From the Department of Woman and Child Health, Neonatal Unit, Astrid Lindgren Children's Hospital,
Karolinska Hospital and Department of Clinical Microbiology, MTC, Karolinska Institute

Ureaplasma urealyticum Induced Pulmonary Inflammation in the Development of Chronic Lung Disease of Prematurity

Yinghua Li, M.D.

李英华

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The only way is to be diligent among the mountains of books;
The unique boat is to study hard in the endless sea of knowledge.

To my parents and my wife and daughter

献给我的父母，妻子和女儿

Contents

Abstract

Chronic lung disease (CLD) of prematurity is a prolonged respiratory failure in very-low-birthweight neonates. This disorder is multifactorial and associated with an early increase in the numbers of neutrophils and alveolar macrophages, together with morphological abnormalities in epithelial and endothelial cells. Infection and associated inflammatory responses in the lungs play important roles in the development of CLD. *Ureaplasma urealyticum* has been postulated to be of importance in this context, but this is still a matter for debate. Treatment of neonates with steroids provides some improvement.

Proinflammatory cytokines have been implicated as being involved in the development of CLD. In the present study, production of TNF-α and IL-6 by both a human and a rat macrophage cell line was found to increase after stimulation with *U. urealyticum* antigen. This induction was down-regulated by dexamethasone, budesonide and recombinant IL-10 (rIL-10) in the human macrophage cell line. In tracheobronchial aspirate fluid (TAF) macrophages, *U. urealyticum* antigen enhanced the production of TNF-α 14-84% and IL-6 46-268%. In the rat alveolar macrophage cell line, steroids inhibited the increases in IL-6 and TNF-α production caused by *U. urealyticum* antigen, whereas rat rIL-10 was without effect.

Nitric oxide (NO) has been suggested to be an important mediator of inflammation associated with pulmonary infections. We discovered that *U. urealyticum* antigen stimulates alveolar macrophages directly to increase their production of NO in a dose- and time-dependent manner. This effect was further enhanced by IFN-γ, while being attenuated by budesonide and dexamethasone. The NO was regulated at transcriptional levels as measured by inducible NO synthase (iNOS). The NO formed in response to *U. urealyticum* caused a 6-fold reduction in the growth rate of this organism itself after 10 hours of infection.

Vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1) may be involved in both early and later pathological changes in the lung during the development of CLD. We found that *U. urealyticum* antigen enhances expression of VEGF, cell surface and soluble ICAM-1 by human macrophages in a dose-dependent manner, an effect which is also inhibited by budesonide and

dexamethasone. The extent of this up-regulation of ICAM-1 was reduced by 86% when TNF-α was prevented from exerting its action by an anti-TNF-α antibody.

In addition, *U. urealyticum* antigen triggered activation of nuclear factor -κB (NF-κB). This observation suggests a possible mechanism for the increases in cytokine production, and expression of iNOS, growth factor and cellular adhesion molecule evoked by this antigen.

In our clinical study, levels of TGF-β1 in TAF from infants who developed CLD were found to be significantly elevated during the first week of postnatal life and remain elevated at 2 weeks and even beyond 4 weeks of age. These levels of TGF-β1 were not significantly decreased by treatment with steroids in 6 infants with CLD. IL-10 was detected in 12/44 (27%) TAF samples from 24 infants who developed CLD, compared to 6/57 (11%) TAF samples from 22 preterm infants who did not develop this disease.

These findings indicate that infection by *U. urealyticum* may be an important causative factor in the development of CLD. NO is probably involved in host defenses against such infection. The down-regulatory effect by steroids might in part explain the beneficial results of treating neonates with CLD with these agents. TGF-β1 might play an important role in the fibrotic response observed in the CLD lung.

List of original papers

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I. **Li YH**, Brauner A, Jónsson B, van der Ploeg I, Söder O, Holst M, Skov Jensen J, Lagercrantz H, Tullus K. *Ureaplasma urealyticum*-induced production of proinflammatory cytokines by macrophages. ***Pediatric Research*** 48**:**114-119, 2000.

II. **Li YH,** Brauner A, Jónsson B, van der Ploeg I, Söder O, Holst M, Skov Jensen J, Lagercrantz H, Tullus K. Inhibition of macrophage proinflammatory cytokine expression by steroids and recombinant IL-10. ***Biology of the Neonate*** 80:124-132, 2001.

III. **Li YH,** Yan Z-Q, Skov Jensen J, Tullus K, Brauner A. Activation of Nuclear Factor kappa B and Induction of Inducible Nitric Oxide Synthase by *Ureaplasma urealyticum* in Macrophages. ***Infection and Immunity*** 68**:**7087-7093, 2000.

IV. **Li YH**, Brauner A, Skov Jensen J, Tullus K: Induction of human macrophage VEGF and ICAM-1 by *Ureaplasma urealyticum* and down-regulation by steroids. Submitted for publication.

V. Jónsson B, **Li YH,** Noack G, Brauner A, Tullus K. Down-regulatory Cytokines in Tracheobronchial Aspirate Fluid from Infants with Chronic Lung Disease of Prematurity. ***Acta Pediatrica*** 89**:**1375-1380, 2000.

Abbreviations

AP-1, active protein-1

BLP, bombesin-like peptide

BPD, bronchopulmonary dysplasia

CCU, color-changing units

CFU, colony-forming units

CHX, cycloheximide

CLD, chronic lung disease

CMV, cytomegalovirus

CONS, coagulase-negative staphylococci

D-NMMA, N^G-monomethyl-D-arginine

ELISA, enzyme-linked immunosorbent assay

EMSA, electrophoretic mobility shift assay

FITC, fluorescein isothiocyanate isomer 1

GR, glucorticoid receptor

GRE, glucocorticoid response element

HMD, hyaline membrane disease

ICAM-1, intercellular adhesion molecule-1

IFN-γ, interferon gamma

IKK, IκB kinases

IL, interleukin

iNOS, inducible nitric oxide synthase

IRAK, IL-1 receptor accessory protein kinase

L-NMMA, N^G-monomethyl-L-arginine

LPS, lipopolysaccaride

MFI, the mean fluorescence intensity

MMP, matrix metalloproteinase

NCPAP, nasal continuous positive airway pressure

NF-κB, nuclear factor-κB

NIK, NF-κB-inducing kinase

NO, nitric oxide

PDA, patent ductus arterious

PMA, phorbol 12-myristate 13-acetate

PROM, premature rupture of membrane

PRRs, pattern-recognition receptors

RDS, respiratory distress syndrome

rIL-10, recombinant IL-10

RIP, receptor-interacting protein

RT-PCR, reverse transcriptase polymerase chain reaction

TAF, tracheobronchial aspirate fluid

TGF-β, transforming growth factor-β

TLRs, Toll-like receptors

TNF-α, tumor necrosis factor-α

TNFR1, type 1 TNF receptor

TRADD, TNFR1-associated death domain protein

TRAF, TNF-receptor-associated factor

U. urealyticum, *Ureaplasma urealyticum*

VEGF, vascular endothelial growth factor

VLBW, very-low-birthweight

Introduction

1. Chronic lung disease of prematurity

Bronchopulmonary dysplasia (BPD) or chronic lung disease of prematurity (CLD), is the most common chronic lung disorder among infants. During the last decade, the survival of very preterm infants has increased and CLD has become a growing problem among these survivors (Manktelow, Draper et al. 2001). The risk for development of this disorder in these survivors is high. Thus prevention of CLD in preterm babies has become a major subject in neonatology (Jobe and Bancalari 2001). The frequency of this disease is inversely related to gestational age at birth, indicating that disturbance of the maturation of the lung is a major cause. An increased understanding of the mechanisms underlying the development of CLD should lead to novel and more focused therapeutic strategies, resulting in decreased morbidity and reduced health care costs.

1.1. History

In 1967, Northway and co-workers defined the disease as "bronchopulmonary dysplasia", a term that has since gained widespread use, and BPD has become an umbrella term for CLD (Northway, Rosan et al. 1967). At that time, this disease was only seen in preterm infants born at 32-34 weeks gestational age treated for hyaline membrane disease (HMD) with mechanical ventilation and supplemental oxygen. The classical form of BPD described by Northway involves four stages, identifiable on the base of a sequence of radiological changes. These stages, however, do not occur consistently, and diagnosing CLD only by radiographic pattern alone is difficult and inappropriate (Lundstrom, Shaw et al. 2000).

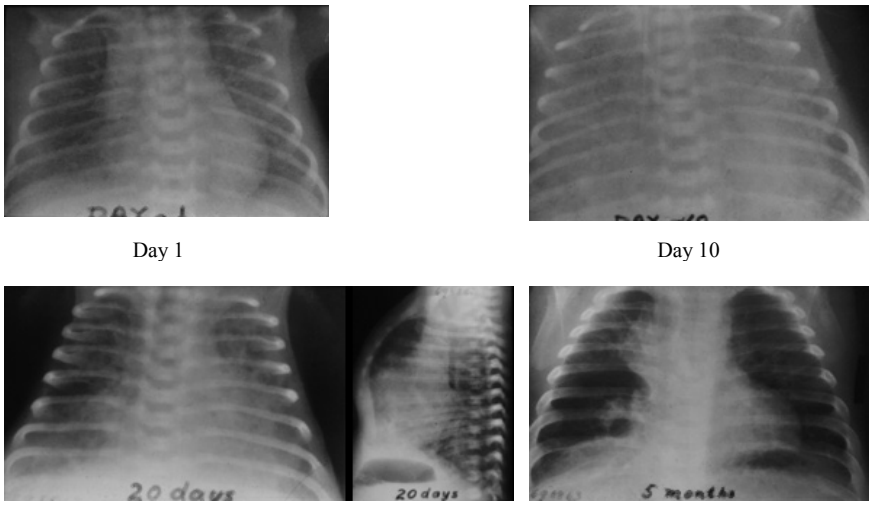
1.2. Clinical criteria for diagnosis

A number of clinical diagnostic criteria for BPD are now generally used. The clinical definition of BPD most frequently employed (Bancalari, Abdenour et al. 1979) includes 1) requirement for mechanical ventilation for 72 hours or more for HMD during the first week of postnatal life, 2) requirement for supplemental oxygen later than 28 days of life, 3) characteristic respiratory symptoms and 4) characteristic

radiological findings at 28 days of life. This set of criteria was later modified to define CLD as all forms of acute pulmonary injury during the first week of life, leading to a dependency on supplemental oxygen at a later stage (36 weeks of postconceptional age), since it has become apparent that conditions other than respiratory distress syndrome (RDS) can lead to the changes and symptoms associated with BPD (O'Brodovich and Mellins 1985; Lundstrom, Shaw et al. 2000; Jobe and Bancalari 2001; Jobe and Ikegami 2001).

1.3. Radiological criteria for diagnosis

Four classical radiological stages of BPD were described by Northway: Stage I (occurring 1-3 days after birth) consists of an initial picture of HMD. In stage II (4-10 days) the lung demonstrates increasing radio-opacity. In stage III (10-20 days) there are increasing signs of chronicity with radiological changes consistent with fibrosis and emphysema, alternating with areas of atelectasis-bubbly pattern. Stage IV (> 30 days) is associated with hyperexpansion and a cystic appearance of the lungs (Figure 1). The corresponding histopathological progression involves an initial appearance of hyaline membrane formation, followed by necrotic bronchiolitis, with focal areas of atelectasis and disturbances in alveolar architecture. As a result of this process, the lungs exhibit emphysematous areas, bullae, fibrosis, bronchial smooth muscle hypertrophy, decreased vascularization and arterial smooth muscle hypertrophy (Northway, Rosan et al. 1967). Today, CT scanning of the lungs of patients with CLD



Day 1 Day 10

Day 20 5 month

Figure 1. Characteristic radiological findings on the lungs of infants with CLD.

is performed extensively and provides valuable information concerning the status of pulmonary parenchyma.

1.4. Incidence

During the past three decades, treatment of preterm infants with RDS has improved considerably, leading to a higher incidence of the milder forms of BPD. CLD has been proposed as a more descriptive term than BPD, which should be used, only for the most severe form of this disease (Greenough 1990).

The incidence of CLD among preterm infants (defined as having a gestational age of <28 weeks or birth weight of <1500 g) varies widely from less than 5% to as much as 40% between different centers (Lundstrom, Shaw et al. 2000). This variation is due in part to the differences in the diagnostic criteria employed, the general health status, differences in the respiratory care and perhaps even genetic factors, rendering comparisons between different centers difficult. Apparently, the incidence of CLD among very-low-birthweight (VLBW) infants continues to be high. One explanation for this may be that the general survival rate for infants with a birthweight of < 1000 g has increased (Northway 1992; Manktelow, Draper et al. 2001).

2. Etiology of CLD

Whereas classical BPD may be a manifestation of delayed recovery form of RDS, CLD represents diseases with many associated etiologies (Jobe and Bancalari 2001). The most important factors involved in the development of CLD are 1) pulmonary immaturity, 2) oxygen toxicity and 3) baro/volutrauma as a consequence of mechanical ventilation and/or infections (Northway, Rosan et al. 1967; Robertson 1989; Northway 1990). Classically, it has been believed that a combination of these factors over an extented period of time will result in the lung pathology seen in CLD. Other important factors which may contribute to the development of this disease, often by increasing the requirement for mechanical ventilation, include increased vascular permeability, circulatory abnormalities (e.g. due to fluid overload), patent ductus arterious (PDA) and surfactant abnormalities.

A North-American multi-center study revealed that the center that applied nasal continuous positive airway pressure (NCPAP), rather than mechanical ventilation, to

infants soon after birth had a lower incidence of CLD (Avery, Tooley et al. 1987). When we also applied NCPAP to preterm infants (with a gestational age of $\geq$ 27 weeks), the incidence of CLD was decreased compared to the use of mechanical ventilation (Jonsson, Katz-Salamon et al. 1997). Furthermore, it has been reported that infants who develop CLD exhibit very low levels of surfactant protein-A in airway specimens (Hallman, Merritt et al. 1991). As a consequence of surfactant therapy, the incidence of RDS has decreased while the smallest infants survive and later develop CLD (Hallman, Glumoff et al. 2001). Recently, increasing attention has been focused on the role of post and antenatal infection and inflammation in the pathogenesis of CLD (Hallman, Glumoff et al. 2001). On a cellular level, inflammatory processes are increasingly recognized as playing an important role in aggravating the initial injury and causing remodeling of the lung parenchyma (Bancalari 1998), hallmarks in the development of CLD (Lundstrom, Shaw et al. 2000) (Figure 2).

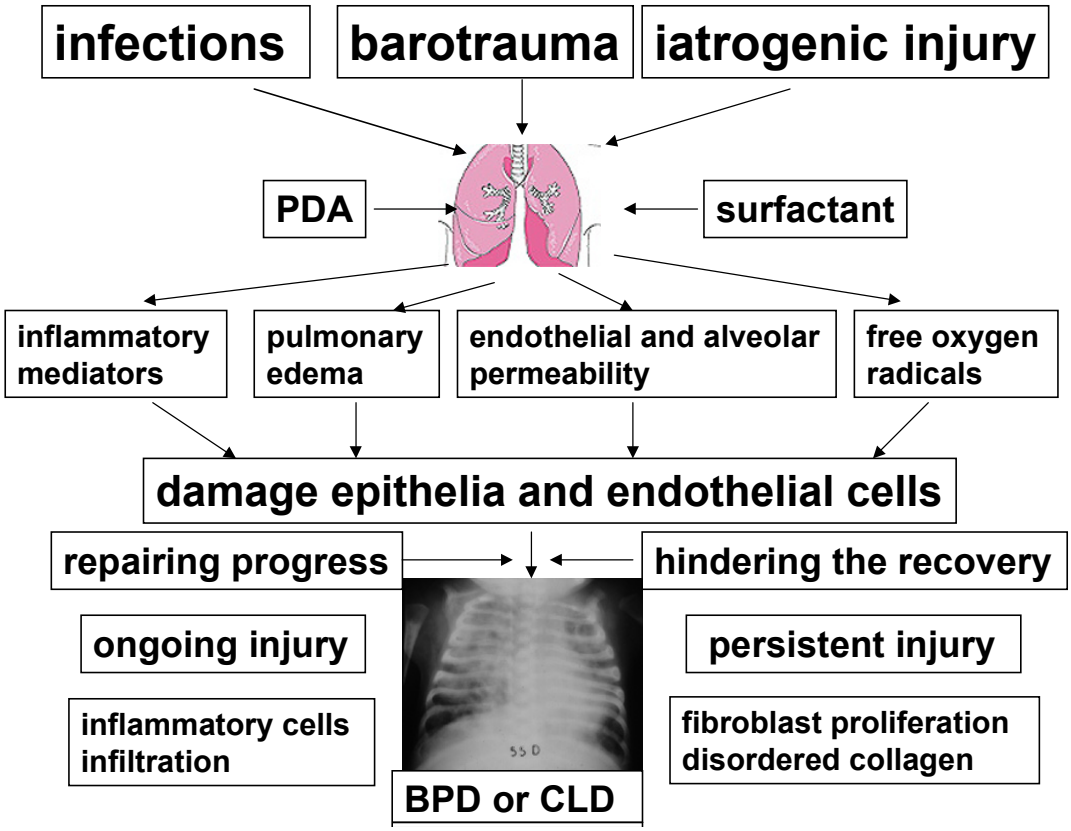


Figure 2 Etiology of CLD.

3. Contribution of infection to the development of CLD

3.1. Bacterial infection

Despite the significant progress made with respect to ventilation techniques and controlling oxygen delivery, there are nonetheless still many cases of CLD. It appears that infection and the inflammatory response play significant roles in the development of CLD. The combination of barotrauma and oxygen toxicity can quite rapidly lead to epithelial cell damage, rendering the normal mechanisms for clearance of the airways ineffective (Sherman, Evans et al. 1988). The frequency of ciliary beating and the efficiency of mucociliary transport are markedly decreased when either the temperature or humidity of inspired gas decreases. Such changes can lead to increased susceptibility of the airways to nosocomial infections (Goodwin, Graves et al. 1985; Tarnow-Mordi, Sutton et al. 1986). During ongoing mechanical ventilation, early colonization of the airways by coagulase-negative staphylococci (CONS) was frequently replaced by non-CONS microorganisms, i.e., predominantly Gram-negative bacteria. This pattern of bacterial airway colonization was also observed in a baboon model of CLD (Coalson, Gerstmann et al. 1991; deLemos and Coalson 1992). A recent study demonstrated that BPD occurred more often in neonates whose airways had a concomitant colonization with both Gram-positive cocci and Gram-negative bacteria compared to those colonized only with Gram-positive cocci (Cordero, Ayers et al. 1997). It was also observed that in ventilated preterm infants Gram negative bacterial airway colonization was found to be associated with the severity of CLD (Cordero, Ayers et al. 1997).

Perterm delivery is a risk factor for the development of CLD. Infection of the maternal genital tract and the associated inflammatory response are involved in the pathogenesis of preterm labor and premature rupture of membrane (PROM). As many as 80% of woman giving birth prior to 30 weeks of gestation exhibit histological chorioamnionitis (Goldenberg and Andrews 1996). Increased incidences of neonatal infection and perinatal mortality have been found to be related to chorioamnioitis in case of both preterm and term pregnancies (Seo, McGregor et al. 1992). Antibiotic treatment of women with preterm PROM has resulted in reduced rates of preterm delivery (Hauth, Goldenberg et al. 1995) and infant morbidity (Mercer and Arheart 1995; Egarter, Leitich et al. 1996).

3.2. Viral infection

Viral agents have also been reported to play a role in the development of CLD in preterm infants. Cytomegalovirus (CMV) is commonly detected in amniotic fluid in connection with abnormal pregnancies, particularly in association with fetal hydrops (Van den Veyver, Ni et al. 1998). Colonization of the lungs with CMV has been reported to be associated with an increased risk for developing CLD (Sawyer, Edwards et al. 1987). However, CMV infection in infants that developed BPD is believed to occur postnatally.

Adenovirus is a double-stranded DNA virus first isolated from primary cultures of human adenoid cells and from airway secretions during an epidemic of respiratory disease in military recruits. A recent screening for adenovirus employed the polymerase chain reaction in tracheal aspirate samples obtained within the 1st week of life demonstrated a significant increase in the frequency of adenovirus genome in BPD patients compared with controls, suggesting that congenital adenovirus infection might be of importance in the development of BPD (Couroucli, Welty et al. 2000).

3.3. Infection by *Ureaplasma urealyticum*

U. urealyticum, a common commensal of the urogenital tract of humans, is gaining recognition as an important opportunistic pathogen during pregnancy. This organism is a member of Mollicutes, commonly referred to as the mycoplasma, and is able to produce urease and thereby hydrolyze urea (Glass, Lefkowitz et al. 2000). The genus Ureaplasma consists of a single species (*U. urealyticum*) and 14 serovars, of which serovar 3 is the most commonly isolated. *U. urealyticum* requires a pH of 6.0 for optimal growth. The morphological characteristics of *U. urealyticum* in liquid medium cultures are similar to those of other mycoplasmas. These organisms are generally round-to-ovoid in shap with in diameter of approximately 330 nm ranging from 100 - 850 nm. Rod-shaped and filamentous structures also occur, the latter exhibiting a length of 2 μm and a width of 50 to 300 nm. In clinical material, short, bacillary forms with a singlepointed end are frequently observed. *U. urealyticum* is surrounded by a single trilaminar membrane approximately 10 nm thick with a pilus-like structure radiating from the surface. The major surface antigen of serovar 3 of this

organism consists of a signal peptide, 120 amino acid conserved region and a variable region consisting of tandem 6 amino acid repeats.

U. urealyticum is present in the lower genital tract of 40-80% of pregnant women (Eschenbach 1993; van Waarde, Brus et al. 1997) and is strongly associated with chorioamnionitis during pregnancy (Cassell, Waites et al. 1986). The rate of vertical transmission from the mother to full term infants and preterm infants ranges from 18-55% and 29-55% respectively (Sanchez 1993). *U. urealyticum* has been cultured from open lung biopsy specimens from infants with CLD (Walsh, Stanley et al. 1991) and shown to cause pneumonitis in experimental animals (Crouse, Cassell et al. 1990; Walsh, Butler et al. 1993). This organism has also been isolated from the blood, cerebrospinal fluid, TAF and lung tissue of preterm neonates (Waites, Crouse et al. 1993) and evidence exists that it can cause invasive disease in such infants. Respiratory tract colonization by *U. urealyticum* is associated with increased incidences of pneumonia (Crouse, Odrezin et al. 1993), RDS, intraventricular hemorrhage and CLD (Abele-Horn, Peters et al. 1997). Such colonization of premature baboons experimentally leads to acute bronchiolitis with epithelial ulceration and polymorphonuclear infiltration (Walsh, Butler et al. 1993). Furthermore, newborn mice inoculated with *U. urealyticum* developed acute interstitial pneumonia (Rudd, Cassell et al. 1989). These findings suggest that *U. urealyticum* may also elicit an inflammatory response in preterm infants.

An association between airway colonisation by this organism and the development of CLD has been suggested as well (Cassell, Davis et al. 1983). The frequency of CLD in neonates colonized with *U. urealyticum* is twice as high as in non-colonized infants (Agarwal, Rajadurai et al. 2000). Colonisation or infection with ureaplasma predisposes to development of CLD, independent of gestational age (Hannaford, Todd et al. 1999). A prospective study in a cohort of infants born after 24–33 weeks of gestation revealed a significant relationship between perinatal *U. urealyticum* infection and subsequent need for hospital care during the first year of life. In addition, CLD is an important factor resulting in subsequent hospital treatment as assessed by the number of hospital days required in children with perinatal *U. urealyticum* infection, suggesting that *U. urealyticum* infection influences the severity of CLD and complicates the later natural course (Ollikainen 2000). These findings support the hypothesis that vertically transmitted colonization and infection with *U.*

urealyticum constitutes an important risk factor in connection with CLD (Cassell, Waites et al. 1988; Wang, Frayha et al. 1988; Abele-Horn, Genzel-Boroviczeny et al. 1998; Perzigian, Adams et al. 1998). A meta-analysis has provided additional support for the existence of a causal relationship between colonization by this organism and the development of CLD (Wang, Ohlsson et al. 1995). However, other studies employed correction for gestational age by logistic regression analysis, have failed to confirm such an association (van Waarde, Brus et al. 1997). Thus, the possible contribution of *U. urealyticum* to the development of CLD remains controversial (van Waarde, Brus et al. 1997).

4. Inflammation and the development of CLD

A variety of inflammatory responses have been described as hallmarks in the development of CLD, and these responses may differ, depending on the etiology of the disease. Exposure of the immature, underdeveloped lung to supplemental oxygen and mechanical ventilation can result in tissue damage that triggers a number of events at the cellular level, e.g. epithelial and endothelial cell damage, increased permeability, surfactant inactivation, activation of inflammatory cells and release of inflammatory mediators (deLemos and Coalson 1992; Groneck, Schmale et al. 2001; Hallman 2001) (Figure 3).

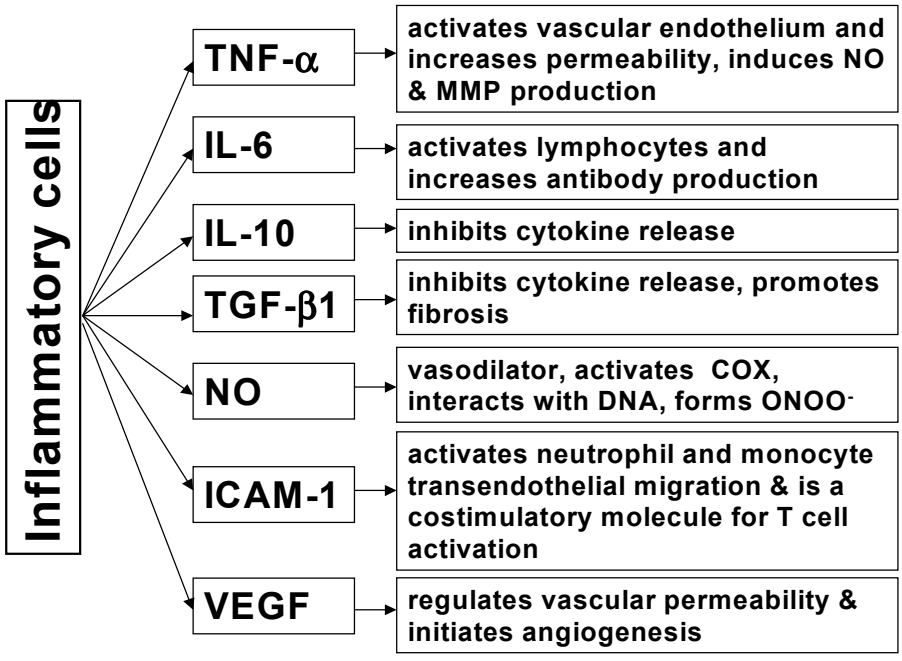


Figure 3.Various aspects of macrophage-mediated inflammation.

4.1. Inflammatory cells

Pulmonary neutrophils are predominately seen during the first week of life and a persistence of these cells has been observed in infants who develop CLD (Merritt, Stuard et al. 1981; Arnon, Grigg et al. 1993; Murch, Costeloe et al. 1996). Alveolar macrophages, important cells for resolution, from approximately 4 days of postnatal age, have been shown to reach the maximum concentration in the airway samples of infants with RDS, and to persist in infants with CLD (Clement, Chadelat et al. 1988). They become the predominant inflammatory cells in the lung of CLD infants after the first week of life (Alenghat and Esterly 1984; Tullus, Noack et al. 1996; Speer 2001). Airway epithelial cells, endothelial cells, T lymphocytes, B lymphocytes, NK cells, leukocytes and fibroblasts also appear to contribute to the inflammatory reaction (Ozdemir, Brown et al. 1997).

4.2. Inflammatory mediators

During the inflammatory response, cytokines, along with a number of other factors including cellular adhesion molecules, lipid mediators, proteases, chemoattractants, nitric oxide, nitric oxide-derived oxidant, mitogenic growth factor and complement are released in response to the inflammatory process.

4.2.1. Cytokines

Cytokines are small soluble proteins secreted by one cell that can alter the behavior or properities of the cell itself or of other cells. These signalling molecules are secreted by many different types of cells and are involved in local or systemic responses to tissue injury, inflammation and tissue repairment. Monocytes/macrophages, T lymphocytes, neutrophils, epithelial and endothelial cells and fibroblasts all produce cytokines in response to cellular injury or exposure to endotoxin, hypoxia or hyperoxia. These cytokines bind to specific receptors on the surface of cells and their most important function appears to be the establishment of a local complex paracrine network involving adjacent cells (Kelley 1990; Janeway and Travers 1997).

Cytokines can be classified on the basis of their primary effects in the inflammatory responses (Kelso 1998). The proinflammatory cytokines TNF-α, IL-1β and IL-6 promote the inflammatory response by exerting direct cell toxicity and recruiting

additional inflammatory cells. The down-regulatory cytokines IL-4, IL-10 and TGF-β are secreted in response to increased levels of the proinflammatory cytokines and to tissue injury. They promote termination of the acute phase reaction by down-regulating the expression of proinflammatory cytokines and inducing tissue repair, which often leads to fibrosis (Border and Noble 1994).

The lung fluid of preterm infants who develop CLD contains elevated levels of proinflammatory cytokines and chemokines as early as day 1 of life. These levels even further increase during the first week of life, reaching a peak level at approximately 2 weeks, and remaining elevated for several weeks thereafter (Bagchi, Viscardi et al. 1994; Little, Dean et al. 1995; Kotecha 1996; Tullus, Noack et al. 1996). Increased levels of IL-6 and Il-1β are associated with chorioamnionitis and bacterial colonisaton of the airways of preterm infants at birth (Groneck, Goetze-Speer et al. 1996; Kotecha, Wilson et al. 1996; Watterberg, Demers et al. 1996). IL-8, which is probably the most important chemotactic factor preceding the marked neutrophil influx in the lung, is produced upon stimulation by alveolar macrophages, fibroblasts, type II pneumocytes and endothelial cells. The level of this cytokine in infants who developed CLD has been shown to be elevated (Kotecha, Chan et al. 1995; Munshi, Niu et al. 1997). In addition, elevated levels of TGF-β1 have been reported as early as day 2 of life (Kotecha, Wangoo et al. 1996). Two reports concerning the levels of the TAF IL-10 in CLD infants have been published. Jones and coworkers (Jones, Cayabyab et al. 1996) examined samples collected from five preterm infants during their first 96 hours of life and found a decreased ability to produce IL-10, which might indicate that lack of IL-10 has importance for an increased tendency to development of CLD. However, McColm and colleages (McColm, Stenson et al. 2000) demonstrated that 16 of 17 preterm infants exhibited measurable levels of IL-10 during the first 10 postnatal days. Most of these infants developed CLD and the investigators saw no relation between IL-10 levels and the outcome in this respect. They thus concluded that the possible influence of IL-10 on the pathogenesis of CLD remains to be elucidated.

4.2.2. Nitric oxide

NO is generated from L-arginine by three different NO synthases. Of these, two are constitutive isoforms; the third, inducible and Ca^{2+}-independent NO synthase (iNOS),

is expressed only following transcriptional activation of its gene (Xie, Cho et al. 1992; Lorsbach, Murphy et al. 1993), as in acute and chronic inflammation (Grisham, Jourd'Heuil et al. 1999) (Figure 4).

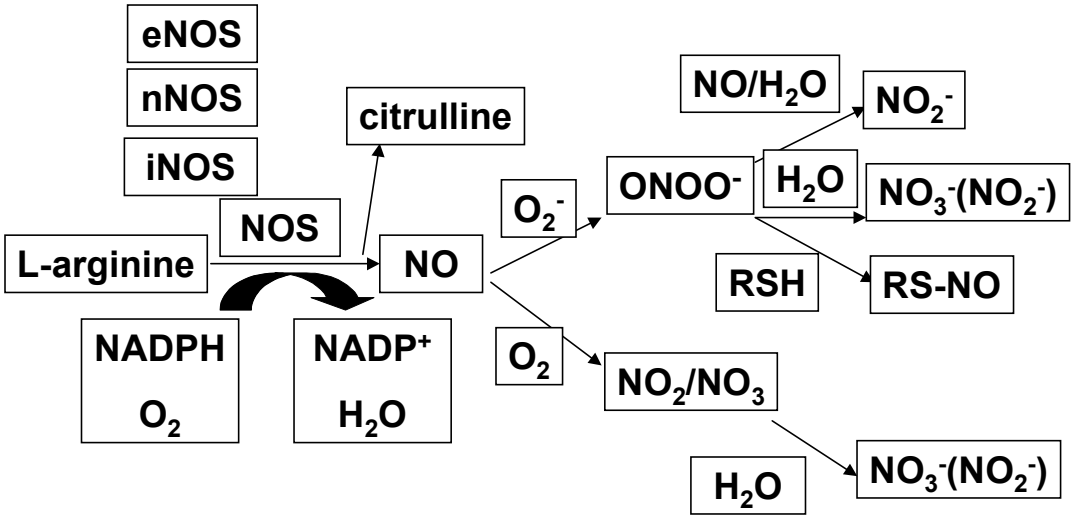


Figure 4. Pathways for the formation and degradation of NO. eNOS, endothelial nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; RSH, thiols; RS-NO, S-nitrosothiols.

It is being increasingly recognized that NO acts as an important intra- and intercellular messenger molecule in vascular relaxation, platelet activation and immunological responses by human mononuclear cells (Sonoda, Kobayashi et al. 1997). Clinically, this simple inorganic compound also plays important roles in the pathogenesis of Gram-negative septic shock and other infectious diseases (Weinberg 1998). Although VLBW infants with CLD have normal plasma levels of arginine, increased ratios of citrulline / arginine and citrulline / ornithine in the serum of infants with severe CLD have been observed, which might be explained by a shift of arginine conversion from urea cycle to nitric oxide synthesis (Heckmann, Kreuder et al. 2000). The plasma content of 3-nitrotyrosine, an oxidant derived from NO, is elevated during the first month of life in infants who developed CLD (Banks, Ischiropoulos et al. 1998). Vyas and co-workers (Vyas, Currie et al. 1999) have demonstrated that the nitrate

concentration in TAF from CLD, RDS and control infants is very similar during the first week of life. However during the second week of life, this nitrate concentration remains high in the infants who progressed to CLD, while decreasing in the RDS and control groups.

4.2.3. Nuclear factor -κB

Nuclear factor -κB (NF-κB) is an ubiquitous transcription factor that governs the expression of genes coding for cytokines, chemokines, enzymes, growth factors, cellular adhesion molecules and some acute phase proteins. This factor is activated by a number of agents, including bacterial and viral products (Chen, Castranova et al. 1999). To date, five members of the NF-κB family have been identified in mammals. These include NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), RelB and c-Rel.

4.2.4. Cellular adhesion molecules

Cellular adhesion molecules, predominantly ICAM-1 (CD54), which promote recruitment of neutrophil to the lung, has been detected in lung fluid from infants at risk for developing CLD (Little, Dean et al. 1995). In addition, serum concentrations of ICAM-1 were found to be higher in infants that developed CLD than those who did not (Ramsay, O'Brian Smith et al. 1998). L-selectin and E-selectin the other important cell adhesion molecules which mediate the margination or “rolling” of leucocytes on the endothelial cell surface, has been found increased in TAF and serum in CLD infants (Kotecha, Silverman et al. 1998; Ramsay, O'Brian Smith et al. 1998).

4.2.5. Lipid mediators

Lipid mediators are released rapidly from cell membranes in response to tissue injury. Leukotrienes (LTB_4), thromboxane A_2 and platelet-activting factor act as chemoattractants for neutrophils, as well as increasing vascular permeability and platelet aggregation (Ozdemir, Brown et al. 1997). In CLD, the levels of LTB_4 prostacylin was found to be elevated (Groneck, Gotze-Speer et al. 1994; Lundstrom, Shaw et al. 2000).

4.2.6. Complement

The anaphylatoxin C5a, an important chemoattractant for human neutrophils, has been detected in bronchoalveolar fluid from CLD infants, where its concentration was significantly higher than in babies with RDS (Groneck, Oppermann et al. 1993).

4.2.7. Proteases

A possible role of elastase, a powerful neutral proteinase stored in the azurophilic granules of neutrophils, in the pathogenesis of acute lung disease and CLD in preterm infants has been considered. Pulmonary tissue elastin is the primary substrate of neutrophil elastase. Under physiological conditions, elastase is rapidly bound and inactivated by α_1-proteinase, which thus protects the alveolar-capillary unit from autolytic proteolysis (Speer, Ninjo et al. 1986). An imbalance in relative amounts of the protease and antiprotease, as well as toxic oxygen radicals released by phagocytes or generated by tissue-bound xanthine oxidase, may play a central role in lung injury. Indeed, such an imbalance of proteases and antiproteases has been proposed to be an important factor in the development of CLD (Ohlsson, Calvert et al. 1992). For example, in tracheobronchial secretions of infants with BPD, increased concentrations of free elastase and low activities of α_1-proteinase have been detected (Yoder, Chua et al. 1991; Ohlsson, Sveger et al. 1992). Furthermore, an alpha1-proteinase inhibitor has been reported to decrease the risk of development of CLD, suggesting that proteinase may contribute to the progression of lung injury (Stiskal, Ito et al. 1999).

Elastase and collagenase released from neutrophils and macrophages can damage collagen, elastin and release glycosaminoglycans in the preterm lung (Bruce, Schuyler et al. 1992; Murch, Costeloe et al. 1996). Furthermore nosocomial pulmonary infection in CLD infants enhances this effect (Walti, Tordet et al. 1989). The maturation of elastic tissue in the lung during fetal development is tightly controlled. Normally, the volume density and the absolute quantity of pulmonary elastic tissue increase progressively during the peroid from 22-50 weeks. After birth in infants with CLD, the volume density and absolute quantity of elastic tissue in the lung are significantly elevated (Thibeault, Mabry et al. 2000). An increase in proteolytic enzyme content contributes to the disruption of the cellular matrix in the lung (Lundstrom, Shaw et al. 2000). For instance, it has been shown that the level of matrix

metalloproteinase (MMP)-9, which can degrade type IV collagen, the major constituent of basement membranes, is increased in TAF fluid from babies who subsequently develop CLD. Furthermore, the level of MMP-8, which can degrade type I collagen, the main structural protein of extracellular matrix of the lung, is higher in neonates who later develop CLD (Sweet, McMahon et al. 2001).

4.2.8. Fibronectin

Fibronectin is a potent chemoattractant for fibroblasts and plays an important role in proliferation of these cells in the lung. Elevated concentrations of fibronectin have been detected in tracheal aspirates from infants who develop CLD (Gerdes, Yoder et al. 1986).

4.2.9. Bombesin-like peptides

Bombesin-like peptide (BLP) synthesized by neuroendocrine cells is known to cause bronchoconstriction, as well as functioning as growth factors for pulmonary fibroblasts and epithelial cells. Thus, early overproduction of BLP could mediate the peribronchiolar and interstitial fibrosis and reactive airway disease which are hallmarks of BPD. A marked elevation in BLP mRNA has been found in lung sections, suggesting that BLP might be implicated in the earliest stages of the development of BPD (Sunday, Kaplan et al. 1988). Human infants and baboons with BPD exhibit elevated numbers of neuroendocrine cells, mast cells and eosinophils in their lungs, and furthermore, treatment of preterm baboons with an anti-BLP antibody decreases the numbers of these “immunologic” cells and results in less lung injury (Sunday, Yoder et al. 1998). Together with other factors, BLP may elicit and/or promote proinflammatory responses that progress to BPD. Urinary levels of BLP are correlated to the severity of BPD in preterm baboons. Similarly, human infants destined to develop BPD also show increased urinary levels of BLP. Thus, BLP may be a useful early indicator for the identification of infants at risk for developing BPD.

4.2.10. Apoptosis

Apoptosis, the well-characterized form of active programmed cell death, is a common phenomenon in developing organs during embryonal and fetal life. Apoptosis may play a role in the etiology of human lung disease in at least two different ways: in the

first place, tissue repair following acute lung injury requires the elimination of proliferating mesenchymal and inflammatory cells from alveolar airspace or walls. Failure to remove such undersirable cells by apoptosis will prolong the inflammation, due to the release of their toxic contents. Secondly, excessive apoptosis may also result in a diseased state, as exemplified by pulmonary fibrosis in mice (Polunovsky, Chen et al. 1993; Greenhalgh 1998; Cooper 2000). Recently, apoptosis in the lung of preterm neonates with acute and chronic lung disease has been examined. This process was found to occur in type 2 pneumocytes, indicating its involvement in the disappearance of these cells during the late subacute stage of BPD. In addition, apoptosis of mesenchymal cells was detected in the chronic stage of BPD (Hargitai, Szabo et al. 2001).

4.3. Role of inflammation in the development of CLD

The presence of increased numbers of inflammatory cells and elevated levels of mediators of both acute and chronic lung injury indicate that inflammation plays an important role in the pathogenesis of BPD. Complex interactions between cytokines, their receptors and inhibitors, and other mediators of inflammation involved in the regulation of this process appear to predispose to lung tissue destruction and fibrosis. Characterization of the inflammatory mediators and their regulation during this period of development will enhance our understanding of their role(s) in the development of CLD.

The damage caused to the immature lung by the primary insult (often RDS) and by iatrogenic injury (barotrauma, hyperoxia and infection) is probably additive and can lead to the development of necrotizing alveolitis. The activation of inflammatory cascades can result in a vicious cycle of inflammation, which subsides only slowly. After the initial damage, repair processes that favor fibroproliferative changes in the lung are begun. Dysregulation of the responses of cytokines and other inflammatory mediators in the preterm infants could help slow down this process. This results in remodeling of lung tissue with disrupted lung morphology and architecture. The final outcome may be a lung parenchyma with areas of atelectasis that alternate with areas of overexpansion or large bullae. The physiological conequences are an increase in dead space, alterations in pulmonary mechanics and a mismatch of ventilation-perfusion that leads to poor ventilation and chronic hypoxia. The inflammatory

response may contribute not only to the development of CLD, but perhaps also to adverse neonatal outcomes such as neurological damage, cerebral palsy and necrotizing enterocolitis (Jobe and Bancalari 2001).

5. Prevention and treatment of CLD

5.1. General

The ultimate prevention of CLD could be achieved by eliminating preterm birth, which is the single most important risk factor for development of this disease. Since achievement of this goal appears to be impossible for the foreseeable future, direct efforts must be made to prepare the immature lung for breathing air, e.g., by enhancing lung maturity with prenatal corticosteroids; treating RDS more effectively with exogenous surfactant; improving respiratory care and the prevention and treatment of infections; administration of antioxidant therapy and steroid treatment to infants at high risk for developing CLD; plus focusing more attention on aspects of growth and nutrition that have been found to influence the development and outcome of CLD.

5.2. Steroid therapy

Corticosteroids can be employed as highly effective pharmacological agents for controlling inflammation, even though the exact mechanism(s) by which these compounds suppress inflammation is not entirely understood. Corticosteroids inhibit the transcription of a number of genes encoding proinflammatory cytokines, including TNF-α, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8 and GM-CSF (Barnes 1997). These effects may be mediated by interaction of the glucocorticoid receptor (GR) with a negative glucocorticoid recognition element (GRE), resulting in a direct reduction in gene transcription. Increased breakdown of mRNA has also been demonstrated for cytokines. Corticosteroids may not only block the synthesis of cytokines, but may block their effects in several other ways as well (Barnes 1997). Thus corticosteroids may down-regulate certain cytokine receptors; inhibit the activation of transcription factors, such as active protein-1 (AP-1) and NF-κB; and, furthermore, directly counteract the action of cytokines on cells. These steroids may also increase the synthesis of lipocortin-1 (a protein that has an inhibitory effect on phospholipase A2)

and thereby inhibit the production of lipid mediators such as leukotrienes, prostaglandins and platelet activation factor (Barnes and Adcock 1993).

5.2.1. Prenatal steroid therapy

Treatment of mothers at high risk for giving preterm birth with glucocorticosteroid has led to a highly significant reduction in the incidence of early neonatal death and RDS (Bancalari 1998). A randomized placebo control study demonstrated that treatment of such mothers with dexamethasone 1-14 days prior to preterm delivery decreased the requirement of their infants for exogenous surfactant, supplementary oxygen and mechanical ventilation and tended to increase survial without the development of severe CLD (Kari, Hallman et al. 1994).

5.2.2. Prophylactic steroid treatment of infants at risk

As discussed above, evidence that inflammation plays a significant role in the development of CLD is accumulating rapidly. Clinical administration of corticosteroids to preterm neonates results in significant short-term improvements in their lung functions (Anonymous 1991; Ohlsson, Calvert et al. 1992). These agents can either be adminisered systemically (dexamethasone) or by inhalation (budesonide). An early 3-day course of dexamethasone therapy increases survival without CLD, reduces CLD and reduces the need for late dexamethasone therapy in high risk, low birth weight infants who receive surfactant therapy for respiratory distress syndrome (Garland, Alex et al. 1999). A 42-day tapering course of dexamethasone decreases the duration of ventilator and oxygen dependency in VLBW infants (Kothadia, O'Shea et al. 1999). A meta-analyses revealed a significant reduction in risk of CLD by using early dexamethasone treatment for infants with RDS (Bhuta and Ohlsson 1998). Recently a randomized, double-blind study concerning the oxygen requirement of infants at high risk for developing CLD demonstrated that such infants who received an aerosolized corticosteroid (budesonide) are more often extubated during the study period and have a greater relative change from baseline in their oxygenation index (budesonide decreased 26% while placebo increased 60%). Subsequent administration of intravenous dexamethasone or inhaled budesonide to infants in the treatment group is significantly less. At 36 weeks of postconceptual age, 61% of the infants in the budesonide group

need supplemental O_2 as opposed to 79% in the placebo group (Jonsson, Eriksson et al. 2000).

Corticosteroids efficiently inhibit the proliferation of T lymphocytes induced by mitogens or antigens by the way of acting selectively at distinct sites of the T cells and monocyte-macrophage triggering processes (Gordon and Nouri 1981; Kronke, Leonard et al. 1985; Reed, Nowell et al. 1985; Barnes and Pedersen 1993). Steroids increase the activity of antioxidant enzymes and concentration of surfactant protein and stabilize lysosomal membranes. These compounds decrease vascular permeability and pulmonary edema and can induce the closure of PDA. Furthermore, they promote diuresis by way of their anti-vasopressin effect and increase pulmonary levels of beta-adrenergic receptors. Steroid treatment has been found to improve pulmonary mechanics, reduce the proteolytic activity in lung fluid, alternate leukocyte recruitment and decrease concentrations of cytokines, leukotrienes and certain growth factors (Yoder, Chua et al. 1991; Groneck, Reuss et al. 1993; Kari, Raivio et al. 1994; Durand, Sardesai et al. 1995).

Aims of the present study

The overall aim of the present investigation was to evaluate the possible contribution of *U. urealyticum* to the development of CLD and to characterize the inflammatory process that occurs in the premature lung during that development.

The specific aims were to examine the following:

1. whether *U. urealyticum* can initiate inflammation by stimulating macrophages to produce proinflammatory mediators, such as cytokines, nitric oxide and cellular adhesion molecules *in vitro* and the influence of *U. urealyticum* infection on the expression of vascular endothelial growth factor;
2. possibilities for down-regulating these inflammatory responses;
3. the possible mechanisms underlying these inflammatory responses;
4. NO production and the defense response of the host; and
5. the significance of down-regulatory cytokines present in tracheobronchial aspirate fluid collected from infants with CLD.

Material and Methods

1. Cell cultures (Papers I, II, III & IV)

1.1. Cell lines

The human monocytic cell line THP-1 (ATCC, Rockville, Maryland, USA) was maintained in RPMI 1640 medium (Sigma chemical Co, St. Louis, Missouri, USA) supplemented with 2 mM L-glutamine (Sigma), 5 mM mercaptoethanol, 10 mM Hepes (GibcoBRL, Gaithersburg, MD, USA) and 10% heat-inactivated fetal bovine serum myclone (GibcoBRL) at 37°C under 5% CO_2. Prior to use, cell cultures were split, seeded into fresh medium for 48 hours and then differentiated with 10^{-8} M PMA (phorbol 12-myristate 13-acetate, Sigma) with 5×10^5cells/ml in teflon-coated bottles for 48 hours. In order to confirm the population of macrophages, flow cytometry analysis was employed. The expression of macrophage-specific cell surface antigens showed that 96.2% of the cells expressed CD14 and CD45 and 99.9% expressed HLA-DR, indicating a full differentiation of macrophages.

The rat alveolar macrophage cell line Nr.8383 (ATCC) was maintained in Ham's F-12 medium (GibcoBRL) supplemented with 15% heat-inactivated fetal bovine serum (GibcoBRL).

1.2. Human TAF macrophages (Paper I & II)

This portion of the study was performed with the approval of the local ethics committee of the Karolinska Hospital and the parents gave their informed consent. Lung macrophages were collected from 4 premature infants receiving mechanical ventilation. The aspirates were collected in connection with routine endotracheal suctioning. At this time, 0.5 ml 0.9% NaCl was instilled into the endotracheal tube and the infant then reconnected to the ventilator. After disconnection of ventilator, the suction catheter was inserted past the tip of the endotracheal tube and aspiration was performed during withdrawal of this catheter. Subsequently, the catheter was flushed employing an additional 0.5 ml 0.9% NaCl into a sterile specimen trap. Thereafter, the TAF was filtered through a special cell strainer (Falcon, New Jersey, USA), centrifuged and the supernatant discarded. The cells thus obtained were cultured in 96

micro-well plates (Costar, Cambridge, MA, USA) at concentration of 5×10^5 cells/ml in RPMI 1640 medium containing 2mM L-glutamine, 10% heat-inactivated fetal bovine serum (GibcoBRL) and penicillin (100 units/ml)-streptomycin (100 μg/ml) (Sigma). Macrophages were allowed to adhere to the plastic during a 2-hour pre-incubation period at 37°C under 5% CO_2 and the supernatant together with non-adhensive cells was then removed and discarded.

2. Preparation of U. urealyticum antigen

U. urealyticum serotype standard strain 8 (T960) (ATCC) was cultured at 37°C in 1.5 l of ureaplasma broth medium containing 22.5 g of trypticase soy broth per l; 16.5% horse serum; 7.5% of a fresh 25% yeast extract; 0.36% urea; 380,000 U penicillin G per l and phenol red. Cells were harvested in late log phase growth by centrifugation at 30,000 x g for 90 min at 4°C. The pellet obtained was washed three times by resuspension in phosphate buffered saline (PBS) and centrifugation as above for 30 min. After the final wash, the ureaplasma were resuspended in PBS to give a total volume of 2 ml. The color-changing units (CCU) of this concentrated suspension quantitated in duplicate employing 10-fold titration in ureaplasma broth. The *U. urealyticum* in this suspension was subsequently heat-killed by incubation in a water bath at 56°C for 20 min. Complete killing was confirmed by incubating 25 μl of the suspension on ureaplasma agar, as well as in ureaplasma broth without urea supplement followed by subculture on agar after 1, 3, and 7 days. The latter procedure was employed since the suspension of heat-killed *U. urealyticum* produced a prompt change in the color of the ureaplasma broth due to urease activity. The limulus amebocyte lysate (LAL)(Charles River Endosafe, SC, USA) test revealed that the endotoxin level in the 4×10^8 CCU/ml of *U. urealyticum* antigen was less than 20 pg/ml. The *U. urealyticum* antigen was stored at -70°C in 0.1ml aliquots until use.

3. Experimental protocols

3.1. Cytokine production and its down-regulation (Papers I and II)

Macrophages obtained from human and rat cell lines were distributed at a concentration of 1×10^6 cells/ml onto 24 micro-well plates and stimulated with 100 ng lipopolysaccaride (LPS) (O55:B5, Sigma) or 4×10^6-4×10^8 CCU of *U. urealyticum*

antigen per ml for 24 hours at 37°C under 5% CO_2. Various doses of dexamethasone (Sigma), budesonide (Astra, Stockholm, Sweden) or recombinant human or rat IL-10 (R&D Systems, Abingdon, Oxon, UK) were then added to the stimulated cells and thereafter incubated for 24 hours. When rat IL-6 and TNF-α mRNA were to be analyzed, the stimulation was carried out for only 4 hours, and as controls, the same cells were incubated with medium alone. All experiments were repeated 4-6 times. Human TAF macrophages were incubated with *U. urealyticum* antigen (4×10^8 CCU/ml) or medium alone for 24 hours at 37°C under 5% CO_2.

3.2. NO production, iNOS expression and NF-κB avtivation (Paper III)

Rat alveolar macrophages were distributed at a concentration of 1×10^6 cells/ml in serum and phenol red-free medium onto 24 micro-well plates and stimulated with 4×10^6-4×10^8 CCU of *U. urealyticum* antigen or 100 ng of lipopolysaccaride (LPS, O55:B5) (Sigma) or in combination with 100 IU IFN-γ (Genzyme, Cambridge, MA, USA) per ml for 24 hours at 37°C under 5% CO_2. To evaluate the effects of steroids, rat macrophages were incubated with 4×10^8 CCU of *U. urealyticum* per ml in the presence of dexamethasone (10^{-4}-10^{-6} M) or budesonide (10^{-4}-10^{-6} M). In order to dertermine wheather *U. urealyticum* antigen could directly stimulate NO production, the macrophages were incubated with 4×10^8 CCU of *U. urealyticum* per ml together with the inhibitor of protein synthesis cycloheximide (1 μg/ml) (CHX, Sigma) for 24 hours. These experiments were repeated 4-8 times.

3.3. Effects of NO on the growth of *U. urealyticum* (paper III)

Rat alveolar macrophages at a concentration of 1×10^6 cells/ml in serum and phenol red-free medium were distributed onto 24 micro-well plates and incubated with 10^5 or $2x10^5$ colony-forming units (CFU) of live *U. urealyticum* per ml (ATCC 1484) alone or in combination with 3 mM the inhibitor of NO synthase N^G-monomethyl-L-arginine (L-NMMA) or its inactive enantiomer N^G-monomethyl-D-arginine (D-NMMA) for 10, 14 and 24 hours. The supernatant was then collected from each well and 100 μl cultured on ureaplasma agar plates (containing 29.1 g trypticase soy broth, 242 ml horse serum, 12 ml IsoVitalex enrichment, 12 ml 25% yeast extract, 1.2 ml 20% urea and 12 ml penicillin 100,000 IU per ml). After 4 days of culture, the CFU of

U. urealyticum was counted and photographed under the light microscope (Nikon, Japan). These experiments were repeated four times.

3.4. Expression of ICAM-1 and VEGF (Paper IV)

Human macrophages at a concentration of 1×10^6 cells/ml were distributed onto 24-micro-well plates and stimulated with 4×10^8 CCU of *U. urealyticum* antigen per ml in the absence and presence dexamethasone (10^{-4} M) or budesonide (10^{-4} M). All of these experiments were repeated 4-6 times.

5. Patients (Paper V)

The study described in paper V was approved by the local ethics committee at the Karolinska Hospital and informed parental consent was obtained. Mechanically ventilated infants were recruited for inclusion in this study at the neonatal intensive care and pediatric intensive care units at the Karolinska Hospital in Stockholm, Sweden. The study group consisted of 69 infants and included preterm infants with RDS (n=22), infants who developed CLD (n=24) and infants ventilated post-operatively (n=23). RDS was diagnosed on the basis of characteristic radiographic findings, respiratory distress and an increasing FiO_2 requirement. Infants with RDS and requiring a $FiO_2 > 0.6$ received rescue treatment with natural porcine surfactant (200 mg/kg) (Curosurf, Serono, Italy). Infants who had been ventilated during the first week of life, exhibited signs of chronic respiratory symptoms (tachypnea, retractions and/or rales), required for supplementary oxygen for more than 28 days in order to maintain a $PaO_2 > 6.5$ kPa (50 torr), and demonstrated characteristic changes with respect to chest radiography, were diagnosed as having CLD. At the time of this study, the criteria for initiation of mechanical ventilation of preterm infants with RDS were a FiO_2 requirement > 0.6, a $PCO_2 > 8.5$ kPa (60 mm Hg) or base excess < -10. Infants were mechanically ventilated using a Sechrist 100B infant ventilator (Sechrist Corp., Anaheim, CA). In the case of three infants, who, despite conventional ventilation and surfactant administration, exhibited an increasing PCO_2 value, hypoxia and acidosis, high frequency oscillatory ventilation was employed (Sensor Medics 3100A; Sensor Medics Corp., CA, USA.).

6. Assays procedures

6.1. ELISA for cytokine determination

Following stimulation of cells, the supernatants were collected and stored at -70°C until being analyzed for cytokines. ELISA kits for human TNF-α, IL-6, TGF-β1, IL-10, IL-4, IL-12, VEGF and soluble ICAM-1 as well as for rat TNF-α were obtained from R & D. The levels of cytokines in the supernatants were determined using the quantitative "sandwich ELISA" technique, employing monoclonal antibodies specific for the cytokines interest. The limits of detection for human IL-6, TNF-α, TGF-β1, IL-10, IL-4, IL-12, VEGF and soluble ICAM-1 and for rat TNF-α were 0.7, 4.4, 31.2, 7.8, 31.2, 7.8, 5.0, 350 and 10 pg/ml, respectively.

6.2. The specific bioassay

Rat IL-6 bioactivity analysis was performed by a specific bioassay using an IL-6 dependent cell line-7TD1 hybridoma cell line as described previously (Hagberg, Gilland et al. 1996). In brief, these cells were cultured in an IL-6-containing medium and then "starved" at a lower level of IL-6 prior to introduction of the bioassay. The viability of the cells was 70%-95%. The cells were subsequently resuspended in medium without IL-6 to obtain 20000 cells / ml and 100-µl aliquals of this suspension was added to serial dilutions of the sample in the same final volume. Recombinant mouse IL-6 (Genzyme; Novakemi AB, Stockholm, Sweden) was used as a standard. After incubation for 72 hours at 37°C under 5% CO_2, the cultures were pulsed with 1 µCi [H^3]-thymidine in 10 µl NaCl and then incubated for additional 6 hours. These cultures were then harvested onto glass fiber strips, which were dried and punched into vials, to which scintillation liquid was added. The radioactivity was determined. The IL-6 concentration in the samples was finially determined by comparison to standard curve.

6.3. Nitrite assay

Following stimulation of cells, the supernatants were collected and stored at -70°C until being analyzed for NO. Accumulation of NO_2^-, a stable end product formed from NO in conditioned media, was measured as an indicator of NO production. 100 µl

cell-free conditioned medium was incubated for 10 min with 100 µl Griess reagent at room temperature and the absorbance at 540 nm subsequently measured in a microplate reader. The NO_2^- level in the samples was determined by comparison to a standard curve constructed using sodium nitrite.

6.4. Western blot analysis

Macrophages were lysed with Laemmli sample buffer and the protein denatured by boiling for 5 min. The protein concentration in this lysate was determined using a bicinchoninic acid kit (Pierce, Oud Beijerlands, The Netherlands). For Western blot analysis, 10 mg protein per lane was separated on sodium dodecyl sulfate–7.5% polyacrylamide gels and subsequently electroblotted onto hydrophobic polyvinylidene difluoride (PVDF) membranes (Amersham, Little Chalfont, Buckshire, United Kingdom). These membranes were blocked in 5% non-fat dry milk dissolved in TTBS (150 mM NaCl, 10 mM Tris-HCl, 0.1% Tween 20, pH 7.4) and incubated thereafter for 1 h at room temperature with a monoclonal antibody against macrophage iNOS (Transduction Lab, Lexington, Ky.), followed by incubation for 1 h with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (Amersham). Immunoreactive bands were visualized by utilizing an enhanced chemiluminescence kit (Amersham).

6.5. Electrophoretic mobility shift assay (EMSA)

Cells grown in serum-free medium were stimulated with 4×10^8 CCU of *U. urealyticum* antigen per ml for 30 min without and with pretreatment with dexamethasone (10^{-4} M) or budesonide (10^{-4} M) for 1 hour. Nuclear extracts were then prepared as described earlier (Yan, Sirsjo et al. 1999) and nuclear protein concentrations determined using the BCA method (Pierce, Rockford, IL). The nuclear extract (3 µg nuclear protein) was preincubated for 10 min in the reaction buffer (10 mM HEPES, pH 7,9, 10% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mg poly (dI-dC)), followed by incubation for 30 min at room temperature with 50,000 cpm of ^{32}P-labeled NF-κB probe (double-stranded oligonucleotides containing an NF-κB consensus binding site: 5'-AGT TGA GGG GAC TTT CCC AGG C-3', Promega, Madison, WI, USA). After 30 min at room temperature, samples were separated on

4% native polyacrylamide gels in low ionic strength buffer (22.3 mM Tris-borate, 0.5 mM EDTA, pH 8). After drying, the gels were autoradiographed employing intensive screens at -80^{o}C. In some cases, the incubation of nuclear extracts with ^{32}P-labeled probe for NF-κB was carried out in the presence of a 25- and 50-fold excess of unlabeled probe or of an unlabeled irrelevant oligonucleotide probe for AP-1 (Promega). In the case of supershift analysis, rabbit anti-p50 and anti-p65 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with the nuclear extracts for 15 min prior to the addition of radiolabeled probe.

6.6. Immunolocalization of NF-κB and iNOS

Cells (3000/well) were plated onto glass cover slips, and incubated with 4×10^{8} CCU of *U. urealyticum* per ml in the absence or presence of dexamethasone (10^{-4} M) or budesonide (10^{-4} M) for 24 hours for examination of iNOS or for 30 min for investigation of NF-κB. After treatment, the cells were fixed with cold methanol and acetone. Intracellular p65 and iNOS were visualized by indirect immunofluorescence using polyclonal rabbit-anti-p65 antibody (Santa Cruz Biotechnology) and polyclonal rabbit-anti-macrophage iNOS antibody (Affiniti BioReagents, Golden CO, USA) followed by FITC (fluorescein isothiocyanate isomer 1)-labeled goat-anti-rabbit-IgG (Daco, Denmark)**.**

6.7. RT-PCR

Following the various treatments, total RNA was extracted from 4 x 10^{6} cells employing 1 ml ice-cold RNAzol^{TM}B (Biotecx Laboratories; Houston, TX, USA) according to the manufacturer's instructions. The final RNA pellet was air-dried and thereafter resuspended in 20 µl diethyl-pyrocarbonate-treated water. 10 µl of this solution was used for spectrophotometric determination of the RNA concentration at 260 nm and quality by assessing the 230-320 nm profile. The remaining 10 µl was stored at -70°C.

Synthesis of first strand cDNA from this total RNA was performed using SuperScript RNase H^{-} Reverse Transcriptase (GibcoBRL) and random hexamer primers (pd(N)$_6$, Amersham Pharmacia Biotech, Uppsala, Sweden). In brief, 1 µg RNA was heated together with 100 pmol pd(N)$_6$ at 70°C for 10 minutes, after which 0.1 mmol DTT, 10

μmol dNTP, 60 U RNasin (Boehringer Mannheim, Mannheim, Germany) and 300 U superscript were added and incubation continued at 45°C for 2 hours.

The specific oligonucleotide primers for human IL-6, human TNF-α (Scandinavian Gene Synthesis, Köping, Sweden) and for the ”housekeeping” gene human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (CyberGene AB, Huddinge, Sweden) were synthesized. The corresponding primers for human VEGF and ICAM-1 and for rat IL-6, TNF-α and G3PDH were purchased from Innovagen (Lund, Sweden), while those for rat iNOS were obtained from Clontech (Palo Alto, CA, USA) (see Table 1).

Table 1. Primers used in the RT-PCR reactions

Name	sequence (5’- 3’)	PCR product length (bp)	Reference
5’-hu IL-6	TGAACTCCTTCTCCACAAGC	315	(Pisa, Pisa et al. 1992)
3’-hu IL-6	ATCCAGATTGGAAGCATCCA		
5’-hu TNF-α	TGAGCACTGAAAGCATGATC	363	(Pisa, Pisa et al. 1992)
3’-hu TNF-α	TTATCTCTCAGCTCCACGCC		
5’-hu G3PDH	TGAAGGTCGGAGTCAACGGATTTGGT	983	(Agular-Santelises, Rottenberg
3’-hu G3PDH	CATGTGGGCCATGAGGTCCACCAC		et al. 1996)
5’-rat IL-6	GAAATACAAAGAAATGATGG	489	(Hagberg, Gilland et al. 1996)
3’-rat IL-6	GTGTTTCAACATTCATATTGC		
5’-rat TNF-α	CTCAAGATTGTCAGCAATGC	409	(Estler, Grewe et al. 1992)
3’-rat TNF-α	CAGGATGCCCTTTAGTGGGC		
5’-rat G3PDH	CTCAAGATTGTCAGCAATGC	404	(Williams and Coleman 1995)
3’-rat G3PDH	CAGGATGCCCTTTAGTGGGC		
5’-hu VEGF	CGGAATTCTCACCGCCTCGGCTTGTCACA		(Hoper, Voelkel et al. 1997)
3’-hu VEGF	TGGGATCCATGAACTTTCTGCTG		
5’-hu ICAM-1	CATTCAGCGTCACCTTGG	238	(Takizawa, Kamijo et al. 1999)
3’-hu ICAM-1	TATGGCAACGACTCCTTCT		
3’-rat iNOS	GGGCTCCTCCAAGGTGTTGCCC	473	(Sirsjo, Soderkvist et al. 1994)
5’-rat iNOS	CCCTTCCGAAGTTTCTGGCAGCAG		

The PCR using Taq polymerase (final concentration 0.025 U/µl; GibcoBRL) was performed in a final volume of 25 µl containing 2 µl of the cDNA for TNF-α and G3PDH and 4µl for IL-6 cDNA in a DNA Thermocycler 480 (Perkin Elmer, Norwalk, CT, USA). The other reaction components included $MgCl_2$ (2 mM), dNTP (0.2 mM), PCR buffer and 3'- and 5'-primers (0.5 µM each). PCR was conducted for 40 cycles in the case of human IL-6, 35 cycles for human TNF-α and 27 cycles for human G3PDH utlizing the following conditions: 1 min denaturation at 94ºC, 1 min annealing at 54ºC, in the case of human IL-6, 56ºC for human TNF-α and at 60ºC for human G3PDH, and 1 min extension at 72ºC, followed by a final extension for 5 min at 72ºC and cooling to 4ºC. PCR was conducted for 40 cycles in the case of rat IL-6 and rat TNF-α, with 1 min at 94ºC; 30 s at 54ºC for rat IL-6 and at 58ºC for rat TNF-α, and 3 min at 72ºC; 35 cycles were employed for rat G3PDH, with 1 min at 94ºC, 1 min at 60ºC and 1 min at 72ºC, followed by a final extension for 5 min at 72ºC and cooling to 4ºC. PCR was conducted for 33 cycles for rat iNOS under the following conditions: 1 min denaturation at 94ºC, 1 min annealing at 60ºC and 2 min extension at 72ºC. In the case of human VEGF, PCR was conducted 33 cycles under the following conditions: 1 min denaturation at 95ºC, 1 min annealing at 60ºC and 1 min extension at 72ºC. For human ICAM-1, 35 cycles with 1 min denaturation at 95ºC, 1 min annealing at 60ºC and 2 min extension at 72ºC were employed.

The PCR products were subsequently separated by electrophoresis on a 1.5 % agarose gel (GibcoBRL). Following staining with ethidium bromide, the gels were photographed under UV-light using the DC120 Digital Zoom Camera (Eastman Kodak Company, Rochester, NY, USA) and the net intensities of the PCR products were analyzed with the Kodak Digital Science™ Electrophoresis Documentation and Analysis System 120 (Eastman Kodak).

6.8. Flow cytometric analysis

After treatment, cells were detouched from the plates by trypsinization, washed with PBS and then incubated with FITC-labeled mouse anti-human CD54 antibody (Serotec Ltd, Oxford, UK) for 15 min at 4°C. Following an additional wash with PBS, the fluorescence associated with 10,000 cells in each sample was analyzed employing a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and the CellQuest software, the data being expressed as the mean fluorescence intensity

(MFI) in the FL1 channel. These MFI values were corrected for unspecific staining by substracting the fluorescence of cells stained with unspecific antibody (mouse IgG1).

7. Statistical analysis of the data

In Papers I-IV, the pooled data from ELISA, bioassay and Griess reaction are reported as means and 95% confidence intervals or as means ± SEMs. Data were analyzed for statistical significance utilizing student's t-test or one-way ANOVA test. Non-normality paired data were analyzed by Wilcoxon matched pairs test. In Paper V, non-parametric statistical analysis was used. For the analysis of cytokine levels in TAF, only one measurement / day / infant was used. Kruskal-Wallis analysis was used to test for intra- and intergroup differences in cytokine levels. For comparison of the clinical variables between groups, the Mann-Whitney U-test was employed. Spearman rank correlation was used to test for the effect of gestational age on cytokine levels. Differences in frequencies were examined with the chi-square test. P values less than 0.05 were considered to be significant.

Summary of the results

1. U. urealyticum-stimulated cytokines production in macrophages (Paper I)

It has been convincingly demonstrated that secretions in the airways of infants with RDS contain high levels of proinflammatory cytokines, and, moreover, those infants who develop CLD exhibit an enhanced proinflammatory cytokine response. In order to investigate wheather *U. urealyticum* can provoke an inflammatory response *in vitro*, we used *U. urealyticum* antigen to stimulate macrophages and we found that the production of TNF-α and IL-6 increased after stimulation with *U. urealyticum* both in the human (Figure 5) and rat macrophage cell lines (Figure 6) in a dose dependent

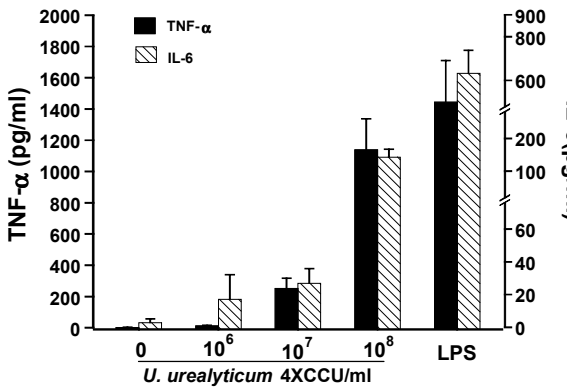


Figure 5. TNF-α and IL-6 production in the THP-1 cell line (PMA differentiated monocytes) stimulated by different doses of *U. urealyticum* antigen or LPS for 24 hrs.

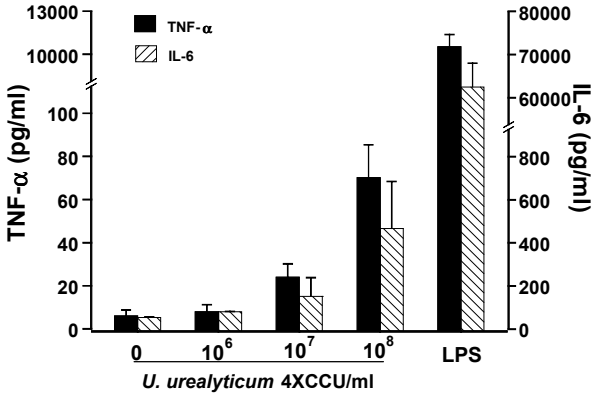


Figure 6. TNF-α and IL-6 production in the rat alveolar macrophage cell line stimulated by different doses of *U. urealyticum* antigen or LPS for 24 hrs.

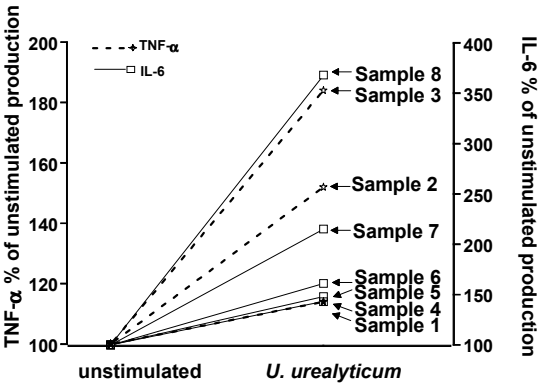


Figure 7. TNF-α and IL-6 production in the TAF macrophages from preterm infants stimulated by *U. urealyticum* antigen for 24 hrs.

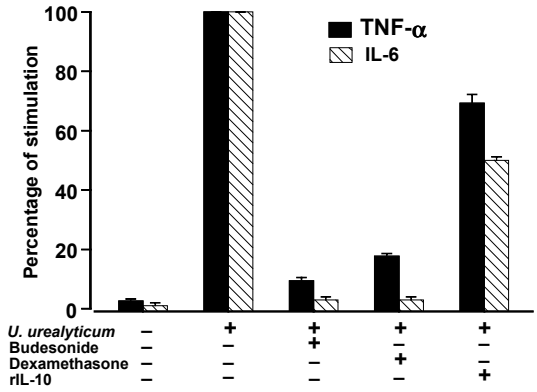


Figure 8. Inhibition of *U. urealyticum* antigen ($4x10^8$ CCU/ml) stimulated prodiction of IL-6 and TNF-α by THP-1 cell line upon addition of budesonide (10^{-4} M), dexamethasone (10^{-4} M) and human rIL-10 (25 ng/ml).

manner. To further examine this cytokine induction under conditions more closely related to the clinical situation, primary cultured TAF macrophages were stimulated with *U. urealyticum* antigen and *U. urealyticum* increased their production of TNF-α by 14% - 84% and of IL-6 by 46% - 268% (Figure 7). Furthermore, the *U. urealyticum* induced production of TNF-α and IL-6 was regulated at the trancription level.

2. Down-regulation of cytokine levels by steroids and rIL-10 (Paper II)

A number of studies have demonstrated that glucocorticoid therapy can be effective in treating CLD. In order to understand the basis for the clinically beneficial effects of steroids as well as to search for new therapeutic drugs, we have tried to use dexamethasone, budesonide and rIL-10 in down-regulating LPS and *U. urealyticum* induced cytokine production in macrophages. We found that dexamethasone, budesonide and rIL-10 significantly inhibited both the IL-6 and TNF-α production in the THP-1 cell line stimulated by LPS or *U. urealyticum* antigen (Figure 8). Similar effects were observed in the case of primary cultures of TAF macrophages collected from newborn infants. With the rat alveolar macrophage cell line, steroids inhibited the IL-6 and TNF-α production, while rIL-10 was without effect. In both cell lines stimulation of TNF-α and IL-6 gene expression by LPS and *U. urealyticum* was down-regulated by the steroids.

3. U. urealyticum-stimulated expression of iNOS and activation of NF-κB (Paper III)

NO is an important inflammatory mediator in several types of infections in humans. Furthermore, production of this molecule may be increased in association with certain diseases, such as pneumonia and sepsis. NO may thus play an important role in the pathophysiology of respiratory disease, but the possible involvement in the pathogenesis of CLD is currently unknown. In the present study *U. urealyticum* antigen was shown to stimulate production of NO by alveolar macrophages *in vitro* in a dose-(Figure 9) and time-dependent manner. This effect was further enhanced by IFN-γ, but was attenuated by budesonide and dexamethasone (Figure 9). This increase in NO production was regulated at the trancriptional level, as indicated by the

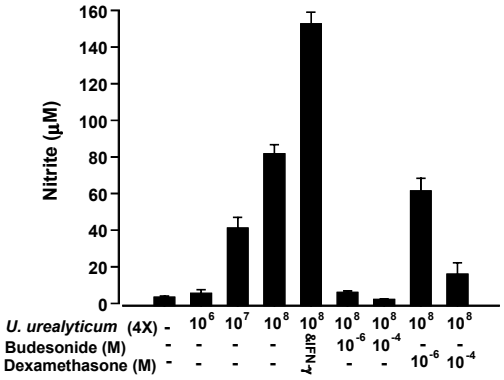


Figure 9. *U. urealyticum* antigen induced NO production and down-regulation by steroids in alveolar macrophages. Macrophages were stimulated with *U. urealyticum* (4×10^6-4×10^8 CCU/ml) and LPS (100 ng/ml) or in combination with IFN-γ (100 IU/ml) or different doses of budesonide and dexamethasone.

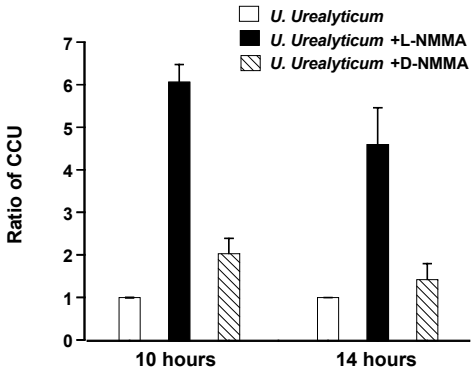


Figure 10. Inhibition of *U. urealyticum* growth by macrophage producing NO. *U. urealyticum* was cultured for 4 days from the supernatant after 10 hrs and 14 hrs incubation with macrophages alone, or in combination with 3 mM L-NMMA and D-NMMA.

induction of iNOS mRNA and protein levels in response to *U. urealyticum* which were also inhibited by steroids.

Many of the proinflammatory cytokines, including TNF-α, IL-1β and IL-6, can up-regulate the expression of iNOS. Therefore the inhibitor of protein synthesis CHX was employed to block de novo synthesis of cytokines and *U. urealyticum* caused a direct effect on iNOS expression which was not dependent on cytokine production.

We further investigated the regulation of *U. urealyticum* stimulated iNOS expression. *U. urealyticum* antigen triggered NF-κB activation, a possible mechanism responsible for the iNOS expression, which also was inhibited by steroids. We also found that the NO induced by *U. urealyticum* caused as much as a 6-fold reduction in the growth of this organism itself after 10 hours of infection (Figure 10).

4. Stimulation of ICAM-1 expression by U. urealyticum (Paper IV)

ICAM-1, an inducible cell adhesion glycoprotein, is important for transendothelial migration of leukocytes to sites of inflammation and functions as a costimulatory molecule for T cell activation. ICAM-1 is expressed constitutively on the surface of the cell but can also be up-regulated in response to a variety of inflammatory mediators, including proinflammatory cytokines, hormones, cellular stress and infection. In order to investigate wheather ICAM-1 is one of the inflammatory

mediators that are produced in response to *U. urealyticum* infection, we stimulated the macrophages with *U. urealyticum*. We found a production of sICAM-1 protein that was dose dependent (Figure 11) and cell surface ICAM-1 expression that was increased compared to unstimulated cells. This expression was regulated at the transcriptional level. As shown previously, *U. urealyticum* can induce the production of TNF-α by macrophage. In order to determine whether the expression of ICAM-1 was a direct response to *U. urealyticum* infection or to the production of TNF-α, we used an anti-TNF-α antibody to block the production of TNF-α, which gave an 85.5% reduction of ICAM-1 expression.

Inhibition of such elevated production of ICAM-1 by blocking its mRNA expression or activity might turn out to be a useful strategy for treatment of inflammatory disorders such as CLD. In this context we demonstrated here that both budesonide and dexamethasone could down-regulate macrophage ICAM-1 mRNA expressions and their protein production (Figure 11) stimulated by *U. urealyticum.*

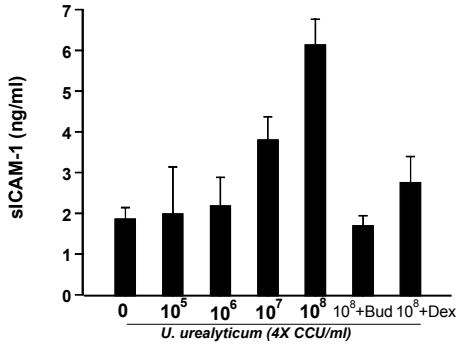


Figure 11. sICAM-1 production in the THP-1 cell line (PMA differentiated monocytes) stimulated by 4×10^5 - 4×10^8 CCU/ml of *U. urealyticum* antigen and 4×10^8 CCU/ml of *U. urealyticum* in combination with 10^{-4} M of budesonide or dexamethasone for 24 hrs.

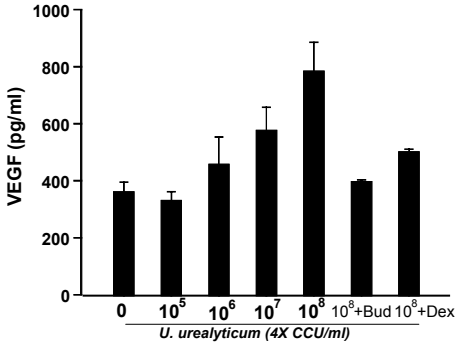


Figure 12. VEGF production in the THP-1 cell line (PMA differentiated monocytes) stimulated by 4×10^5 - 4×10^8 CCU/ml of *U. urealyticum* antigen and 4×10^8 CCU/ml of *U. urealyticum* in combination with 10^{-4} M of budesonide or dexamethasone for 24 hrs.

5. Stimulation of VEGF expression by U. urealyticum (Paper IV)

The vascular endothelial growth factor (VEGF) is a major regulator of both physiological and pathological neovascularization. It participates in angiogenesis during embryogenesis, wound healing, ovulation and is an important growth factor in

lung development. The present study revealed that *U. urealyticum* stimulates the production of VEGF by macrophages in a dose dependent manner at 24 hrs (Figure 12). Semi-quantitative RT-PCR demonstrated the presence of enhanced levels of VEGF mRNA in such stimulated macrophages.

Both budesonide and dexamethasone were found to down-regulate the levels of macrophage VEGF mRNA expressions and their protein production (Figure 12) stimulated by *U. urealyticum.*

6. Levels of down-regulatory cytokines in TAF from infants with CLD (Paper V)

TGF-β1, IL-10, IL-4 and IL-12 are potentially important in down-regulating the pro-inflammatory response and/or induction of lung fibrosis in infants with developing or established CLD. Consequently, we investigated levels of these cytokines in TAF samples. TGF-β1 in infants who developed CLD was significantly elevated during the first week of life, remained elevated at 2 weeks and even beyond 4 weeks of age. Treatment of 6 infants with CLD with steroid did not significantly reduce these elevated levels of TGF-β1. IL-10 was detected in 18 of 100 (18%) TAF samples collected from preterm infants, with infants with CLD or RDS being more likely to have measurable TAF levels of this cytokine (see Table 2), compared with the postoperative infants without lung disease. TAF levels of IL-4 or IL-12 were below the detection limits in all samples.

Table 2**.** Presence of IL-10 levels in TAF from infants

Study group	Total Sample(N)	Positive sample (N)	% of positive
CLD infants (N=24)	44	12	27
No CLD (N=22)	56	6	11
Term infants (N=23)	29	0	0

General discussion

1. Role of U. urealyticum - induced inflammatory responses in the development of CLD

CLD is the chronic phase of neonatal lung injury, characterized by delayed alveolarization, chronic inflammation and fibrosis that occur in susceptible VLBW preterm infants. Although barotrauma and oxidant injury are important factors in the pathogenesis of CLD, accumulating epidemiological and biological information indicates that intrauterine and postnatal infections also contribute to the initiation and exacerbation of lung inflammation and injury. Thus, it is now an important task to determine the roles of specific infectious organisms and the mechanisms of injury involved in the development of CLD.

U. urealyticum is one of the most common infectious organisms which can be isolated from infected amniotic fluid and placentae as well as from the respiratory tract of preterm infants (Sanchez 1993; Waites, Crouse et al. 1993). A number of studies have indicated that *U. urealyticum* is involved in the development of CLD, but the exact mechanism(s) is not yet understood. The overall aim of present thesis was to help elucidate this mechanism(s).

Our present investigation demonstrated that the *U. urealyticum* antigen stimulates production of proinflammatory cytokines (IL-6 and TNF-α) and expression of iNOS, growth factor (VEGF) and cellular adhesion molecule (ICAM-1) by macrophages, showed a down-regulatory effect of steroids (budesonide and dexamethasone) as well as rIL-10 on the inflammatory responses and presents the biological effect of iNOS activation on the *U. urealyticum* growth *in vitro*. Activation of NF-κB was found to be a possible molecular mechanism for this up-regulation of these inflammatory mediators by *U. urealyticum*. Clinically, enhanced levels of down-regulatory cytokines TGF-β1 and IL-10 in the development of CLD have been demonstrated. Our findings support the clinical notion that *U. urealyticum* plays an important role in the development of CLD.

2. Role of U. urealyticum-induced proinflammatory cytokines in the development of CLD

The initial pathological changes of CLD include an increase in the number of macrophages and polymorphonuclear lymphocytes in the lung and elevated levels of proinflammatory cytokines in TAF (Jones, Cayabyab et al. 1996). Lung lavage fluid from premature infants who develop CLD contains elevated levels of proinflammatory cytokines and chemokines (Tullus, Noack et al. 1996; Jonsson, Tullus et al. 1997; Ozdemir, Brown et al. 1997). In the present study we observed a dose-dependent increase in levels of expression of IL-6 and TNF-α protein and mRNA *in vitro* in macrophages stimulated with *U. urealyticum* antigen. This phenomenon was not only observed in human and rat macrophage cell lines but also in primary cultures of TAF macrophages from preterm infants, even though these latter cells had already been activated *in vivo*.

The exact mechanistic relationship between the increased TNF-α and IL-6 levels and the development of CLD is not presently known. These cytokines can potentiate the inflammatory response by exiting direct cell toxicity and by recruiting additional inflammatory cells. These mediators can activate vascular endothelium and increase its permeability, as well as activating lymphocytes and increasing antibody production. TNF-α can also induce the expression of other inflammatory mediators, e.g., NO and ICAM-1, and mediate apoptosis (Figure 3). Furthermore, recent study has revealed that infection by *U. urealyticum* enhances the proinflammatory response to a second infection (LPS), thus predisposing the preterm infant to prolonged and dysregulated inflammation, lung injury and impaired clearance of secondary infections (Manimtim, Hasday et al. 2001). Our present results, in combination with previous findings, do demonstrate that the imbalance in the complex network of inflammatory mediators may lead to irreversible destruction of lung tissue and fibrosis.

3. Mechanism of induction of NO and iNOS by U. urealyticum in connection with CLD

NO has now been implicated as participating in many different physiological processes. This molecule plays dual roles in infection e.g., as a key component of host defences, but also as a central mediator of pathogenesis. iNOS is absent in resting cells, but its gene is rapidly expressed in response to various stimuli. Once expressed, iNOS synthesises NO 100–1000-fold more rapidly than do the constitutive enzymes and continues to do so for prolonged periods of time. The resulting high concentration of NO may inhibit a large variety of microbes, but can also potentially damage the host, thereby contributing to the pathology (Burgner, Rockett et al. 1999).

It has been reported that NO may be involved in the pathogenesis of CLD. Not only is the nitrate concentration in TAF from infants who progressed to CLD higher than in RDS and control infants (Vyas, Currie et al. 1999), but VLBW infants with CLD also exhibit elevated TAF levels of citrulline, a metabolite of NO (Heckmann, Kreuder et al. 2000) and of plasma 3-nitrotyrosine, an oxidant derived from NO (Banks, Ischiropoulos et al. 1998). However, the possible role of iNOS in the development of CLD had not yet been investigated. In the present study we found that *U. urealyticum* antigen directly stimulates iNOS expression by alveolar macrophages which is independent of cytokine induction, followed by a dose and time dependent production of NO. Although the physiological production of NO plays a role in many host reactions to the changed environment, its overproduction may be responsible in part for the pathophysiology of infection. Overproduction of NO is likely to contribute to the hemodynamic instability in overwhelming sepsis in humans (Evans, Carpenter et al. 1993). Animal studies suggest that the high-output NO pathway is responsible for escalating the inflammatory response (Hierholzer, Harbrecht et al. 1998). When the production of NO is left unattenuated, especially under conditions of oxidative stress, direct cytotoxic effects of NO can emerge through the formation of peroxynitrite ($OONO^-$) (Figure 4), as a result of a reaction between NO and superoxide (Beckman, Beckman et al. 1990), which is an important mediator of tissue injury and organ dysfunction (Grisham, Jourd'Heuil et al. 1999). The oxidative stress, DNA damage

and disruption caused by excess NO can lead to cell death by apoptosis or necrosis (Murphy 1999). When the damaged lung starts remodeling, pulmonary fibrosis may occur (Hagimoto, Kuwano et al. 1997; Chapman 1999). Pulmonary fibrosis, as a final pathological change of CLD, begins in the alveolus and develops in definable stages over time: a) epithelial cell injury and alveolar inflammation; b) organization of the resultant alveolar exudate and c) incorporation of the alveolar fibroproliferative process into alveolar walls. Repeating cycles of this series of events gives rise to the characteristic signature of a distorted and dysfunctional lung.

We have also found evidence that NO contributes to host defenses. Presence of the iNOS inhibitor L-NMMA enhanced the growth rate of *U. urealyticum*. This anti-microbial effect does not appear to be exerted by NO itself, but rather by reactive nitrogen intermediates formed by the oxidation of NO (Burgner, Rockett et al. 1999). For example, as mentioned above, reaction between NO and the free radical superoxide results in the formation of the unstable molecule $OONO^-$, while reaction of NO with thiol groups produces nitrosothiols. These reactive nitrogen intermediates inactivate key microbial enzymes, such as ribonucleotide reductase and aconitase, by reacting with iron-containing groups in these enzymes, damaging microbial DNA, protein and lipids (Fang 1997; Wheeler, Smith et al. 1997).

4. Contribution of U. urealyticum-stimulated ICAM-1 to CLD

An early increase in the number of neutrophils is one of the pathophysiological characteristics of CLD lung. These neutrophils transmigrate from peripheral blood. The process of neutrophil adhesion to the vascular endothelium and subsequent transendothelial migration is complex. Two mechanisms that play key roles in the recruitment of neutrophils to sites of inflammation are the induction of cellular adhesion molecules and the release of chemokines. Our present investigation revealed that not only the cell surface ICAM-1, but also the soluble form of this protein is induced in response to *U. urealyticum* infection, probably as a secondary effect of the increased production of proinflammatory cytokines associated with such infection.

ICAM-1 has been reported early to be associated with the development of CLD (Little, Dean et al. 1995; Ramsay, O'Brian Smith et al. 1998). During infection it is

important for neutrophils and monocytes to be able to migrate from the circulation to the site of inflammation (Sorkness, Mehta et al. 2000). ICAM-1 can also serve as a costimulatory molecule for T-cell activation and for cytotoxic T-cell function (Paine and Ward 1999). Furthermore, ICAM-1 may also influence the functioning of neutrophils. Adherence to pulmonary endothelial ICAM-1 is associated with a respiratory burst in neutrophils during endotoxin infusion (Minamiya, Motoyama et al. 1998). Neutrophils are capable of injuring epithelial cells, most effectively during firm adherence (Simon, DeHart et al. 1986). The infected pulmonary epithelial cells exhibit increased neutrophil adherence that is due in part to ICAM-1 interaction (Tosi, Stark et al. 1992). Thus, inflammatory cells, which migrate to the lungs, may adhere to and injure lung parenchymal cells. After such initial injury, interactions of extracellular matrix molecules with both epithelial cells and fibroblasts may lead to the disordered healing process, resulting in fibrosis (Paine and Ward 1999).

5. Contribution of U. urealyticum-mediated VEGF to CLD

We have also studied the *U. urealyticum* infection and the expression of VEGF in macrophages, showing that *U. urealyticum* can stimulate macrophages to express higher levels of VEGF. It is well known that VEGF is one of the most potent regulators of endothelial permeability, also called vascular permeability factor. Only minutes after administration of VEGF, small capillaries and venules display endothelial fenestration and opening of tight junctions (Roberts and Palade 1995). VEGF is also an endothelial mitogen which promotes embryonal vascular development and neovascularization (Veikkola and Alitalo 1999). Secretion of VEGF by human neutrophils increases when these cells are infected by *Streptococcus pneumoniae*, suggesting this factor in mediating vascular permeability and formation of edema in pneumococcal disease (van Der Flier, Coenjaerts et al. 2000). CLD has some distinct characters, including early endothelial cell damage, delayed alveolarization and lung fibrosis. Integral to recovery from experimental lung injury is repair of microvascular endothelium, which correlates with increased expression of VEGF in alveolar epithelial cells (Maniscalco, Watkins et al. 1995). In the lung, endogenous VEGF appears to play an important role in modulating the extent of

blood vessel remodeling. It has been shown that VEGF can enhance the activation and migration of monocytes through the *flt* receptor *in vitro* (Tolnay, Kuhnen et al. 1998), which is presumably an initial pathophysiological step in the development of CLD, and also mediate angiogenic activity during the proliferative phase of wound healing (Nissen, Polverini et al. 1998), which is similar to the later phase of CLD.

6. Activation of NF-κB by U. urealyticum in the regulation of inflammation

We have demonstrated that *U. urealyticum* can induce the production of proinflammatory cytokines, stimulate iNOS expression and subsequently synthesis of NO and enhance expression of VEGF and ICAM-1 by macrophages. In attempt to elucidate the underlying molecular mechanism(s), we examined the activation of NF-κB. Our data demonstrate that *U. urealyticum* is a potent activator of NF-κB, as evidenced by its rapid and intense NF-κB activation in macrophages. NF-κB is a member of the Rel family of transcription factors and composed of two groups of structurally related, interacting proteins that bind DNA recognition sites as homo- or hetero-dimers and whose activities are regulated by their subcellular location (Barnes 1997). NF-κB /Rel family members of the first group include NF-κB1 (p50) and NF-κB2 (p52), which are synthesized as precursor proteins with molecular weights of 105 (p105) and 100 (p100) kDa, respectively. The second group includes Rel A (p65), Rel B and c-Rel, which are synthesized as mature proteins containing one or more potent activation domains. In resting cells, latent NF-κB is complexed to a class of cytoplasmic retention proteins called inhibitors of NF-κB (IκB). Signals that induce NF-κB activity result in the phosphorylation of IκB on specific serine residues, marking the protein inhibitor for ubiquitination and subsequent proteolytic degradation by the ATP-dependent 26S proteasome complex (Chen, Castranova et al. 1999). The NF-κB released is then free to translocate to the nucleus, where it binds to its recognition site and activates gene transcription (Figure 13). NF-κB is a critical transcription factor involved in the production of cytokines with important roles in the immune and inflammatory responses. NF-κB binds to the κB binding motifs in the

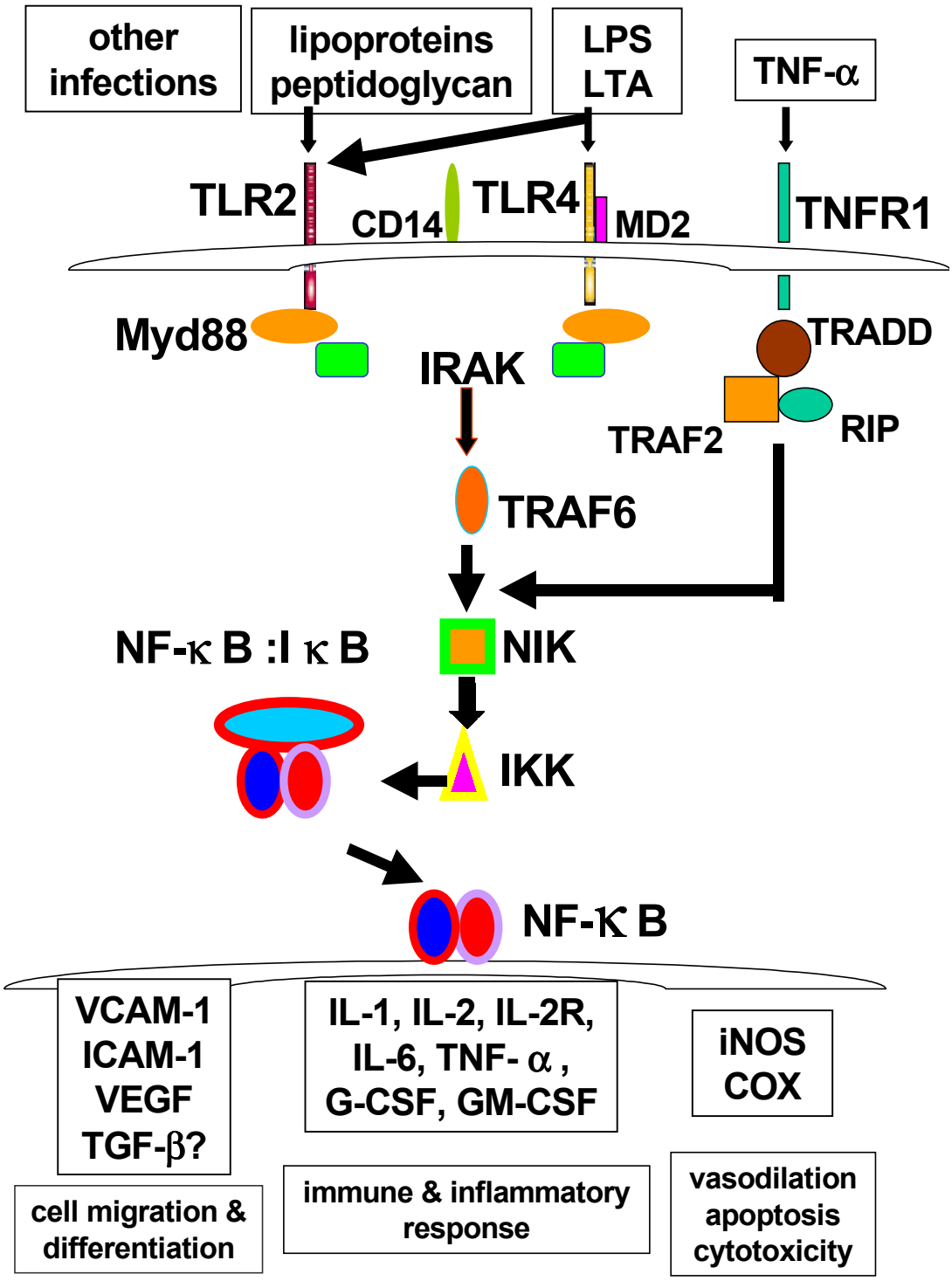


Figure 13. The immune response pathway involving TLRs and NF-κB. LPS, lipopolysaccharide; LTA, lipoteichoic acid; TLR, Toll-like receptors; IRAK, interleukin-1 receptor accessory protein kinase; TRAF, tumour necrosis factor receptor-associated factor; NIK, NF-κB-inducing kinase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; TRADD, TNFR1-associated death domain protein; RIP, the receptor-interacting protein; IKK, IκB kinases.

promoter or enhancers of the genes encoding, e.g., TNF-α and IL-6 (Lee and Burckart 1998). Furthmore, TNF-α is one of the most important activators of NF-κB, contributing to a positive feedback loop for NF-κB activation. It binds to the type-1 TNF receptor (TNFR1), which results in an association with the TNFR1-associated death domain protein (TRADD), the receptor-interacting protein (RIP) and the TNF receptor-associated factor-2 (TRAF-2). Subsequently these cytoplasmic proteins form an active signalling complex that interacts with NF-κB-inducing kinase (NIK). Activation of NIK results in phosphorylation of IκB kinases (IKK), which in turn phosphorylates IκB (Christman, Lancaster et al. 1998). When NF-κB is translocated into the nucleus, the genes coding for proteins which mediate inflammatory responses are activated (Figure 13). In this manner, TNF-α positively regulates NF-κB activation for continuous up-regulation of the cytokine network (Lee and Burckart 1998).

The role of NF-κB in the expression of iNOS gene has been extensively elucidated (Christman, Lancaster et al. 1998). Stimulation of macrophages with LPS or cytokines such as IL-1β leads to activation of NF-κB and its subsequent binding to the κB response element of the iNOS promoter (Schroeder, Cai et al. 1999). Activation of NF-κB is an essential mechanism responsible for LPS or oxidative stress-induced NO production (Xie, Kashiwabara et al. 1994). This suggests that NF-κB activation may also be of great importance for the *U. urealyticum* induced iNOS expression.

However, the potential role of NF-κB in inflammation and immune modulation in *U. urealyticum* infection is not limited to the induction of proinflammatory cytokines and transcriptional activation of iNOS. In fact, NF-κB has been shown to exert a crucial role in the inducible expression of numerous other genes encoding for mediators of inflammatory responses, including transcription factors, adhesion molecules and growth factors (Chen, Castranova et al. 1999).

Regulation of the ICAM-1 gene has been well investigated. The architecture of the ICAM-1 promoter is complex, containing a large number of binding sites for inducible transcription factors, the most important of which is NF-κB. NF-κB acts in concert with other transcription factors and co-activators via specific protein-protein

interactions, which facilitate the assembly of distinct stereo-specific transcription complexes on the ICAM-1 promoter. These complexes presumably mediate the induction of ICAM-1 expression in different cell types and in response to different stimuli (Roebuck and Finnegan 1999). Proteosome inhibitors known to block NF-κB activity have also been shown to inhibit the induction of ICAM-1 expression by TNF-α in endothelial cells (Kalogeris, Laroux et al. 1999). This observation indicates that the activation of NF-κB might have a key role for the induction of ICAM-1 expression by *U. urealyticum*.

It is known that colonic epithelial cells induce endothelial cell expression of ICAM-1 and VCAM-1 by a NF-κB-dependent mechanism (Maaser, Schoeppner et al. 2001). In addition, transactivation of NF-κB-dependent genes by oligodeoxynucleotides causes significant inhibition of UV-induced secretion of VEGF (Abeyama, Eng et al. 2000). Mutated IκBα transfection, which blocks NF-κB activation, suppresses the synthesis of VEGF both under *in vitro* and *in vivo* conditions (Huang, Robinson et al. 2000). The significant decrease in VEGF promoter activity found in the mutated IκBα-transfected cells suggested that the regulation of VEGF by NF-κB probably occurred at the transcriptional level.

In summary, *U. urealyticum*-induced activation of NF-κB in macrophages may represent a key event responsible for the inflammatory reaction associated with CLD.

7.The pathway underlying the down-regulatory effects of steroids and rIL-10

The down-regulatory effects on inflammatory mediators by steroids or rIL-10 have been shown in our studies. Dexamethasone and budesonide or rIL-10 could attenuate the increases in proinflammatory cytokine, iNOS, ICAM-1 and VEGF mRNA and protein induced by *U. urealyticum*. This observation might explain why treatment with these agents has beneficial effects in CLD infants or infants at risk for developing CLD. It has been reported that infants who have developed CLD exhibit lower basal and stimulated cortisol values than those who recover. This finding indicates that some infants have a decreased ability to synthesize cortisol, leading to physiological disruptions including amplified inflammatory responses, thereby

resulting in CLD (Watterberg, Gerdes et al. 2001). Corticosteroids are commonly used as highly effective pharmacological agents to control inflammation. Because corticosteroids are lipid-soluble molecules, they diffuse easily across cell membranes and subsequently bind to a specific GR in the cell cytoplasm (Spahn and Kamada 1995). This complex is then transported into the nucleus, where it binds to a specific GRE within the promoter region of the target genes, resulting in down-regulation of gene transcription. In addition, the GR can also inhibit gene expression in a DNA-independent manner, via a transrepression mechanism involving protein–protein interactions between the GR and the intra-cytoplasmic transcription factors, active protein-1 (AP-1) and NF-κB (Barnes 1997). These transcription factors are known to be involved in the positive regulation of a number of genes whose products play central roles in inflammation. This transrepression mechanism involving the transcription factors appears to be due to the formation of inactive GR/AP-1 and GR/NF-κB complexes. Most recently, it has been demonstrated that corticosteroids can block the NF-κB pathway by inducing protein synthesis of its cytoplasmic inhibitor, IκBα (Auphan, DiDonato et al. 1995; Lee and Burckart 1998). We have also shown here that budesonide and dexamethasone inhibit the activation of NF-κB induced by *U. urealyticum,* which is possibly one of the key mechanisms underlying the ability of these steroids to decrease inflammatory response to infection by this organism.

8. Role of down-regulatory cytokines in CLD

In connection with tissue repair, TGF-β1 is released, first locally by platelets, then by activated macrophages and later on by other cells such as epithelial cells and fibroblasts. TGF-β1 initially down-regulates the inflammatory response, while excessive secretion of this factor at a later stage may give rise to an exaggerated healing response, leading to fibrosis (Border and Noble 1994). TGF-β1 has been reported to play a role in progressive kidney fibrosis (Yamamoto, Noble et al. 1994), as well as in bleomycin-induced pulmonary fibrosis in rats (Westergren-Thorsson, Hernnas et al. 1993). Our present findings reveal that TGF-β1 are significantly enhanced in infants with CLD. Furthermore, our observations suggest that the same

situation may apply to the preterm human lung. The positive correlation between early high levels of TGF-β1 and degree of immaturity might explain why the fibrotic healing response is more dominant in the more immature infants. The fact that these elevated levels of TGF-β1 were not reduced by steroid treatment is in agreement with what was observed with respect to alveolar macrophages from rats with bleomycin-induced pulmonary fibrosis, where high doses of corticosteroids effectively decrease IL-1β levels, but fail to suppress TGF-β1 secretion (Khalil, Whitman et al. 1993). This might provide part of the explaination as to why steroids exert only moderately beneficial effects in CLD patients.

IL-10 inhibits cytokine synthesis and can exert either immunosuppressive or immunostimulatory effects on a variety of cell types. This cytokine is a potent modulator of monocyte/macrophage function (de Waal Malefyt, Abrams et al. 1991; Fiorentino, Zlotnik et al. 1991; Niiro, Otsuka et al. 1994). Interleukin-10 inhibits macrophage production of reactive oxygen intermediates and nitric oxide, while down-regulating the proinflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8 (Bogdan, Paik et al. 1992; Gerard, Bruyns et al. 1993; Moore, O'Garra et al. 1993; Thomassen, Divis et al. 1996). In case of endotoxemia, the elevated levels of proinflammatory cytokines can be reduced by hypersecretion of IL-10 and administration of this cytokine to mice provides protection from lethality in shock (Howard, Muchamuel et al. 1993; Marchant, Deviere et al. 1994). Thus, IL-10 has powerful antiinflammatory properties.

We found measurable levels of IL-10 in TAF from both preterm infants with RDS who resolved and infants who later developed CLD, but not from postoperative infants without lung disease. This finding is in disagreement with those of Jones et al (Jones, Cayabyab et al. 1996), but consistent with those of McColm and co-workers (McColm, Stenson et al. 2000). The exact explanation as to why IL-10 is expressed in CLD infants but not in term control infants remains unclear. However, this difference reflects the different stimuli experienced by these two groups of infants. At present, there is little evidence that IL-10 is an important anti-inflammatory cytokine in the pathogenesis of chronic lung disease. IL-10-knockout mice do not develop lung disease spontaneously, although these animals do manifest chronic enterocolitis,

probably as a consequence of inability to resolve inflammation in the gut (Kuhn, Lohler et al. 1993). Therefore, the possible role of IL-10 in the development of CLD requires further investigation.

9. Interaction of inflammatory factors in CLD

As discussed above, *U. urealyticum* can stimulate the production of proinflammatory cytokines and expression of iNOS followed by NO production, increase formation of VEGF and ICAM-1 by macrophages. These factors interact with each other (see the figure on the cover). TNF-α can activate expression of iNOS, promote production of ICAM-1, induce apoptosis and up-regulate activation of NF-κB. Elevated levels of iNOS not only cause injury to host cell and induce apoptosis directly, but are also involved in host defenses against infection. This reaction is regulated by NF-κB, which is activated by *U. urealyticum*. Down-regulatory cytokines can resolve inflammation by inhibition of proinflammatory cytokine, but at same time promote the fibrosis.

Conclusions

1. *U. urealyticum* may be an important causative factor in the development of CLD due to its ability to induce production or expression of proinflammatory cytokines, nitric oxide, cellular adhesion molecules and vascular endothelial growth factor by alveolar macrophages.
2. Steroids and human rIL-10 can down-regulate these responses to *U. urealyticum* which may explain the beneficial effects of steroids in CLD patients.
3. The increased expression of iNOS caused by *U. urealyticum* appears to be mediated via activation of NF-κB.
4. The defensive response of host to *U. urealyticum* infection may be influenced by NO.
5. The high levels of TGF-β1 present in TAF from preterm infants who develop CLD and absence or irregular secretion of the other down-regulatory cytokines IL-10 and IL-4 in the same patients indicate that there is a dysregulation in the production of down-regulatory cytokines.

Future perspectives

1. Apoptosis

Cell death in living organisms is classified either as necrosis or apoptosis, also referred to "programmed cell death". Apoptosis is an important physiological process in developing organs during embryonal and fetal life. This process may also play a role in the development of lung disease in humans. Macrophages play essential roles in host defense mechanisms of both preterm and newborn infants against infection. Slightly impaired macrophage functions, in combination with some defects in humoral defense mechanisms, may contribute directly to the increased susceptibility of preterm infants to pulmonary and systemic nosocomial infections (Speer 2001). At the same time, the phagocytosis of "unwanted" neutrophils will be impaired, leading to prolonged inflammation. Pulmonary epithelial cells are essential in maintaining the integrity of the alveolar–capillary barrier and serve as a first-line defense against a variety of insults including infection. Furthermore, these cells play central roles in modulation of the inflammatory response and in the repair of damaged lung tissue. Compromised epithelial cell function may lead to the leakage of fluid and macromolecules into the air spaces that then can result in clinical respiratory failure and even death. Therefore, the death and proliferation of pulmonary epithelial cells are under stringent control in order to maintain the homeostasis under normal condition (Mantell and Lee 2000). To study the role of apoptosis in response to *U. urealyticum* infection may help us to further understand the possible mechanism of *U. urealyticum* in the development of CLD.

2. Toll like receptors

Recently, the role of toll like receptors (TLRs) in the innate immunity to infection has been focus of increasing attention. It is vital that the host detects the pathogen and rapidly mounts a defense. Recognition of pathogens is mediated by a set of germline-encoded receptors referred to as pattern-recognition receptors (PRRs). TLRs function as the PRRs in mammals, playing an essential role in the recognition of microbial components and activating the inflammatory response when stimulated (Aderem 2001; Akira, Takeda et al. 2001).

TLR2 and TLR4 are the Toll-like receptors that have been shown to be responsive to microbial ligands. TLR4 is clearly the main receptor for LPS, a major component of Gram-negative bacteria. TLR-2 mediates cellular responses to peptidoglycans and lipoteichoic acid, both of which are stimulatory components of Gram-positive bacteria. TLR5 has been found to recognize the flagellin present in Gram-positive and Gram-negative bacteria (Akira, Takeda et al. 2001). The N-termini of these various microbial lipoproteins share a common triacyl motif, which is responsible for their stimulatory properties. Like many of these bacterial ligands, microbial lipoproteins are potent activators of macrophages, increasing the production of cytokines and up-regulating inflammatory events (Brightbill and Modlin 2000; Schuster and Nelson 2000).

It has been demonstrated that human TLR4 can induce the activation of NF-κB. The TLR signaling complex involves a heterodimer complex of homologous proteins, i.e., multiple TLRs. Other mediators have been implicated in this context, including MD-2, which may be involved in TLR4 signaling in response to LPS. MyD88, an intracellular adapter molecule for the TLR/IL-1R complex, was recently shown to be required for the cellular response to all of the cell wall components of Gram-positive and Gram-negative bacteria that are tested. MyD88 recruits IRAK (IL-1 receptor accessory protein kinase) through interactions of the death domain on both proteins, resulting in autophosphorylation of IRAK. TNF-receptor-associated factor (TRAF6) is then recruited, leading to the activation of NF-κB-inducing kinase (NIK) and subsequent expression of genes regulated by NF-κB (Brightbill and Modlin 2000) (Figure 13).

The ontogeny of TLR2 and TLR4 in mice has been investigated (Harju, Glumoff et al. 2001). In the lung of immature fetuses, TLRs are expressed at very low levels, these levels increase 8-fold during the last trimester of murine pregnancy and exhibit an additional 2.5-fold increase after birth. The expression levels of TLR2 and TLR4 were similar.

To investigate the role of TLRs in response to *U. urealyticum* infection might improve our understanding of the mechanism underlying development of CLD in preterm infants, as well as aid in designing new strategies for prevention and treatment of this disease.

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References

Abele-Horn, M., O. Genzel-Boroviczeny, T. Uhlig, A. Zimmermann, J. Peters and M. Scholz (1998). "Ureaplasma urealyticum colonization and bronchopulmonary dysplasia: a comparative prospective multicentre study." European Journal of Pediatrics **157**:1004-11.

Abele-Horn, M., J. Peters, O. Genzel-Boroviczeny, C. Wolff, A. Zimmermann and W. Gottschling (1997). "Vaginal Ureaplasma urealyticum colonization: influence on pregnancy outcome and neonatal morbidity." Infection **25**:286-91.

Abeyama, K., W. Eng, J. V. Jester, A. A. Vink, D. Edelbaum, C. J. Cockerell, P. R. Bergstresser and A. Takashima (2000). "A role for NF-kappaB-dependent gene transactivation in sunburn." Journal of Clinical Investigation **105**:1751-9.

Aderem, A. (2001). "Role of Toll-like receptors in inflammatory response in macrophages." Critical Care Medicine **29**:S16-8.

Agarwal, P., V. S. Rajadurai, V. K. Pradeepkumar and K. W. Tan (2000). "Ureaplasma urealyticum and its association with chronic lung disease in Asian neonates." Journal of Paediatrics and Child Health **36**:487-90.

Agular-Santelises, M., M. E. Rottenberg, N. Lewin, H. Mellstedt and M. Jondal (1996). "Bcl-2, Bax and p53 expression in B-CLL in relation to in vitro survival and clinical progression." International Journal of Cancer **69**:114-9.

Akira, S., K. Takeda and T. Kaisho (2001). "Toll-like receptors: critical proteins linking innate and acquired immunity." Nature Immunology **2**:675-80.

Alenghat, E. and J. R. Esterly (1984). "Alveolar macrophages in perinatal infants." Pediatrics **74**:221-3.

Anonymous (1991). "Dexamethasone therapy in neonatal chronic lung disease: an international placebo-controlled trial. Collaborative Dexamethasone Trial Group." Pediatrics **88**:421-7.

Arnon, S., J. Grigg and M. Silverman (1993). "Pulmonary inflammatory cells in ventilated preterm infants: effect of surfactant treatment." Archives of Disease in Childhood **69**:44-8.

Auphan, N., J. A. DiDonato, C. Rosette, A. Helmberg and M. Karin (1995). "Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis." Science **270**:286-90.

Avery, M. E., W. H. Tooley, J. B. Keller, S. S. Hurd, M. H. Bryan, R. B. Cotton, M. F. Epstein, P. M. Fitzhardinge, C. B. Hansen, T. N. Hansen and et al. (1987). "Is chronic lung disease in low birth weight infants preventable? A survey of eight centers." Pediatrics **79**:26-30.

Bagchi, A., R. M. Viscardi, V. Taciak, J. E. Ensor, K. A. McCrea and J. D. Hasday (1994). "Increased activity of interleukin-6 but not tumor necrosis factor-alpha in lung lavage of premature infants is associated with the development of bronchopulmonary dysplasia." Pediatric Research **36**:244-52.

Bancalari, E. (1998). "Corticosteroids and neonatal chronic lung disease." European Journal of Pediatrics **157**:S31-7.

Bancalari, E., G. E. Abdenour, R. Feller and J. Gannon (1979). "Bronchopulmonary dysplasia: clinical presentation." Journal of Pediatrics **95**:819-23.

Banks, B. A., H. Ischiropoulos, M. McClelland, P. L. Ballard and R. A. Ballard (1998). "Plasma 3-Nitrotyrosine Is Elevated in Premature Infants Who Develop Bronchopulmonary Dysplasia." Pediatrics **101**:870-874.

Barnes, P. J. (1997). "Molecular mechanisms of glucocorticoid action in asthma." Pulmonary Pharmacology & Therapeutics **10**:3-19.

Barnes, P. J. (1997). "Nuclear factor-kappa B." International Journal of Biochemistry & Cell Biology **29**:867-70.

Barnes, P. J. and I. Adcock (1993). "Anti-inflammatory actions of steroids: molecular mechanisms." Trends in Pharmacological Sciences **14**:436-41.

Barnes, P. J. and S. Pedersen (1993). "Efficacy and safety of inhaled corticosteroids in asthma. Report of a workshop held in Eze, France, October 1992." American Review of Respiratory Disease **148**:S1-26.

Beckman, J. S., T. W. Beckman, J. Chen, P. A. Marshall and B. A. Freeman (1990). "Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide." Proceedings of the National Academy of Sciences of the United States of America **87**:1620-4.

Bhuta, T. and A. Ohlsson (1998). "Systematic review and meta-analysis of early postnatal dexamethasone for prevention of chronic lung disease." Archives of Disease in Childhood Fetal & Neonatal Edition **79**:F26-33.

Bogdan, C., J. Paik, Y. Vodovotz and C. Nathan (1992). "Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10." Journal of Biological Chemistry **267**:23301-8.

Border, W. A. and N. A. Noble (1994). "Transforming growth factor beta in tissue fibrosis." New England Journal of Medicine **331**:1286-92.

Brightbill, H. D. and R. L. Modlin (2000). "Toll-like receptors: molecular mechanisms of the mammalian immune response." Immunology **101**:1-10.

Bruce, M. C., M. Schuyler, R. J. Martin, B. C. Starcher, J. F. Tomashefski, Jr. and K. E. Wedig (1992). "Risk factors for the degradation of lung elastic fibers in the ventilated neonate. Implications for impaired lung development in bronchopulmonary dysplasia." American Review of Respiratory Disease **146**:204-12.

Burgner, D., K. Rockett and D. Kwiatkowski (1999). "Nitric oxide and infectious diseases." Archives of Disease in Childhood **81**:185-8.

Cassell, G. H., R. O. Davis, K. B. Waites, M. B. Brown, P. A. Marriott, S. Stagno and J. K. Davis (1983). "Isolation of Mycoplasma hominis and Ureaplasma urealyticum from amniotic fluid at 16-20 weeks of gestation: potential effect on outcome of pregnancy." Sexually Transmitted Diseases **10**:294-302.

Cassell, G. H., K. B. Waites, D. T. Crouse, P. T. Rudd, K. C. Canupp, S. Stagno and G. R. Cutter (1988). "Association of Ureaplasma urealyticum infection of the lower respiratory tract with chronic lung disease and death in very-low-birth-weight infants." Lancet **2**:240-5.

Cassell, G. H., K. B. Waites, R. S. Gibbs and J. K. Davis (1986). "Role of Ureaplasma urealyticum in amnionitis." Pediatric Infectious Disease **5**:S247-52.

Chapman, H. A. (1999). "A Fas pathway to pulmonary fibrosis." Journal of Clinical Investigation **104**:1-2.

Chen, F., V. Castranova, X. Shi and L. M. Demers (1999). "New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases." Clinical Chemistry **45**:7-17.

Christman, J. W., L. H. Lancaster and T. S. Blackwell (1998). "Nuclear factor kappa B: a pivotal role in the systemic inflammatory response syndrome and new target for therapy." Intensive Care Medicine **24**:1131-8.

Clement, A., K. Chadelat, A. Sardet, A. Grimfeld and G. Tournier (1988). "Alveolar macrophage status in bronchopulmonary dysplasia." Pediatric Research **23**:470-3.

Coalson, J. J., D. R. Gerstmann, V. T. Winter and R. A. Delemos (1991). "Bacterial colonization and infection studies in the premature baboon with bronchopulmonary dysplasia." American Review of Respiratory Disease **144**:1140-6.

Cooper, J. A., Jr. (2000). "Pulmonary fibrosis: pathways are slowly coming into light." American Journal of Respiratory Cell & Molecular Biology **22**:520-3.

Cordero, L., L. W. Ayers and K. Davis (1997). "Neonatal airway colonization with gram-negative bacilli: association with severity of bronchopulmonary dysplasia." Pediatric Infectious Disease Journal **16**:18-23.

Couroucli, X. I., S. E. Welty, P. L. Ramsay, M. E. Wearden, F. J. Fuentes-Garcia, J. Ni, T. N. Jacobs, J. A. Towbin and N. E. Bowles (2000). "Detection of microorganisms in the tracheal aspirates of preterm infants by polymerase chain reaction: association of adenovirus infection with bronchopulmonary dysplasia." Pediatric Research **47**:225-32.

Crouse, D. T., G. H. Cassell, K. B. Waites, J. M. Foster and G. Cassady (1990). "Hyperoxia potentiates Ureaplasma urealyticum pneumonia in newborn mice." Infection & Immunity **58**:3487-93.

Crouse, D. T., G. T. Odrezin, G. R. Cutter, J. M. Reese, W. B. Hamrick, K. B. Waites and G. H. Cassell (1993). "Radiographic changes associated with tracheal isolation of Ureaplasma urealyticum from neonates." Clinical Infectious Diseases **17**:S122-30.

de Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor and J. E. de Vries (1991). "Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes." Journal of Experimental Medicine **174**:1209-20.

deLemos, R. A. and J. J. Coalson (1992). "The contribution of experimental models to our understanding of the pathogenesis and treatment of bronchopulmonary dysplasia." Clinics in Perinatology **19**:521-39.

Durand, M., S. Sardesai and C. McEvoy (1995). "Effects of early dexamethasone therapy on pulmonary mechanics and chronic lung disease in very low birth weight infants: a randomized, controlled trial." Pediatrics **95**:584-90.

Egarter, C., H. Leitich, H. Karas, F. Wieser, P. Husslein, A. Kaider and M. Schemper (1996). "Antibiotic treatment in preterm premature rupture of membranes and neonatal morbidity: a metaanalysis." American Journal of Obstetrics & Gynecology **174**:589-97.

Eschenbach, D. A. (1993). "Ureaplasma urealyticum and premature birth." Clinical Infectious Diseases **17**:S100-6.

Estler, H. C., M. Grewe, R. Gaussling, M. Pavlovic and K. Decker (1992). "Rat tumor necrosis factor-alpha. Transcription in rat Kupffer cells and in vitro posttranslational processing based on a PCR-derived cDNA." Biological Chemistry Hoppe-Seyler **373**:271-81.

Evans, T., A. Carpenter, H. Kinderman and J. Cohen (1993). "Evidence of increased nitric oxide production in patients with the sepsis syndrome." Circulatory Shock **41**:77-81.

Fang, F. C. (1997). "Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity." Journal of Clinical Investigation **99**:2818-25.

Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard and O. G. A (1991). "IL-10 inhibits cytokine production by activated macrophages." Journal of Immunology **147**:3815-22.

Garland, J. S., C. P. Alex, T. H. Pauly, V. L. Whitehead, J. Brand, J. F. Winston, D. P. Samuels and T. L. McAuliffe (1999). "A three-day course of dexamethasone therapy to prevent chronic lung disease in ventilated neonates: a randomized trial." Pediatrics **104**:91-9.

Gerard, C., C. Bruyns, A. Marchant, D. Abramowicz, P. Vandenabeele, A. Delvaux, W. Fiers, M. Goldman and T. Velu (1993). "Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia." Journal of Experimental Medicine **177**:547-50.

Gerdes, J. S., M. C. Yoder, S. D. Douglas, M. Paul, M. C. Harris and R. A. Polin (1986). "Tracheal lavage and plasma fibronectin: relationship to respiratory distress syndrome and development of bronchopulmonary dysplasia." Journal of Pediatrics **108**:601-6.

Glass, J. I., E. J. Lefkowitz, J. S. Glass, C. R. Heiner, E. Y. Chen and G. H. Cassell (2000). "The complete sequence of the mucosal pathogen Ureaplasma urealyticum." Nature **407**:757-62.

Goldenberg, R. L. and W. W. Andrews (1996). "Intrauterine infection and why preterm prevention programs have failed." American Journal of Public Health **86**:781-3.

Goodwin, S. R., S. A. Graves and C. M. Haberkern (1985). "Aspiration in intubated premature infants." Pediatrics **75**:85-8.

Gordon, D. and A. M. Nouri (1981). "Comparison of the inhibition by glucocorticosteroids and cyclosporin A of mitogen-stimulated human lymphocyte proliferation." Clinical & Experimental Immunology **44**:287-94.

Greenhalgh, D. G. (1998). "The role of apoptosis in wound healing." International Journal of Biochemistry & Cell Biology **30**:1019-30.

Greenough, A. (1990). "Bronchopulmonary dysplasia: early diagnosis, prophylaxis, and treatment." Archives of Disease in Childhood **65**:1082-8.

Grisham, M. B., D. Jourd'Heuil and D. A. Wink (1999). "Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation." American Journal of Physiology **276**:G315-21.

Groneck, P., B. Goetze-Speer and C. P. Speer (1996). “Inflammatory bronchopulmonary response of preterm infants with microbial colonisation of the airways at birth.” Archives of Disease in Childhood. Fetal and Neonatal Edition **74**:F51-5.

Groneck, P., B. Gotze-Speer, M. Oppermann, H. Eiffert and C. P. Speer (1994). “Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of high-risk preterm neonates.” Pediatrics **93**:712-8.

Groneck, P., M. Oppermann and C. P. Speer (1993). “Levels of complement anaphylatoxin C5a in pulmonary effluent fluid of infants at risk for chronic lung disease and effects of dexamethasone treatment.” Pediatric Research **34**:586-90.

Groneck, P., D. Reuss, B. Gotze-Speer and C. P. Speer (1993). “Effects of dexamethasone on chemotactic activity and inflammatory mediators in tracheobronchial aspirates of preterm infants at risk for chronic lung disease.” Journal of Pediatrics **122**:938-44.

Groneck, P., J. Schmale, V. Soditt, H. Stutzer, B. Gotze-Speer and C. P. Speer (2001). “Bronchoalveolar inflammation following airway infection in preterm infants with chronic lung disease.” Pediatric Pulmonology **31**:331-8.

Hagberg, H., E. Gilland, E. Bona, L. A. Hanson, M. Hahin-Zoric, M. Blennow, M. Holst, A. McRae and O. Soder (1996). “Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats.” Pediatric Research **40**:603-9.

Hagimoto, N., K. Kuwano, H. Miyazaki, R. Kunitake, M. Fujita, M. Kawasaki, Y. Kaneko and N. Hara (1997). “Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen.” American Journal of Respiratory Cell & Molecular Biology **17**:272-8.

Hallman, M. (2001). “Inflammatory pathways between placenta and foetus.” Acta Paediatrica **90**:1-2.

Hallman, M., V. Glumoff and M. Ramet (2001). “Surfactant in respiratory distress syndrome and lung injury.” Comparative Biochemistry and Physiology A: Moecularl Integration of Physiology **129**:287-94.

Hallman, M., T. A. Merritt, T. Akino and K. Bry (1991). “Surfactant protein A, phosphatidylcholine, and surfactant inhibitors in epithelial lining fluid. Correlation with surface activity, severity of respiratory distress syndrome, and outcome in small premature infants.” American Review of Respiratory Disease **144**:1376-84.

Hannaford, K., D. A. Todd, H. Jeffery, E. John, K. Blyth and G. L. Gilbert (1999). “Role of ureaplasma urealyticum in lung disease of prematurity.” Archives of Disease in Childhood. Fetal and Neonatal Edition **81**:F162-7.

Hargitai, B., V. Szabo, J. Hajdu, A. Harmath, M. Pataki, P. Farid, Z. Papp and B. Szende (2001). “Apoptosis in various organs of preterm infants: histopathologic study of lung, kidney, liver, and brain of ventilated infants.” Pediatric Research **50**:110-4.

Harju, K., V. Glumoff and M. Hallman (2001). “Ontogeny of Toll-like receptors Tlr2 and Tlr4 in mice.” Pediatric Research **49**:81-3.

Hauth, J. C., R. L. Goldenberg, W. W. Andrews, M. B. DuBard and R. L. Copper (1995). "Reduced incidence of preterm delivery with metronidazole and erythromycin in women with bacterial vaginosis." New England Journal of Medicine **333**:1732-6.

Heckmann, M., J. Kreuder, C. Spenneberg, I. Reiss and L. Gortner (2000). "Arginine and Citrulline Concentrations in Very Low Birth Weight Infants (VLBWI) with Bronchopulmonary Dysplasia (BPD)." Biology of the Neonate **78**:153.

Hierholzer, C., B. Harbrecht, J. M. Menezes, J. Kane, J. MacMicking, C. F. Nathan, A. B. Peitzman, T. R. Billiar and D. J. Tweardy (1998). "Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock." Journal of Experimental Medicine **187**:917-28.

Hoper, M. M., N. F. Voelkel, T. O. Bates, J. D. Allard, M. Horan, D. Shepherd and R. M. Tuder (1997). "Prostaglandins induce vascular endothelial growth factor in a human monocytic cell line and rat lungs via cAMP." American Journal of Respiratory Cell & Molecular Biology **17**:748-56.

Howard, M., T. Muchamuel, S. Andrade and S. Menon (1993). "Interleukin 10 protects mice from lethal endotoxemia." Journal of Experimental Medicine **177**:1205-8.

Huang, S., J. B. Robinson, A. DeGuzman, C. D. Bucana and I. J. Fidler (2000). "Blockade of Nuclear Factor-{{kappa}}B Signaling Inhibits Angiogenesis and Tumorigenicity of Human Ovarian Cancer Cells by Suppressing Expression of Vascular Endothelial Growth Factor and Interleukin 8." Cancer Research **60**:5334-5339.

Janeway, J. C. and P. Travers (1997). Immunobiology. London, Current Biology Limited.

Jobe, A. H. and E. Bancalari (2001). "Bronchopulmonary Dysplasia." American Journal of Respiratory and Critical Care Medicine **163**:1723-1729.

Jobe, A. H. and M. Ikegami (2001). "Prevention of bronchopulmonary dysplasia." Current Opinion in Pediatrics **13**:124-9.

Jones, C. A., R. G. Cayabyab, K. Y. Kwong, C. Stotts, B. Wong, H. Hamdan, P. Minoo and R. A. deLemos (1996). "Undetectable interleukin (IL)-10 and persistent IL-8 expression early in hyaline membrane disease: a possible developmental basis for the predisposition to chronic lung inflammation in preterm newborns." Pediatric Research **39**:966-75.

Jonsson, B., M. Eriksson, O. Soder, U. Broberger and H. Lagercrantz (2000). "Budesonide delivered by dosimetric jet nebulization to preterm very low birthweight infants at high risk for development of chronic lung disease." Acta Paediatrica **89**:1449-55.

Jonsson, B., M. Katz-Salamon, G. Faxelius, U. Broberger and H. Lagercrantz (1997). "Neonatal care of very-low-birthweight infants in special-care units and neonatal intensive-care units in Stockholm. Early nasal continuous positive airway pressure versus mechanical ventilation: gains and losses." Acta Paediatrica. Supplement **419**:4-10.

Jonsson, B., K. Tullus, A. Brauner, Y. Lu and G. Noack (1997). "Early increase of TNF alpha and IL-6 in tracheobronchial aspirate fluid indicator of subsequent chronic lung disease in preterm infants." Archives of Disease in Childhood. Fetal & Neonatal Edition **77**:F198-201.

Kalogeris, T. J., F. S. Laroux, A. Cockrell, H. Ichikawa, N. Okayama, T. J. Phifer, J. S. Alexander and M. B. Grisham (1999). "Effect of selective proteasome inhibitors on TNF-induced activation of primary and transformed endothelial cells." American Journal of Physiology **276**:C856-64.

Kari, M. A., M. Hallman, M. Eronen, K. Teramo, M. Virtanen, M. Koivisto and R. S. Ikonen (1994). "Prenatal dexamethasone treatment in conjunction with rescue therapy of human surfactant: a randomized placebo-controlled multicenter study." Pediatrics **93**:730-6.

Kari, M. A., K. O. Raivio, P. Venge and M. Hallman (1994). "Dexamethasone treatment of infants at risk for chronic lung disease: surfactant components and inflammatory parameters in airway specimens." Pediatric Research **36**:387-93.

Kelley, J. (1990). "Cytokines of the lung." American Review of Respiratory Disease **141**:765-88.

Kelso, A. (1998). "Cytokines: principles and prospects." Immunology & Cell Biology **76**:300-17.

Khalil, N., C. Whitman, L. Zuo, D. Danielpour and A. Greenberg (1993). "Regulation of alveolar macrophage transforming growth factor-beta secretion by corticosteroids in bleomycin-induced pulmonary inflammation in the rat." Journal of Clinical Investigation **92**:1812-8.

Kotecha, S. (1996). "Cytokines in chronic lung disease of prematurity." European Journal of Pediatrics **155**:S14-7.

Kotecha, S., B. Chan, N. Azam, M. Silverman and R. J. Shaw (1995). "Increase in interleukin-8 and soluble intercellular adhesion molecule-1 in bronchoalveolar lavage fluid from premature infants who develop chronic lung disease." Archives of Disease in Childhood. Fetal & Neonatal Edition **72**:F90-6.

Kotecha, S., M. Silverman, R. J. Shaw and N. Klein (1998). "Soluble L-selectin concentration in bronchoalveolar lavage fluid obtained from infants who develop chronic lung disease of prematurity." Archives of Disease in Childhood. Fetal and Neonatal Edition **78**:F143-7.

Kotecha, S., A. Wangoo, M. Silverman and R. J. Shaw (1996). "Increase in the concentration of transforming growth factor beta-1 in bronchoalveolar lavage fluid before development of chronic lung disease of prematurity." Journal of Pediatrics **128**:464-9.

Kotecha, S., L. Wilson, A. Wangoo, M. Silverman and R. J. Shaw (1996). "Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity." Pediatric Research **40**:250-6.

Kothadia, J. M., T. M. O'Shea, D. Roberts, S. T. Auringer, R. G. Weaver, 3rd and R. G. Dillard (1999). "Randomized placebo-controlled trial of a 42-Day tapering course of dexamethasone to reduce the duration of ventilator dependency in very low birth weight infants." Pediatrics **104**:22-7.

Kronke, M., W. J. Leonard, J. M. Depper and W. C. Greene (1985). "Sequential expression of genes involved in human T lymphocyte growth and differentiation." Journal of Experimental Medicine **161**:1593-8.

Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky and W. Muller (1993). "Interleukin-10-deficient mice develop chronic enterocolitis." Cell **75**:263-74.

Lee, J. I. and G. J. Burckart (1998). "Nuclear factor kappa B: important transcription factor and therapeutic target." Journal of Clinical Pharmacology **38**:981-93.

Little, S., T. Dean, S. Bevin, M. Hall, M. Ashton, M. Church, J. Warner and J. Shute (1995). "Role of elevated plasma soluble ICAM-1 and bronchial lavage fluid IL-8 levels as markers of chronic lung disease in premature infants." Thorax **50**:1073-9.

Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Snyder and S. W. Russell (1993). "Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide." Journal of Biological Chemistry **268**:1908-13.

Lundstrom, K. E., N. J. Shaw and M. A. Thomson (2000). "Chronic lung disease-cracking the condition in the 21st century." Biology of the Neonate **78**:233-68.

Maaser, C., S. Schoeppner, T. Kucharzik, M. Kraft, E. Schoenherr, W. Domschke and N. Luegering (2001). "Colonic epithelial cells induce endothelial cell expression of ICAM-1 and VCAM-1 by a NF-kappaB-dependent mechanism." Clinical and Experimental Immunology **124**:208-13.

Manimtim, W. M., J. D. Hasday, L. Hester, K. D. Fairchild, J. C. Lovchik and R. M. Viscardi (2001). "Ureaplasma urealyticum Modulates Endotoxin-Induced Cytokine Release by Human Monocytes Derived from Preterm and Term Newborns and Adults." Infection and Immunity **69**:3906-3915.

Maniscalco, W. M., R. H. Watkins, J. N. Finkelstein and M. H. Campbell (1995). "Vascular endothelial growth factor mRNA increases in alveolar epithelial cells during recovery from oxygen injury." American Journal of Respiratory Cell & Molecular Biology **13**:377-86.

Manktelow, B. N., E. S. Draper, S. Annamalai and D. Field (2001). "Factors affecting the incidence of chronic lung disease of prematurity in 1987, 1992, and 1997." Archives of Disease in Childhood. Fetal and Neonatal Edition **85**:F33-5.

Mantell, L. L. and P. J. Lee (2000). "Signal transduction pathways in hyperoxia-induced lung cell death." Molecular Genetics & Metabolism **71**:359-70.

Marchant, A., J. Deviere, B. Byl, D. De Groote, J. L. Vincent and M. Goldman (1994). "Interleukin-10 production during septicaemia." Lancet **343**:707-8.

McColm, J. R., B. J. Stenson, N. Biermasz and N. McIntosh (2000). "Measurement of interleukin 10 in bronchoalveolar lavage from preterm ventilated infants." Archives of Disease in Childhood. Fetal and Neonatal Edition **82**:F156-9.

Mercer, B. M. and K. L. Arheart (1995). "Antimicrobial therapy in expectant management of preterm premature rupture of the membranes." Lancet **346**:1271-9.

Merritt, T. A., I. D. Stuard, J. Puccia, B. Wood, D. K. Edwards, J. Finkelstein and D. L. Shapiro (1981). "Newborn tracheal aspirate cytology: classification during respiratory distress syndrome and bronchopulmonary dysplasia." Journal of Pediatrics **98**:949-56.

Minamiya, Y., S. Motoyama, M. Kitamura, S. Saito, K. Terada and J. Ogawa (1998). "The requirement of intercellular adhesion molecule-1 for neutrophil respiratory burst in the pulmonary circulation of rats infused with endotoxin." American Journal of Respiratory and Critical Care Medicine **158**:635-42.

Moore, K. W., A. O'Garra, R. de Waal Malefyt, P. Vieira and T. R. Mosmann (1993). "Interleukin-10." Annual Review of Immunology **11**:165-90.

Munshi, U. K., J. O. Niu, M. M. Siddiq and L. A. Parton (1997). "Elevation of interleukin-8 and interleukin-6 precedes the influx of neutrophils in tracheal aspirates from preterm infants who develop bronchopulmonary dysplasia." Pediatric Pulmonology **24**:331-6.

Murch, S. H., K. Costeloe, N. J. Klein and T. T. MacDonald (1996). "Early production of macrophage inflammatory protein-1 alpha occurs in respiratory distress syndrome and is associated with poor outcome." Pediatric Research **40**:490-7.

Murch, S. H., K. Costeloe, N. J. Klein, H. Rees, N. McIntosh, J. W. Keeling and T. T. MacDonald (1996). "Mucosal tumor necrosis factor-alpha production and extensive disruption of sulfated glycosaminoglycans begin within hours of birth in neonatal respiratory distress syndrome." Pediatric Research **40**:484-9.

Murphy, M. P. (1999). "Nitric oxide and cell death." Biochimica et Biophysica Acta **1411**:401-14.

Niiro, H., T. Otsuka, S. Kuga, Y. Nemoto, M. Abe, N. Hara, T. Nakano, T. Ogo and Y. Niho (1994). "IL-10 inhibits prostaglandin E2 production by lipopolysaccharide-stimulated monocytes." International Immunology **6**:661-4.

Nissen, N. N., P. J. Polverini, A. E. Koch, M. V. Volin, R. L. Gamelli and L. A. DiPietro (1998). "Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing." American Journal of Pathology **152**:1445-52.

Northway, W. H., Jr. (1990). "Bronchopulmonary dysplasia: then and now." Archives of Disease in Childhood **65**:1076-81.

Northway, W. H., Jr. (1992). "Bronchopulmonary dysplasia: twenty-five years later." Pediatrics **89**:969-73.

Northway, W. H., Jr., R. C. Rosan and D. Y. Porter (1967). "Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia." New England Journal of Medicine **276**:357-68.

O'Brodovich, H. M. and R. B. Mellins (1985). "Bronchopulmonary dysplasia. Unresolved neonatal acute lung injury." American Review of Respiratory Disease **132**:694-709.

Ohlsson, A., S. A. Calvert, M. Hosking and A. T. Shennan (1992). "Randomized controlled trial of dexamethasone treatment in very-low-birth-weight infants with ventilator-dependent chronic lung disease." Acta Paediatrica **81**:751-6.

Ohlsson, K., T. Sveger and N. Svenningsen (1992). "Protease inhibitors in bronchoalveolar lavage fluid from neonates with special reference to secretory leukocyte protease inhibitor." Acta Paediatrica **81**:757-9.

Ollikainen, J. (2000). "Perinatal Ureaplasma urealyticum infection increases the need for hospital treatment during the first year of life in preterm infants." Pediatric Pulmonology **30**:402-5.

Ozdemir, A., M. A. Brown and W. J. Morgan (1997). "Markers and mediators of inflammation in neonatal lung disease." Pediatric Pulmonology **23**:292-306.

Paine, R., 3rd and P. A. Ward (1999). "Cell adhesion molecules and pulmonary fibrosis." American Journal of Medicine **107**:268-79.

Perzigian, R. W., J. T. Adams, G. M. Weiner, M. A. Dipietro, L. K. Blythe, C. L. Pierson and R. G. Faix (1998). "Ureaplasma urealyticum and chronic lung disease in very low birth weight infants during the exogenous surfactant era." Pediatric Infectious Disease Journal **17**:620-5.

Pisa, E. K., P. Pisa, M. Hansson and H. Wigzell (1992). "OKT3-induced cytokine mRNA expression in human peripheral blood mononuclear cells measured by polymerase chain reaction." Scandinavian Journal of Immunology **36**:745-9.

Polunovsky, V. A., B. Chen, C. Henke, D. Snover, C. Wendt, D. H. Ingbar and P. B. Bitterman (1993). "Role of mesenchymal cell death in lung remodeling after injury." Journal of Clinical Investigation **92**:388-97.

Ramsay, P. L., E. O'Brian Smith, S. Hegemier and S. E. Welty (1998). "Early clinical markers for the development of bronchopulmonary dysplasia: soluble E-Selectin and ICAM-1." Pediatrics **102**:927-32.

Reed, J. C., P. C. Nowell and R. G. Hoover (1985). "Regulation of c-myc mRNA levels in normal human lymphocytes by modulators of cell proliferation." Proceedings of the National Academy of Sciences of the United States of America **82**:4221-4.

Roberts, W. G. and G. E. Palade (1995). "Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor." Journal of Cell Science **108**:2369-79.

Robertson, B. (1989). "The evolution of neonatal respiratory distress syndrome into chronic lung disease." European Respiratory Journal - Supplement **3**:33s-37s.

Roebuck, K. A. and A. Finnegan (1999). "Regulation of intercellular adhesion molecule-1 (CD54) gene expression." Journal of Leukocyte Biology **66**:876-88.

Rudd, P. T., G. H. Cassell, K. B. Waites, J. K. Davis and L. B. Duffy (1989). "Ureaplasma urealyticum pneumonia: experimental production and demonstration of age-related susceptibility." Infection & Immunity **57**:918-25.

Sanchez, P. J. (1993). "Perinatal transmission of Ureaplasma urealyticum: current concepts based on review of the literature." Clinical Infectious Diseases **17**:S107-11.

Sawyer, M. H., D. K. Edwards and S. A. Spector (1987). "Cytomegalovirus infection and bronchopulmonary dysplasia in premature infants." American Journal of Diseases Childhood **141**:303-5.

Schroeder, R. A., C. Cai and P. C. Kuo (1999). "Endotoxin-mediated nitric oxide synthesis inhibits IL-1beta gene transcription in ANA-1 murine macrophages." American Journal of Physiology **277**:C523-30.

Schuster, J. M. and P. S. Nelson (2000). "Toll receptors: an expanding role in our understanding of human disease." Journal of Leukocyte Biology **67**:767-73.

Seo, K., J. A. McGregor and J. I. French (1992). "Preterm birth is associated with increased risk of maternal and neonatal infection." Obstetrics & Gynecology **79**:75-80.

Sherman, M. P., M. J. Evans and L. A. Campbell (1988). "Prevention of pulmonary alveolar macrophage proliferation in newborn rabbits by hyperoxia." Journal of Pediatrics **112**:782-6.

Simon, R. H., P. D. DeHart and R. F. d. Todd (1986). "Neutrophil-induced injury of rat pulmonary alveolar epithelial cells." Journal of Clinical Investigation **78**:1375-86.

Sirsjo, A., P. Soderkvist, T. Sundqvist, M. Carlsson, M. Ost and A. Gidlof (1994). "Different induction mechanisms of mRNA for inducible nitric oxide synthase in rat smooth muscle cells in culture and in aortic strips." FEBS Letters **338**:191-6.

Sonoda, M., J. Kobayashi, M. Takezawa, T. Miyazaki, T. Nakajima, H. Shimomura, K. Koike, A. Satomi, H. Ogino, R. Omoto and T. Komoda (1997). "An assay method for nitric oxide-related compounds in whole blood." Analytical Biochemistry **247**:417-27.

Sorkness, R. L., H. Mehta, M. R. Kaplan, M. Miyasaka, S. L. Hefle and R. F. Lemanske, Jr. (2000). "Effect of ICAM-1 blockade on lung inflammation and physiology during acute viral bronchiolitis in rats." Pediatric Research **47**:819-24.

Spahn, J. D. and A. K. Kamada (1995). "Special considerations in the use of glucocorticoids in children." Pediatrics in Review **16**:266-72.

Speer, C. P. (2001). "New Insights into the Pathogenesis of Pulmonary Inflammation in Preterm Infants." Biology of the Neonate **79**:205-9.

Speer, C. P., A. Ninjo and M. Gahr (1986). "Elastase-alpha 1-proteinase inhibitor in early diagnosis of neonatal septicemia." Journal of Pediatrics **108**:987-90.

Stiskal, J. A., S. Ito, D. W. Cox, A. T. Shennan, K. K. O'Brien, E. N. Kelly, T. B. Longley, M. Rabinovitch and M. S. Dunn (1999). "Functional and antigenic concentrations of alpha-1-proteinase inhibitor after administration for the prevention of chronic lung disease of prematurity." Biology of the Neonate **76**:134-43.

Sunday, M. E., L. M. Kaplan, E. Motoyama, W. W. Chin and E. R. Spindel (1988). "Gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease." Laboratory Investigation **59**:5-24.

Sunday, M. E., B. A. Yoder, F. Cuttitta, K. J. Haley and R. L. Emanuel (1998). "Bombesin-like peptide mediates lung injury in a baboon model of bronchopulmonary dysplasia." Journal of Clinical Investigation **102**:584-94.

Sweet, D. G., K. J. McMahon, A. E. Curley, C. M. O'Connor and H. L. Halliday (2001). "Type I collagenases in bronchoalveolar lavage fluid from preterm babies at risk of developing chronic lung disease." Archives of Disease in Childhood. Fetal and Neonatal Edition **84**:F168-71.

Takizawa, K., R. Kamijo, D. Ito, M. Hatori, K. Sumitani and M. Nagumo (1999). "Synergistic induction of ICAM-1 expression by cisplatin and 5-fluorouracil in a cancer cell line via a NF-kappaB independent pathway." British Journal of Cancer **80**:954-63.

Tarnow-Mordi, W. O., P. Sutton and A. R. Wilkinson (1986). "Inadequate humidification of respiratory gases during mechanical ventilation of the newborn." Archives of Disease in Childhood **61**:698-700.

Thibeault, D. W., S. M. Mabry, Ekekezie, II and W. E. Truog (2000). "Lung elastic tissue maturation and perturbations during the evolution of chronic lung disease." Pediatrics **106**:1452-9.

Thomassen, M. J., L. T. Divis and C. J. Fisher (1996). "Regulation of human alveolar macrophage inflammatory cytokine production by interleukin-10." Clinical Immunology & Immunopathology **80**:321-4.

Tolnay, E., C. Kuhnen, B. Voss, T. Wiethege and K. M. Muller (1998). "Expression and localization of vascular endothelial growth factor and its receptor flt in pulmonary sarcoidosis." Virchows Archiv **432**:61-5.

Tosi, M. F., J. M. Stark, A. Hamedani, C. W. Smith, D. C. Gruenert and Y. T. Huang (1992). "Intercellular adhesion molecule-1 (ICAM-1)-dependent and ICAM-1-independent adhesive interactions between polymorphonuclear leukocytes and human airway epithelial cells infected with parainfluenza virus type 2." Journal of Immunology **149**:3345-9.

Tullus, K., G. W. Noack, L. G. Burman, R. Nilsson, B. Wretlind and A. Brauner (1996). "Elevated cytokine levels in tracheobronchial aspirate fluids from ventilator treated neonates with bronchopulmonary dysplasia." European Journal of Pediatrics **155**:112-6.

Van den Veyver, I. B., J. Ni, N. Bowles, R. J. Carpenter, Jr., C. P. Weiner, J. Yankowitz, K. J. Moise, Jr., J. Henderson and J. A. Towbin (1998). "Detection of intrauterine viral infection using the polymerase chain reaction." Molecular and Genetics Metabolism **63**:85-95.

van Der Flier, M., F. Coenjaerts, J. L. Kimpen, A. M. Hoepelman and S. P. Geelen (2000). "Streptococcus pneumoniae induces secretion of vascular endothelial growth factor by human neutrophils." Infection & Immunity **68**:4792-4.

van Waarde, W. M., F. Brus, A. Okken and J. L. Kimpen (1997). "Ureaplasma urealyticum colonization, prematurity and bronchopulmonary dysplasia." European Respiratory Journal **10**:886-90.

Veikkola, T. and K. Alitalo (1999). "VEGFs, receptors and angiogenesis." Seminars in Cancer Biology **9**:211-20.

Vyas, J. R., A. E. Currie, D. E. Shuker, D. J. Field and S. Kotecha (1999). "Concentration of nitric oxide products in bronchoalveolar fluid obtained from infants who develop chronic lung disease of prematurity." Archives of Disease in Childhood. Fetal and Neonatal Edition **81**:F217-20.

Waites, K. B., D. T. Crouse and G. H. Cassell (1993). "Systemic neonatal infection due to Ureaplasma urealyticum." Clinical Infectious Diseases **17**:S131-5.

Walsh, W. F., J. Butler, J. Coalson, D. Hensley, G. H. Cassell and R. A. deLemos (1993). "A primate model of Ureaplasma urealyticum infection in the premature infant with hyaline membrane disease." Clinical Infectious Diseases **17**:S158-62.

Walsh, W. F., S. Stanley, K. P. Lally, R. E. Stribley, D. P. Treece, F. McCleskey and D. M. Null (1991). "Ureaplasma urealyticum demonstrated by open lung biopsy in newborns with chronic lung disease." Pediatric Infectious Disease Journal **10**:823-7.

Walti, H., C. Tordet, L. Gerbaut, P. Saugier, G. Moriette and J. P. Relier (1989). "Persistent elastase/proteinase inhibitor imbalance during prolonged ventilation of infants with bronchopulmonary dysplasia: evidence for the role of nosocomial infections." Pediatric Research **26**:351-5.

Wang, E. E., H. Frayha, J. Watts, O. Hammerberg, M. A. Chernesky, J. B. Mahony and G. H. Cassell (1988). "Role of Ureaplasma urealyticum and other pathogens in the development of chronic lung disease of prematurity." Pediatric Infectious Disease Journal **7**:547-51.

Wang, E. E., A. Ohlsson and J. D. Kellner (1995). "Association of Ureaplasma urealyticum colonization with chronic lung disease of prematurity: results of a metaanalysis." Journal of Pediatrics **127**:640-4.

Watterberg, K. L., L. M. Demers, S. M. Scott and S. Murphy (1996). "Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops." Pediatrics **97**:210-5.

Watterberg, K. L., J. S. Gerdes and K. L. Cook (2001). "Impaired glucocorticoid synthesis in premature infants developing chronic lung disease." Pediatric Research **50**:190-5.

Weinberg, J. B. (1998). "Nitric oxide production and nitric oxide synthase type 2 expression by human mononuclear phagocytes: a review." Molecular Medicine **4**:557-91.

Westergren-Thorsson, G., J. Hernnas, B. Sarnstrand, A. Oldberg, D. Heinegard and A. Malmstrom (1993). "Altered expression of small proteoglycans, collagen, and transforming growth factor-beta 1 in developing bleomycin-induced pulmonary fibrosis in rats." Journal of Clinical Investigation **92**:632-7.

Wheeler, M. A., S. D. Smith, G. Garcia-Cardena, C. F. Nathan, R. M. Weiss and W. C. Sessa (1997). "Bacterial infection induces nitric oxide synthase in human neutrophils." Journal of Clinical Investigation **99**:110-6.

Williams, C. M. and J. W. Coleman (1995). "Induced expression of mRNA for IL-5, IL-6, TNF-alpha, MIP-2 and IFN-gamma in immunologically activated rat peritoneal mast cells: inhibition by dexamethasone and cyclosporin A." Immunology **86**:244-9.

Xie, Q. W., H. J. Cho, J. Calaycay, R. A. Mumford, K. M. Swiderek, T. D. Lee, A. Ding, T. Troso and C. Nathan (1992). "Cloning and characterization of inducible nitric oxide synthase from mouse macrophages." Science **256**:225-8.

Xie, Q. W., Y. Kashiwabara and C. Nathan (1994). "Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase." Journal of Biological Chemistry **269**:4705-8.

Yamamoto, T., N. A. Noble, D. E. Miller and W. A. Border (1994). "Sustained expression of TGF-beta 1 underlies development of progressive kidney fibrosis." Kidney International **45**:916-27.

Yan, Z., A. Sirsjo, M. L. Bochaton-Piallat, G. Gabbiani and G. K. Hansson (1999). "Augmented expression of inducible NO synthase in vascular smooth muscle cells during aging is associated with enhanced NF-kappaB activation." Arteriosclerosis, Thrombosis, and Vascular Biology **19**:2854-62.

Yoder, M. C., Jr., R. Chua and R. Tepper (1991). "Effect of dexamethasone on pulmonary inflammation and pulmonary function of ventilator-dependent infants with bronchopulmonary dysplasia." American Review of Respiratory Disease **143**:1044-8.

Papers I-V