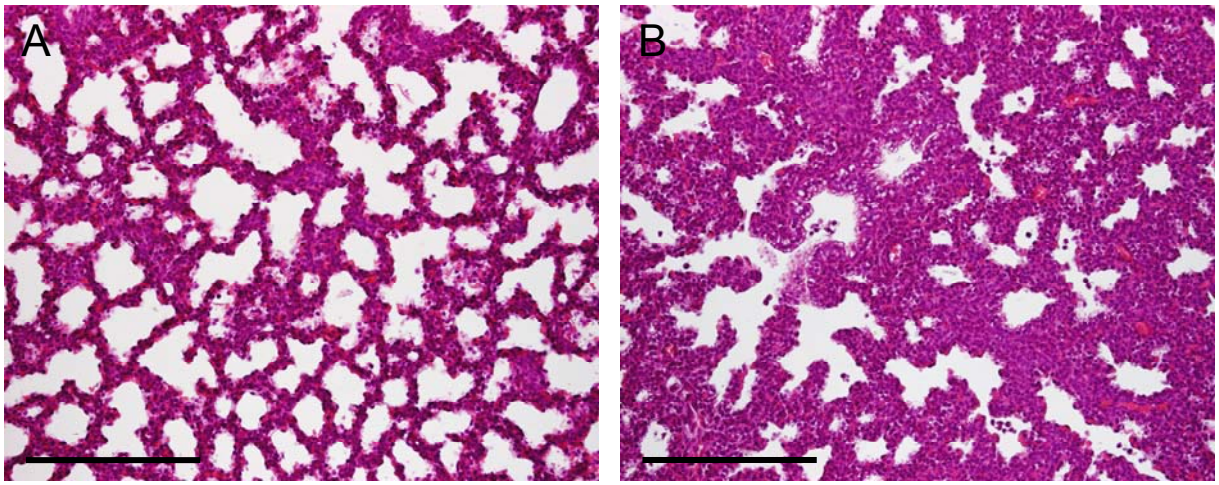

Figure 5. Lung histology at E18.5 of (A) a control mouse and (B) an IL-1β-expressing mouse. IL-1β delayed lung development in fetal mice. Scale bar = 200 μm.

IL-1β changed the expression of surfactant proteins and CCSP

The mRNA expression of the four surfactant proteins, SP-A, -B, -C and -D increased with gestational age (Paper III; Fig. 4A) as expected (Mendelson, 2000). The levels of SP-A did not differ between IL-1β-expressing mice and littermate controls, but IL-1β decreased the expression of SP-B and SP-C, and increased SP-D mRNA expression. The number of cells that produced SP-B or pro-SP-C was decreased in IL-1β-expressing fetuses, indicating that IL-1β suppressed type II cell differentiation (Paper III; Fig. 4B).

In the proximal airways, the number of CCSP-positive cells (Clara cells) was markedly decreased in IL-1β-expressing mice (Paper III; Fig. 6B). In addition, the mRNA expression of CCSP was suppressed by IL-1β (Paper III; Fig. 6A). In contrast, IL-1β upregulated the expression of Ym1, Ym2 and pendrin (Paper III; Fig. 6C), proteins associated with allergic airway inflammation and asthma (Iwashita *et al.*, 2006; Nakagami *et al.*, 2008; Webb *et al.*, 2001).

DISCUSSION

The αvβ6 integrin, a potential pathogenic factor in BPD

The αvβ6 integrin has not previously been investigated in relation to pulmonary disease of the newborn. Paper I demonstrates that absence of the β6 integrin subunit alleviates IL-1β-induced lung injury in a model of BPD in newborn mice.

Alveolarization

The pulmonary pathology in infants with BPD is characterized by lack of alveolar septation, resulting in large alveoli and thick alveolar walls (Bhatt *et al.*, 2001; Coalson, 2003). Alveolar wall thinning occurring during lung development is important for normal gas exchange (Burri, 2006). Perinatal expression of IL-1β results in disrupted alveolar septation in infant mice, and thinning of alveolar walls fails to occur in IL-1β-expressing lungs (Bry *et al.*, 2007 and Papers I, II). Alveolar chord length was shorter and alveolar walls were thinner in IL-1β/$\beta6^{-/-}$ lungs than in IL-1β/$\beta6^{+/+}$ lungs, indicating better alveolar septation in the absence of the β6 integrin subunit in this model of BPD.

Neutrophils and macrophages

The number of septal neutrophils was similar in IL-1β/$\beta6^{+/+}$ and IL-1β/$\beta6^{-/-}$ mice. However, the IL-1β-induced transepithelial migration of neutrophils from lung parenchyma to alveolar spaces was suppressed in β6-deficient mice. The production of KC and MIP-2, essential for maximal neutrophil emigration into the alveolar spaces (Frevert *et al.*, 1995; Schmal *et al.*, 1996), was inhibited in mice lacking the β6 integrin subunit. Blocking S100A8 and S100A9 inhibits the migration of neutrophils and macrophages into the alveolar spaces during Streptococcal pneumonia (Raquil *et al.*, 2008). Possibly the lower expression of these calgranulins in $\beta6^{-/-}$ mice might have reduced the IL-1β-induced transepithelial migration of neutrophils into the airspaces.

Preterm infants developing BPD have elevated levels of chemokines and inflammatory cells in tracheal aspirate fluid soon after delivery and inflammation is thought to be an important contributing factor in lung injury observed in these infants (Speer, 2006). Absence of the β6 integrin subunit suppressed the IL-1β-induced infiltration of macrophages into the lung parenchyma and the alveolar spaces, despite increased expression of the monocyte chemoattractants MCP-1 and MCP-3 compared to IL-1β/$\beta6^{+/+}$ mice. However, the expression of SAA3, S100A8, and S100A9 and the production of osteopontin were reduced in IL-1β/$\beta6^{-/-}$ mice compared to IL-1β/$\beta6^{+/+}$ mice. Since these proteins are chemotactic for e.g. monocytes and macrophages (Liang *et al.*, 2000; O'Regan, 2003; Raquil *et al.*, 2008), the decreased production of these proteins in IL-1β/$\beta6^{-/-}$ mice may have resulted in a less pronounced influx of macrophages into the lungs. Decreased IL-1β-induced infiltration of neutrophils into the alveolar spaces of $\beta6^{-/-}$ mice may have contributed to the lower number of macrophages in the lungs of IL-1β/$\beta6^{-/-}$ mice, since neutrophil infiltration may be important for the subsequent increase in lung monocytes/macrophages (Janardhan *et al.*, 2006).

Spontaneous inflammation in $\beta6^{-/-}$ mice

Adult mice lacking the β6 integrin subunit are protected from bleomycin-induced ALI (Munger *et al.*, 1999; Pittet *et al.*, 2001). Pittet *et al*. suggested that the spontaneous

inflammation observed in adult $\beta6^{-/-}$ mice (Huang *et al.*, 1996) may condition their lungs to resist subsequent bleomycin-induced lung injury (Pittet *et al.*, 2001). A spontaneous inflammation was detected in control/$\beta6^{-/-}$ pups in the present study. At PN7, control/$\beta6^{-/-}$ mice had more alveolar neutrophils and higher expression of several chemokines (i.e. KC, MIP-2, MCP-1, MCP-3, S100A8 and S100A9) than control/$\beta6^{+/+}$ mice. However, at E14, the time point at which the production of hIL-1β first becomes induced in the lungs of bitransgenic mice, the levels of inflammatory cytokines and inflammatory cells were not higher in $\beta6^{-/-}$ fetuses compared to $\beta6^{+/+}$ fetuses. Thus, the partial protection to IL-1β-induced inflammation and injury observed in $\beta6^{-/-}$ infant mice is probably not due to a tolerance of inflammatory injury due to a previous inflammation.

Airway structure

The airways of control/$\beta6^{-/-}$ pups were similar to those of control/$\beta6^{+/+}$ pups and did not display goblet cell hyperplasia. The chitinase-like lectins Ym1 and Ym2 are upregulated in allergic airway inflammation and may play a role in the development of asthma (Iwashita *et al.*, 2006; Webb *et al.*, 2001). Absence of the β6 integrin subunit strongly suppressed the IL-1β-induced expression of Ym1 and Ym2 and reduced goblet cell hyperplasia. In addition to the chemoattractant role of osteopontin, it has recently been suggested that osteopontin is an important factor in promoting the development of asthma in adult mice (Kohan *et al.*, 2009). Osteopontin deficiency resulted in a reduced number of mucus-producing airway cells (goblet cells) and a reduced number of leukocytes recovered in BAL fluid in a mouse model of chronic allergen-induced asthma (Kohan *et al.*, 2009). It is possibly that inhibition of Ym1, Ym2 and osteopontin by the absence of the β6 integrin subunit may have in part prevented the IL-1β-induced airway inflammation and remodeling.

TGF-β1 in pulmonary disease

The αvβ6 integrin is an activator of TGF-β1. IL-1β increased the levels of active TGF-β1, but there was no significant difference in the levels of active TGF-β1 in the lungs of IL-1β/$\beta6^{+/+}$ mice and IL-1β/$\beta6^{-/-}$ mice, consistent with other studies (Ludlow *et al.*, 2005; Munger *et al.*, 1999). Since the binding of latent TGF-β1 to the αvβ6 integrin causes a conformational change in the TGF-β1 complex without causing release of free TGF-β, it is believed that lack of the β6 integrin subunit results in a local deficiency of active TGF-β1 (Munger *et al.*, 1999; Sheppard, 2003). This local decrease in TGF-β1 might be difficult to detect in whole lung homogenates. The protective effect of β6 integrin deficiency in lung fibrosis of adult mice is thought to be a consequence of local decrease in TGF-β1 (Munger *et al.*, 1999). Both IL-1β in the present model and the αvβ6 integrin are expressed solely in epithelial cells. The protection against IL-1β-induced lung injury in $\beta6^{-/-}$ mice might be a result of local inhibition of TGF-β1 activity. Recently, IL-1β-induced increase in vascular permeability and edema in adult mice were shown to be partially dependent on αvβ6 integrin and/or TGF-β signaling (Ganter *et al.*, 2008). Inhibition of the αvβ6 integrin or TGF-β blocks the IL-1β-induced increase in vascular permeability across epithelial monolayers *in vitro* (Ganter *et al.*, 2008).

Preterm infants developing BPD have increased levels of active TGF-β1 in their lungs (Kotecha *et al.*, 1996a; Lecart *et al.*, 2000). Overexpression of TGF-β1 disrupts lung development prior to birth (Zhou *et al.*, 1996b), and when it occurs from PN7, TGF-β1 causes an illness in the lungs of young mice characterized by few alveoli, thick alveolar septa, abnormal vascular appearance, and fibrosis (Vicencio *et al.*, 2004). On the other hand, inhibition of TGF-β1 signaling partially protects against hypoxia- or hyperoxia-induced lung

injury in newborn mice (Ambalavanan *et al.*, 2008; Nakanishi *et al.*, 2007). The role of TGF-β1 in the present mouse model of BPD remains to be determined. The αvβ6 integrin can also bind fibronectin (Busk *et al.*, 1992; Weinacker *et al.*, 1994), vitronectin (Huang *et al.*, 1998a), tenascin (Prieto *et al.*, 1993; Yokosaki *et al.*, 1996) and osteopontin (Yokosaki *et al.*, 2005) and may therefore affect the lung by interacting with other proteins than TGF-β1.

A possible protective role of MMP-9 in BPD

Increased levels of MMP-9 and an imbalance between MMP-9 and its inhibitor TIMP-1 have been associated with the development of BPD in infants (Ekekezie *et al.*, 2004; Fukunaga *et al.*, 2009; Schulz *et al.*, 2004) and in animals models (Tambunting, *et al.*, 2005). Blocking MMP has been suggested as a potential therapeutic target in BPD (Chetty *et al.*, 2008; Ekekezie *et al.*, 2004). However, other studies have not demonstrated an increase in MMP-9 activity in BPD infants compared to non-BPD infants (Cederqvist *et al.*, 2001; Danan *et al.*, 2002). Elevated MMP-9 activity has been correlated with the recovery from RDS in preterm infants, whereas infants who developed BPD did not have elevated MMP-9 activity (Dik *et al.*, 2006). The role of MMP-9 in the development of BPD is unclear and difficult to study in the human infant.

Alveolarization

We studied the lung development in IL-1β-expressing and control mice with wild-type or null MMP-9 loci. Since control/MMP-$9^{-/-}$ mice had normal alveolar development, MMP-9 is not essential for normal alveolar development. In the present animal model of BPD, IL-1β increased the expression and activity of MMP-9. Absence of MMP-9 however, did not improve the IL-1β-induced lung injury. Instead lack of MMP-9 worsened the alveolar hypoplasia in IL-1β-expressing newborn mice, suggesting that MMP-9 has a protective role in inflammatory lung injury. Decreased (Hosford *et al.*, 2004) and increased (Cederqvist *et al.*, 2006) levels of MMP-9 have been associated with hyperoxia-induced lung injury in animal models. MMP-9 deficiency worsens ventilator-induced injury in adult mice (Albaiceta *et al.*, 2008). In contrast, Chetty *et al*. demonstrated that absence of MMP-9 alleviates hyperoxia-induced lung injury in newborn mice (Chetty *et al.*, 2008). The hIL-1β transgene in the present model of BPD is induced during gestation (Bry *et al.*, 2007), whereas mice in the study by Chetty *et al.* (2008) were exposed to hyperoxia from PN5 to PN13, i.e. after the beginning of alveolarization. Possibly, the increased production of MMP-9 during IL-1β-induced lung injury is part of a repair process and thus lack of MMP-9 aggravates the induced injury. MMP-9 may have diverse roles in different types of pulmonary injury caused by different insults and at different times during development.

MMP-9 during repair of injured epithelium

In the CCSP-controlled transgenic mouse model of BPD, the expression of IL-1β, restricted to epithelial cells, is likely to cause injury to epithelial and adjacent cells. After an injury to the epithelium, the epithelial cells at the edge of the wound spread, proliferate, and migrate to cover the de-epithelialized zone. Both the pro-form and the active form of MMP-9 are expressed in human bronchial epithelial cells during repair (Buisson *et al.*, 1996). After wound injury, MMP-9 accumulates in migrating airway epithelial cells and promotes the migration of these cells *in vitro* (Legrand *et al.*, 1999). Activation of MMP-9 is likewise associated with increased migration of hyperoxia-exposed alveolar epithelial cells (Buckley *et al.*, 2001). It is possible that the MMP-9 activity present in IL-1β/MMP-$9^{+/+}$ lungs participates in a repair process initiated as a consequence of the IL-1β-induced lung injury.

MMP-9 and inflammation

MMP-9 has been shown to participate in the migration of neutrophils (Keck *et al.*, 2002; Kim *et al.*, 2006) and can affect the activity of chemokines (Atkinson and Senior, 2003; Chakrabarti and Patel, 2005; Van den Steen *et al.*, 2000). Absence of MMP-9 did not modulate the number of lung neutrophils at PN4 or PN7 in control mice. IL-1β increased the number of neutrophils and macrophages similarly in IL-1β/MMP-$9^{+/+}$ and IL-1β/MMP-$9^{-/-}$ mice at PN4. However, at PN7, the number of neutrophils was lower IL-1β/MMP-$9^{-/-}$ than in IL-1β/MMP-$9^{+/+}$ mice. Absence of MMP-9 increased the IL-1β-induced macrophage infiltration at PN7. This may have been caused by higher levels of chemoattractants. In addition, the increased number of apoptotic cells in IL-1β/MMP-$9^{-/-}$ mice may have stimulated infiltration of macrophages into the lungs of these mice (Savill *et al.*, 1989; Wang *et al.*, 2003).

Antenatal inflammation, surfactant, and lung injury

The presence of inflammatory mediators in the lung soon after birth suggests that inflammation promoting lung injury in premature infants may begin before birth (Murthy and Kennea, 2007). Watterberg *et al*. suggested that newborn infants exposed to chorioamnionitis are protected from RDS but have an increased risk of developing BPD (Watterberg *et al.*, 1996). These correlations between chorioamnionitis and RDS or BPD have been confirmed by other, but not all studies (Been and Zimmermann, 2009). A single intra-amniotic injection of IL-1 (Bry *et al.*, 1997; Willet *et al.*, 2002) or endotoxin (Jobe *et al.*, 2000; Kramer *et al.*, 2001) enhances the production of surfactant components, e.g. SP-B, and improves lung function after preterm delivery. The acute effects of IL-1α on the lung surfactant system studied in rabbit lung explants *in vitro* are dependent on degree of lung maturity (Glumoff *et al.*, 2000).

Since intrauterine infections such as chorioamnionitis are often chronic (Goldenberg *et al.*, 2000; Wenstrom *et al.*, 1998), we sought to determine how a prolonged continuous fetal expression of hIL-1β mainly in type II epithelial cells affects lung development (Paper III). Induced expression of IL-1β under the control of the SP-C promoter disrupted acinar bud formation as demonstrated by fewer distal airspaces occupying a smaller area of the distal lung. IL-1β decreased the production of SP-B, pro-SP-C and CCSP, suggesting that the differentiation of distal and proximal lung epithelial cells was affected (Clark *et al.*, 2001; Harrod *et al.*, 1998; Lin *et al.*, 1999; Zeng *et al.*, 1998; Zhou *et al.*, 1996a). As an indication of severe lung injury, none of the IL-1β-expressing mice survived beyond PN1. Production of lung surfactant is necessary for postnatal survival. Mice lacking SP-B die immediately after birth (Clark *et al.*, 1995). Since the lungs of IL-1β-expressing fetuses had decreased expression of SP-B and fewer SP-B-positive cells, SP-B deficiency may have contributed to the neonatal mortality in these mice. Chronic inflammation may have a more serious effect on surfactant production and lung development than a single exposure to IL-1β.

Transgenic expression of IL-1β, controlled by the SP-C or CCSP promoter

In Papers I and II, the hIL-1β transgene is expressed under the control of the CCSP promoter, whereas IL-1β in Paper III is under the control of the SP-C promoter. Bry *et al*. previously demonstrated that CCSP-controlled IL-1β expression disrupts alveolar and vascular development in newborn mice, thereby causing a lung disease that resembles BPD (Bry *et al.*, 2007). This was confirmed in both Paper I and Paper II. However, the mice appear healthy at birth and do not have disrupted acinar bud formation or inhibited surfactant protein expression

prior to birth (unpublished data) as IL-1β-expressing SP-C mice do. When doxycycline is administered to pregnant dams, the expression of IL-1β is initiated in bitransgenic CCSP-rtTA/$(tetO)_7$-CMV-hIL-1β mice at ~E14 (Bry *et al.*, 2007) whereas target transgene expression in SP-C-rtTA/$(tetO)_7$-CMV-hIL-1β starts at ~E11 (Perl *et al.*, 2002). Previous studies have shown that the target transgene controlled by the SP-C promoter is expressed in the epithelium of the distal lung but not in the proximal airways, whereas when controlled by the CCSP promoter the target transgene is detected in both the distal and proximal lung epithelium (Akeson *et al.*, 2003; Perl *et al.*, 2002; Perl *et al.*, 2009). Possibly a more distal location of target transgene expression in the SP-C-rtTA/$(tetO)_7$-CMV-hIL-1β mice has led to the dissimilar fetal lung structure compared to CCSP-rtTA/$(tetO)_7$-CMV-hIL-1β mice. The more severe phenotype of SP-C-rtTA/$(tetO)_7$-CMV-hIL-1β mice may also be a consequence of the fact that the transgenic expression is induced earlier and is higher than in CCSP mice (Bry *et al*., 2007; Perl *et al*., 2002; Paper III). Differences in timing, concentration and location of IL-1β expression may result in different forms of lung disease.

Apoptosis in inflammatory lung injury

Apoptosis, programmed cell death, occurs normally during lung organogenesis and injury to remove old and damaged cells. Apoptosis of mesenchymal cells at the periphery of distal lung buds is detected during late embryonal lung development and increases at birth (Kresch *et al.*, 1998), but declines rapidly thereafter leading to a nadir around PN7 (Luyet *et al.*, 2002). Both decreased and increased levels of apoptosis have been detected in the lungs of infants with RDS and infants developing BPD (Hargitai *et al.*, 2001; Kotecha *et al.*, 2003; Lukkarinen *et al.*, 2003; Oei *et al.*, 2003). Overexpression of FasL, an apoptosis inducer, disrupts alveolarization in mice (De Paepe *et al.*, 2008). On the other hand, enhanced apoptosis of mesenchymal cells has been associated with improved alveolarization and thinning of airspace walls (Reyburn *et al.*, 2008). Control/$\beta6^{+/+}$ mice had a peak in apoptosis on PN0 that declined to PN7. In IL-1β/$\beta6^{+/+}$ mice, apoptosis was reduced at PN0 but enhanced at PN7 compared to control mice (Paper I). IL-1β/$\beta6^{-/-}$ and IL-1β/MMP-$9^{-/-}$ mice had at PN7 more apoptotic cells than IL-1β/$\beta6^{+/+}$ and IL-1β/MMP-$9^{+/+}$ mice, respectively, possibly contributing to the improved thinning of alveolar walls in $\beta6^{-/-}$ and MMP-$9^{-/-}$ mice. The disrupted lung development observed prenatally in SP-C promoter-controlled IL-1β-expressing mice was not associated with changes in the number of apoptotic cells in late gestation, suggesting that inflammation can arrest lung morphogenesis even in the absence of detectable changes in apoptosis.

CONCLUSIONS

Newborn mice lacking the β6 integrin subunit were partially protected from IL-1β-induced lung injury. IL-1β-expressing $\beta6^{-/-}$ mice had smaller alveoli, thinner alveolar septal walls, fewer macrophages and neutrophils in the alveolar spaces and decreased expression of several inflammatory mediators compared with IL-1β-expressing $\beta6^{+/+}$ mice. In addition, mice lacking the β6 integrin subunit had a milder IL-1β-induced goblet cell hyperplasia in proximal airways. These results suggest for the first time that the β6 integrin subunit may play an important role in the development of BPD.

Increased MMP-9 activity has been suggested to participate in the pathogenesis of BPD. Mice lacking MMP-9 developed a worse alveolar hypoplasia and an increased infiltration of macrophages in response to IL-1β expression than did $MMP\text{-}9^{+/+}$ mice. The increased production of MMP-9 during inflammatory lung injury is not necessarily disadvantageous for the lung. Blocking MMP-9 activity, previously suggested as a potential treatment for infants developing BPD, may be harmful.

IL-1 has been shown to acutely increase surfactant protein production in premature animals. The effects of chronic IL-1β exposure on prenatal lung development and surfactant production have not been studied previously. Chronic expression of IL-1β, controlled by the SP-C promoter, caused a severe prenatal lung injury associated with decreased production of surfactant proteins, leading to 100% mortality at birth. These results suggest that chronic inflammation in the distal lung epithelium severely disrupts lung development and function.

FUTURE STUDIES

Role of TGF-β1 in IL-1β-induced lung injury

Since the αvβ6 integrin is an activator of the cytokine TGF-β1, some differences between IL-1β/β6$^{-/-}$ mice and IL-1β/β6$^{+/+}$ mice may be caused by a local deficiency of TGF-β in mice lacking the β6 integrin subunit. However, the β6 integrin binds other proteins as well and may have unidentified roles in pulmonary diseases. Thus, it would be of interest to investigate whether the improved phenotype in IL-1β/β6$^{-/-}$ mice compared to IL-1β/β6$^{+/+}$ mice is caused by diminished TGF-β1 activity. Projected experiments where IL-1β-expressing wild-type mice are injected with prenatal and/or postnatal antibodies inhibiting TGF-β signaling will study whether inhibition of TGF-β1 activity improves lung development in IL-1β-expressing mice.

Long-term effects of perinatal IL-1β expression

Children with BPD have an enhanced risk of asthma and may have abnormal lung function until adulthood (Doyle *et al.*, 2006; Wong *et al.*, 2008; Vrijlandt *et al.*, 2005; Vrijlandt *et al.*, 2007). Preliminary data using the SP-C transgenic model demonstrate that cessation of doxycycline administration to the pregnant dam at 16 days after vaginal plug (i.e. the fetuses are embryonal day 16) does not cause neonatal mortality of bitransgenic SP-C-rtTA/$(tetO)_7$-CMV-hIL-1β pups. These IL-1β-expressing mice survive at least until PN7 despite structural changes in the distal lung observed postnatally. Using this model it is possible to study how prenatal inflammation affects lung structure and function in adulthood.

ACKNOWLEDGEMENTS

This work was carried out at the Institute of Clinical Sciences at the University of Gothenburg. I would like to express my gratitude to the following people who have enabled me to perform this work.

Kristina Bry, my supervisor, for giving me the opportunity to do my PhD studies in this interesting field of research. Thank you for your encouragement, and for the never-ending scientific and personal support throughout the years.

Urpo Lappalainen, co-author. Thank you for your contribution in all the projects and for being so kind and helpful with everything.

Heikki Lukkarinen, co-author and friend. Thank you for the rewarding collaboration and for discussions about science and life in general.

Dean Sheppard, co-author, thank you for fruitful collaboration.

Erica Bäckström, good friend and fellow PhD student. Thank you for all your support and kindness and for reminding me about the world out there. The lab has been really empty without you these last couple of months.

Ola Hjalmarson, for your support and for improving the content of this thesis.

Anna Bry, for correcting the English usage in the manuscripts and the thesis frame.

Karin Brandt and Sara Jägevall, laboratory members during my first two years. Thank you for showing me the methods when I first came to the lab and for creating a friendly atmosphere.

Nico Cavallini, friend and former fellow PhD student. Thank you for all the fun, interesting discussions and for just being a wonderful person. You have been a true support during the last months during this work.

Muna Elmi, friend and former fellow PhD student. Thank you for your kindness, help with western blotting and for all your comments on the thesis frame.

Daniel Wetterskog, friend and former fellow PhD student. I have enjoyed your company and thank you for showing me the wonderful world of Lumigen.

Mikael Persson, “German” friend. Thank you for your support and for always cheering me up with your stories.

A special thank to other present and former colleagues, especially Alicia, Abtin, Gisela, Daniel, Erik and Noreen, for all the good times.

Madeleine Ingelsten, friend and fellow PhD student. Thank you for your kindness and optimism. I wish both of us have had more time for those lunches. Good luck on your dissertation!

Bertil Ohlsson, my supervisor during my master thesis. Thank you for introducing me to science and for believing in me.

Marlene Mussmann and Eira Brandby, thank you for excellent administrative assistance.

My family, especially my parents Karin and Lennart, thank you for all your encouragement and support.

Johan, thank you for your endless love, support and enthusiasm. You rock!

This work was financially supported by the Swedish Medical Research Council, the Swedish Heart and Lung Foundation, the Frimurare Barnhus Foundation, the Swedish Government Grants for Medical Research, and the Queen Silvia Children's Hospital Research Foundation.

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