Modulation of lung inflammation of preterm ventilated infants: role of IL-6 *trans*-signalling and IL-8 isoforms

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Dedication

This work is dedicated to my dad, who sadly passed away before it was finished, and my mum, both of who have been pivotal in me reaching where I am today.
I also dedicate this work to my daughter Diya and my wife Shikha, who have been very understanding at hard times and have been patient with me.

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Summary

Chronic lung disease (CLD) is a common respiratory sequalae of infants born extremely premature (< 32 weeks gestational age). A persistent and poorly-resolving neutrophilic lung inflammation has been strongly implicated in the development of CLD. Pathways of resolution of lung inflammation have been poorly characterised in preterm infants.

Interleukin-8 (IL-8) is an key neutrophil chemokine implicated in the pathogenesis of CLD. Longer, and less functionally potent, isoforms of IL-8 predominate the preterm circulation but their expression and function in the preterm lungs is not known. The complex of interleukin-6 (IL-6), along with the soluble IL-6 receptor (sIL-6R), initiates IL-6 *trans*-signalling which experimentally has demonstrated downregulation of IL-8 during acute inflammation. Expression of IL-6 *trans*-signalling cytokines and their functional activity in the preterm lungs have not been studied.

My work shows that the concentration of sgp130, a specific inhibitor of IL-6 *trans*-signalling, was significantly increased in the bronchoalveolar lavage fluid (BALF) of preterm ventilated baboons, and human infants developing CLD later, compared to infants who did not. Although total IL-6 activity was detected in a specific functional assay, IL-6 *trans*-signalling activity could not be detected from the BALF samples or by using a complex from recombinant cytokines.

I have shown that the long isoform of IL-8, IL-8_{77} was a minor proportion of total IL-8 in the preterm ventilated BALF, although significant expression of IL-8_{77} was detected *in vitro* from lung cells. Preterm BALF efficiently converted exogenously added recombinant IL-8_{77} to shorter isoforms, mainly by the activity of serine proteases from neutrophils and the clotting cascade. BALF from CLD infants converted significantly more IL-8_{77}, compared to BALF from No-CLD infants. Among the neutrophil serine proteases, proteinase-3 (Pr-3) converted IL-8_{77} to functionally more potent isoforms; Pr-3 antigen and thrombin activity was also significantly increased in BALF from CLD infants, making them attractive targets for intervention.

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Submitted

Serine Proteases Regulate Interleukin-8_{77} Function in the development of Bronchopulmonary Dysplasia in Preterm Ventilated Infants

Chakraborty M, McGreal EP, Williams A, Davies PL, Powell W, Abdulla S, Voitenok NN, Hogwood J, Gray E, Spiller OB, Chambers RC, Kotecha S.

Abbreviations

2ME	2-mercaptoethanol
AA	Amino acid
AAT	Alpha-1 antitrypsin
ATIII	Antithrombin III
BALF	Broncho alveolar lavage fluid
BPD	Bronchopulmonary dysplasia
BSA	Bovine serum albumin
BSF-2	B-cell stimulatory factor-2
CBD	Cytokine binding domain
CF	Cystic fibrosis
CG	Cathepsin-G
CLC	cardiotrophin like cytokine
CLD	Chronic lung disease
CNTF	ciliary neurotrophic factor
COPD	Chronic obstructive pulmonary disease
COPD	Chronic obstructive pulmonary disease
CPAP	Continuous positive airways pressure
CRP	C-reactive protein
CT-1	cardiotrophin-1
CTGF	Connective tissue growth factor
CTGF	Connective tissue growth factor
D	Dalton
DNA	Deoxyribonucleic acid
DS sIL-6R	Differential spliced sIL-6R
E.coli	*Escherichia coli*
ELISA	Enzyme linked immuno sorbent assay
EMAP II	Endothelial monocyte-activating polypeptide II
ENA-78	Epithelial cell-derived neutrophil activating peptide-78
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor

FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
fMLP	N-Formyl-L-methionyl-L-leucyl-L-phenylalanine
FNIII	Fibronectin type-III
FRC	Functional residual capacity
GCP	Granulocyte chemotactic protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GRO	Growth regulated oncogene
GWAS	genome wide association study
H IL-6	Hyper IL-6
HFOV	High frequency oscillatory ventilation
HGF	Hybridoma growth factor
HRPO	Horse radish peroxidase
HSF	Hepatocyte stimulating factor
ICAM	Inter-cellular adhesion molecule
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
IL-8	Interleukin-8
Jak	Janus activated kinase
JNK	c-Jun N-terminal kinase
LFA-1	Lymphocyte function-associated antigen-1
LIF	leukaemia inhibitory factor
LPC	Lipophosphatidyl choline
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MAPK	Mitogen activated protein kinase
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein

mg	milligram
MIP	Macrophage inflammatory protein
ml	millilitre
MTS	[3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
N	Normal
NaCl	Sodium chloride
NE	Human neutrophil elastase
NEC	Necrotising enterocolitis
NF-κB	Nuclear factor-κB
ng	nanogram
NICHD	National Institute of Child Health and Human Development
NK	Natural killer (cells)
nm	nanometre
ONS	Office for National Statistics
OSM	oncostatic-M
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Patent ductus arteriosus
PDGF-A	Platelet derived growth factor-A
PECAM-1	Platelet endothelial cell adhesion molecule-1
PEEP	Positive end expiratory pressure
PES	Phenazine ethosulfate
pg	picogram
PHA	Phytohaemagglutinin
PI3K	Phosphoinositide 3-kinase
PIAS	Protein inhibitor of activated STAT
PICD	Phagocytosis-induced cell death
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear neutrophils

PMSF	Phenylmethanesulfonyl fluoride
Pr-3	Proteinase 3
PS	Phosphatidyl serine
RA	Retinoic acid
RDS	Respiratory distress syndrome
rh	recombinant human
RIA	Radio immuno assay
rm	recombinant mouse
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
sIL-6R	Soluble IL-6R
siRNA	short interfering ribo nucleic acid
SNP	single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TAF	Tracheal aspirate fluid
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TMB	Tetramethyl benzidine
TNF-α	Tumour necrosis factor-α
Tyk	Tyrosine kinase
Tyr	Tyrosine
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VEGFR	Vascular endothelial growth factor receptor
VLBW	Very low birth weight
µg	microgram
µl	microlitre

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1. Introduction

1.1. Introduction

Preterm infants, born before 37 completed weeks of gestation, are about 7-10% in number of all live births in the UK (Office for National Statistics, ONS). Of all live births, about 1-2% are born before 32 completed weeks of gestation (ONS). This group of newborn infants (≤ 32 weeks gestational age) require significant support in the first few weeks to months after birth, resulting is substantial cost to the health care services (Mangham et al., 2009). National epidemiological studies, on infants born at < 27 weeks gestational age, suggest that survival of this group of infants is increasing in the UK, and a large percentage of them continue to require significant resources due to complications of preterm birth (Costeloe et al., 2012). This adds to the health care burden of society.

The most common insufficiency, and long term complication, of preterm infants is that of the respiratory system. In this chapter, I will discuss the most common respiratory complication of preterm infants, chronic lung disease (CLD). I will start with a discussion on the definitions of CLD, and the difficulties and controversies surrounding it. Then I will proceed to provide an overview of the risk factors for CLD, as per current understanding, and argue that non-resolving inflammation of the preterm lungs is the final common pathway involved in the pathogenesis of this condition. My research is on two pathways of modulation of inflammation, and I will introduce these pathways in the following sections. I will end the chapter with the hypothesis for this project, along with detailed aims of the research work that I have carried out.

1.2. Definitions of Bronchopulmonary Dysplasia / Chronic Lung Disease

Preterm infants born at early gestations (≤32 weeks) are surviving in increasing numbers (Fanaroff et al., 2007). This is most evident in the group of infants born with extremely low birth weight (≤1000g) or gestation of less than 28 weeks (Costeloe et al., 2012). Respiratory insufficiency, characterised by Respiratory Distress Syndrome (RDS) – an inflammatory condition in the lungs of preterm infants contributed by inadequate surfactant production resulting in respiratory failure with small volume lungs and

ground-glass appearance in chest radiographs – is the most common challenge faced by these infants. Chronic lung disease (CLD) or bronchopulmonary dysplasia (BPD) is the term used to describe an evolving condition of the lung in infants, the majority of who are born preterm (mainly ≤32 weeks gestation). CLD results in pulmonary insufficiency requiring supplemental oxygen or respiratory support, and with long-term respiratory sequelae (Kotecha and Silverman, 1999). CLD and BPD are commonly used interchangeably, with CLD describing the clinical picture and BPD describing the pathology of the condition.

The clinical definition of CLD has been evolving since the first description of BPD in 1967 by Northway and his colleagues (Northway et al., 1967). Their retrospective case series described four stages of the disease from birth: stage I from two to three days, stage II from four to ten days, stage III from ten to twenty days and stage IV beyond one month after birth. Of note, the infants described in this study were more mature than the current population of preterm infants who are at maximum risk for CLD.

In 1979 Bancalari and colleagues (Bancalari et al., 1979) suggested that the term BPD be used to describe the more advanced stages of the disease, i.e. after 28 days (stage IV in Northway and colleagues' original description), as they were unable to clinically and radiologically distinguish the earlier stages (stages I, II and III) from severe RDS. Also, some of their patients seemed to develop stage IV of the disease without apparently going through the earlier stages. The term "chronic lung disease" was also coined in the same year by Tooley who suggested it be used for preterm infants after 30 days of life with radiological lung abnormalities, respiratory failure and supplemental oxygen requirement (Tooley, 1979).

In a retrospective study of infants born preterm who were followed up for at least two years, Shennan and colleagues (Shennan et al., 1988) concluded that oxygen requirement at 36 weeks corrected gestational age was a better predictor of long term pulmonary outcome, especially in infants born extremely prematurely. They suggested that the 28 day criterion be used for more mature infants born at ≥ 30 weeks gestational age as in the population of Northway's (Northway et al., 1967) original study. For more preterm infants (< 30 weeks gestational age at birth), the modified

criteria seemed to be a better predictor of long-term morbidity. Shennan's study was conducted on infants whose mothers did not receive antenatal corticosteroids to mature the lungs, and the infants did not have exogenous surfactant replacement after birth (these are discussed in later sections). Thus, it may be argued that the current population of preterm infants, most of who have the benefit of antenatal corticosteroids and postnatal surfactant therapy, are not comparable to Shennan's cohort. More recent long-term follow up studies of preterm infants have attempted answer this question. The 36-week corrected gestational age cut-off point was revalidated in the Trial of Indomethacin Prophylaxis in Preterms (TIPP) cohort of infants, where the authors concluded that oxygen requirement at any other time-point (from 32 weeks to 40 weeks corrected gestational age) was not superior as a predictor of CLD, compared to 36 week time-point; indeed, supplemental oxygen requirement at 36 weeks corrected gestational age was found to be an accurate predictor of long term pulmonary and neurodevelopmental outcome in their cohort of patients (Davis et al., 2002). Thus, the definition proposed by Shennan *et al* seemed to be appropriate in preterm infants born in the pre-natal steroid and surfactant era (after early to mid 1990s).

The current population of infants who are at high risk for BPD are born at extremely preterm (< 28weeks gestational age) with very low birth-weight (Coalson, 2003). There are also significant differences in the lung pathology of these infants (Coalson, 2003, Husain et al., 1998) from the ones described in the original description of Northway. They seem to have fewer but larger alveoli (alveolar simplification) and a sparse capillary network (Coalson, 2003). The term "new BPD" was coined by Jobe (Jobe, 1999) to reflect the current pathology.

A modification of the definition of BPD, stratified by gestational age at birth, was proposed in 2001 by the NIH Workshop on BPD (Jobe and Bancalari, 2001b). The authors graded the condition into mild, moderate and severe CLD depending on oxygen requirement, and stratified by gestational age (< 32 or ≥ 32 weeks gestational age at birth). This classification was found to be a valid predictor of adverse long-term pulmonary outcome as the severity of the disease increased (Ehrenkranz et al., 2005). Importantly, this definition acknowledged the group of patients who did not need

supplemental oxygen at 36 weeks corrected gestational age but had residual lung disease (e.g. needing continuous positive airway pressure, or CPAP, in air). One of the important reasons for proposing a unified definition was to allow for comparisons to be made between different units and centres.

A “room air challenge test”, to confirm supplemental oxygen requirement at 36 weeks corrected gestational age, was proposed by Walsh and colleagues (Walsh et al., 2003). Application of this test in a prospective trial resulted in reduction of calculated BPD rates (Walsh et al., 2003, Walsh et al., 2004) when compared to the traditional clinical definitions. It also seemed valid in predicting long-term pulmonary outcomes. The Bronchopulmonary Dysplasia Group, in their summary proceedings in 2006 (Walsh et al., 2006), proposed using the severity based definition (Jobe and Bancalari, 2001b) and augmenting it with the physiologic test to confirm oxygen requirements (Walsh et al., 2003).

In summary, definition of CLD has changed several times to describe the evolving condition more accurately. Currently, respiratory support including supplemental oxygen requirement at 36 weeks corrected gestational age, has become the most common definition used for the condition. This is also the definition most commonly used by researchers to describe the condition, and is used to define CLD in this thesis.

1.3. Epidemiology

As the definition of CLD has evolved, so has the reported incidence of the condition. In the original description of BPD by Northway (Northway et al., 1967), the authors reported a 100% rate of pulmonary disease beyond four weeks of age in survivors. In his report of 14 years of retrospective data from 1969 to 1978, Tooley (Tooley, 1979) published an incidence of 25-40% of BPD in survivors in babies born with a birth weight between 750 and 1500gm. In their review of BPD in 1985, O’Brodovich and Mellins (O'Brodovich and Mellins, 1985) remark that there were no prospective trials until that date which set out to calculate the incidence of BPD. From retrospective studies, the reported range was from 2% to 68% and the incidence

seemed to increase with decreasing birth weight. It needs to be emphasised that both of these reviews are selective, and probably did not represent the true scale of the problem at that time.

In the current population of infants, the incidence rates of CLD vary greatly between centres and regions, but can affect up to 25% of infants born at < 32 weeks gestational age (Gortner et al., 2011) to more than 40% of infants born before 28 weeks gestational age (Stoll et al., 2010). UK data from the Trent and Yorkshire regions (The Neonatal Survey, 2008) report an incidence of CLD (supplemental oxygen at 36 weeks corrected gestational age) of up to 30% in preterm infants ≤ 32 weeks who have received mechanical ventilation. Due to differences in the definition of CLD, the reported incidence rates have varied as well (Fenton et al., 1996, Manktelow et al., 2001, Oh et al., 2005). In addition, the changing pathology of CLD (Husain et al., 1998, Willet et al., 1999) brings into question the validity and applicability of either of the traditional definitions in the current population of preterm infants. Implementing modified definitions (Jobe and Bancalari, 2001b, Walsh et al., 2003) have resulted in decreasing the calculated rates of CLD in the same population of preterm infants from 35% (using oxygen or respiratory support at 36 weeks) to 25% (using the physiologic room-air test) (Walsh et al., 2004). However, if the 36-week corrected gestational age definition is used purely as a predictor of long-term outcome (as in the original study by Shennan *et al*), several studies in the current population of preterm infants have shown its limited applicability (Lefkowitz and Rosenberg, 2008).

Recent reviews suggest that the incidence of CLD may be decreasing. In a large review of all neonatal admissions nationwide (USA) over a 14-year period (1993 – 2006), a steady and statistically significant decline in the incidence of CLD was noted over the study period (Stroustrup and Trasande, 2010). This was accompanied by a decline in the use of invasive ventilation and an increase in the use of non-invasive respiratory support (Figure 1.1). A similar study from Spain, involving infants born between 1997 – 2009, concluded that there was a significant increase in survival of infants (birth-weight < 1000 g) without significant CLD (Botet et al., 2012). Factors reported by the authors to be associated with this outcome included a reduction in

endo-tracheal intubation rate, a reduction in duration of mechanical ventilation and (surprisingly) delivery by Caesarean section.

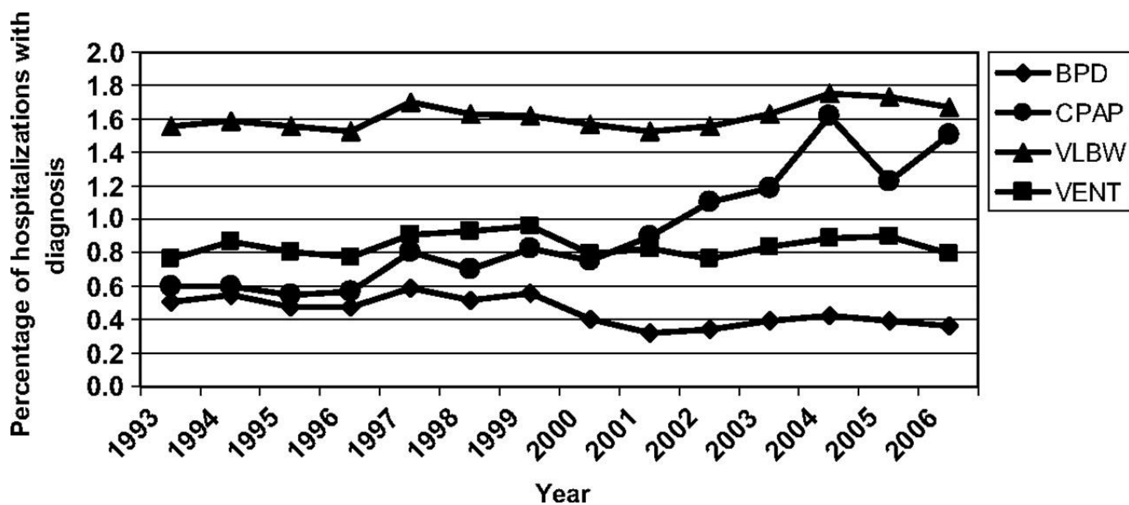


Figure 1-1: Change in Incidence of BPD. Percentage change in the annual incidence of BPD and related management of preterm infants in neonatal units in the USA from 1993 to 2006. With permission from (Stroustrup and Trasande, 2010).

Neurodevelopmental outcome and respiratory morbidity of infants born prematurely is of primary concern for neonatologists. CLD has been shown to be a risk factor for adverse respiratory outcome (Greenough, 2012, Greenough, 2008) with prolonged oxygen requirements, persistent respiratory symptoms leading to repeated hospitalisations and abnormalities in lung function tests. Long term follow-up studies have concluded that severe CLD is an independent risk factor for neurological deficits in preterm infants (Majnemer et al., 2000). The incidence of moderate to severe cerebral palsy (CP), mental development index (MDI) <70, psychomotor development index (PDI) <70 and neurodevelopmental impairment (NDI) are all increased in infants with CLD (Doyle and Anderson, 2009). It needs to be remembered, however, that long-term follow-up studies as above are from cohorts of preterm infants who were born a few decades earlier, and clinical care for preterm infants has changed since then. Thus, application of such studies to the current population of babies may not be entirely appropriate and new cohort studies need to be conducted for data that are more relevant.

A recent systematic review of respiratory morbidity in adult survivors of CLD concluded that impairment of respiratory function (defined variously) continues into

adulthood (Gough et al., 2012). In a meta-analysis, children who were born preterm were found to have significantly impaired lung function, when compared to control infants born at term (Kotecha et al., 2013). However, there is a lack of data on non-pulmonary outcomes of CLD survivors into adulthood. Obvious caveats of such a conclusion are heterogeneity between the different studies. Also, a large number of survivors included in them were born when antenatal corticosteroids and postnatal surfactant replacement were not routine. It has also been suggested that CLD/BPD could be the precursor of adult chronic obstructive pulmonary disease (COPD) (Baraldi and Filippone, 2007). Studies which have followed a current (representative) cohort of preterm infants into adulthood are awaited.

In summary, CLD is a problem mainly of infants born extremely preterm, leading to significant long-term respiratory morbidity and is associated with neurodevelopmental disorders, regardless of the definition used to describe CLD. Respiratory and neurodevelopmental outcomes are used extensively as end-points in research and clinical trials on neonates. Thus, understanding the causes and mechanisms of CLD forms the first steps towards attempts to treat or prevent it in the future.

1.4. Normal Lung Development

Extremely preterm infants, who are born at < 28 weeks gestational age, have lungs which are in the late canalicular or early saccular stages of development process (table 1.1). Further development of their airways and synthesis of surfactant is initiated at later gestations, while the process of alveolarisation and capillary maturation are primarily postnatal events in humans.

Stage	Duration	Key Events
Embryonic	3-7 weeks	Airway: Lung bud arises from ventral foregut endoderm Initiation of branching morphogenesis Bronchi formation Trachea and oesophagus separate Vessels: Pulmonary arteries bud off sixth pair of aortic arches Pulmonary veins develop as outgrowths of left atrium
Pseudoglandular	5-17 weeks	Airway: Acinar tubules and buds form in peripheral lung Ciliated, goblet, neuroepithelial, basal cells differentiate Cartilage, mucous glands, smooth muscle develop Pleuroperitoneal cavity closes Vessels: Pulmonary arteries parallel airway branching
Canalicular	16-26 weeks	Airway: Acinar tubules and buds expand Air-blood barrier forms Alveolar type I and type II cells differentiate Lamellar bodies form in type II cells Vessels: Capillary network form
Saccular	24-38 weeks	Airway: Air spaces expand to form saccules Mesenchyme thins and condenses Elastin deposited at sites of secondary crest formation Surfactant synthesised and secreted by type II cells Foetal lung fluid and foetal breathing Vessels: Septal walls contain double capillary network
Alveolar and microvascular maturation	36 weeks to 2nd postnatal year	Alveolar: Secondary septa form, subdividing saccules into alveoli Alveolar septal walls thin, with loss of connective tissue Alveolar surface area increases Collagen, elastin and fibronectin deposited Surfactant production increases in type II cells Vessels: Double capillary network fuses into single network

Table 1-1: Summary of stages of lung development. [Modified from (Wert, 2004, Snyder, 2004, Tuyl and Post, 2004, Roth-Kleiner and Post, 2003)]

1.5. Lung Pathology in CLD

In the original description of BPD (Northway et al., 1967), four stages of the disease was described. The pathology was characterised by alveolar atelectasis, necrosis of alveolar epithelium and thickening of capillary basement membrane in the early stages, progressing to areas of emphysematous over-inflation, severe airway changes including smooth-muscle hypertrophy, fibrosis and generalised thickening of capillary basement membranes. Vascular changes, indicative of pulmonary hypertension, were also noted. The infants included in these early descriptions were more mature than preterm infants seen now; they did not receive the benefits of antenatal steroids or postnatal surfactant replacement therapy. Clinical care of preterm infants has also changed significantly since then.

CLD is now seen mainly in infants born extremely preterm (< 28 weeks gestation with birth weights < 1000g) (Fanaroff et al., 2007) and the term “new” BPD has been coined to describe the lung condition in this population (Jobe, 1999). Significant differences have been noted in the lung pathology of the current population of preterm infants compared to those reported in early descriptions. Most of the severe changes observed in earlier cohorts were absent in the current population of infants (Coalson, 2006, Coalson, 2003). “New” BPD/CLD is characterised by milder airway changes with less fibrosis but abundance of large airspaces with decreased surface area, suggesting an interruption or dysregulated alveolarisation (Husain et al., 1998), and dysmorphic blood vessels (Figure 1.2). These changes were observed more diffusely all over the lung, suggesting that the use of surfactant keeps the airways open leading to milder but more widespread injury (Husain et al., 1998).

Studies describing lung pathology of CLD in the modern era are only few in number. As mortality in the current population of CLD infants is rare, this reduces the possibility of further similar histological studies. Moreover, infants with CLD who die likely represent the severe end of the spectrum, and their histology may not be representative of less severe CLD. Indirect studies using surrogate markers like pulmonary diffusion capacity have suggested that current infants with CLD may indeed have decreased alveolar surface area but not lung volume (Balinotti et al., 2010). In

spite of a lack of data, currently CLD is considered a disease of lung growth abnormality.

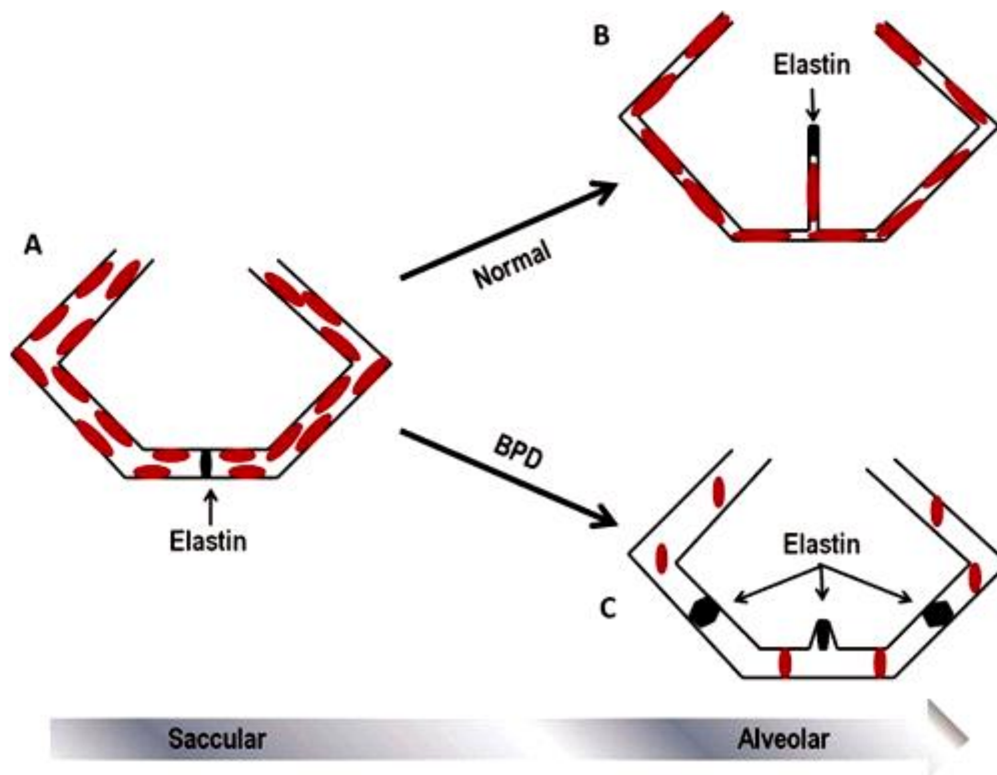


Figure 1-2: Lung Growth abnormalities in CLD. (A) In the saccular stage, the walls of the airspaces are thicker with interstitium and a double network of capillaries. (B) Normal alveolarisation results in thinning of the interstitium, development of a single layer of capillaries in the walls and elongation of secondary septa, which increase the surface area for gas exchange. (C) In CLD, the interstitium remains thick and the capillary network in underdeveloped and dysmorphic. The secondary septa are blunted, resulting in large and simplified alveoli. Reproduced with permission from (Ahlfeld and Conway, 2012)

1.6. Risk Factors for CLD

Northway and colleagues (Northway et al., 1967) suggested that possible mechanisms leading to CLD could include impaired pulmonary healing after severe RDS, oxygen toxicity during that process, mechanical ventilation and poor bronchial drainage due to intubation. Bancalari and colleagues (Bancalari et al., 1979) implicated

RDS with positive pressure ventilation along with the toxic effects of oxygen as important pathogenetic factors in the development of CLD. A review of CLD by O'Brodovich and Mellins (O'Brodovich and Mellins, 1985) suggested several mechanisms contributing to the development of lung injury including (a) quantitative and qualitative inadequacy of surfactant (b) pulmonary oedema and inflammation including release of neutrophil derived proteases and generation of toxic oxygen radicals (c) oxygen toxicity playing a role in the genesis of chronic lung injury, inhibiting normal lung development and promoting dysplasia and (d) assisted mechanical ventilation.

The pathology of BPD has undergone change since these descriptions with a shift from persistent inflammation, fibrosis and small airway disease ("old" BPD) to dilated distal airways and decreased alveolarisation ("new" BPD) (Jobe and Ikegami, 1998). Presented below is a short review of the current understanding about the main risk factors implicated in the development of CLD (Figure 1.3).

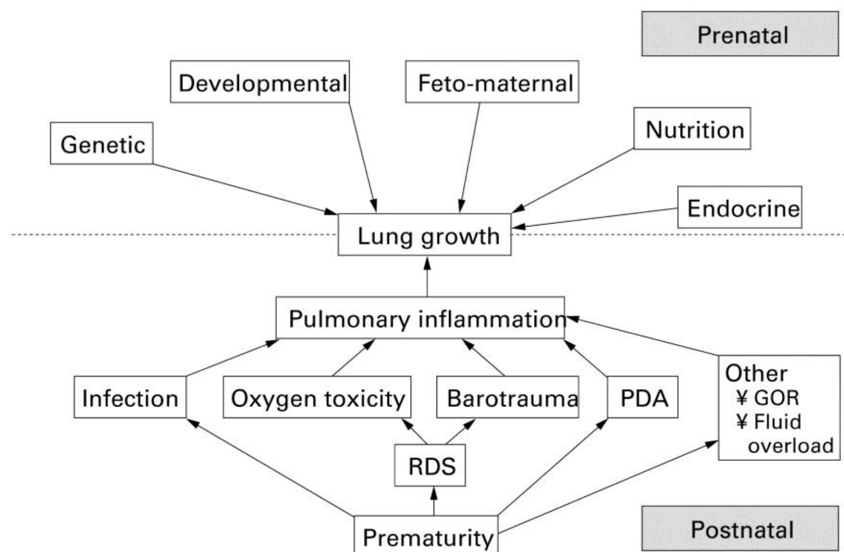


Figure 1-3: Factors affecting lung growth. Summary of factors which have an impact on lung growth ante-natally (top half), and post-natally after preterm delivery, that can contribute to the development of CLD. Reproduced with permission from (Kotecha, 2000).

1.6.1. Antenatal Factors

Infection

About 50 - 80% of preterm births are spontaneous in origin (Goldenberg et al., 2000) and infection/inflammation of the uterus has been widely suggested as the triggering cause. Intrauterine infections have been noted to increase in incidence with decreasing gestation (Goldenberg et al., 2000, Goncalves et al., 2002, Yoon et al., 2000). Bacteria causing infection in the uterus are most often of upper-vaginal origin and can lead to preterm labour in early gestations (Goldenberg et al., 2000, Goncalves et al., 2002). Bacteria act on decidua and foetal membranes through pattern recognition receptors (Kim et al., 2004b, Harju et al., 2005) leading to production of cytokines and chemokines. They can also stimulate production of prostaglandins. Leucocytes attracted to these sites of inflammation are activated and release proteases. Prostaglandin stimulates uterine contraction and proteases can rupture membranes (Athayde et al., 1998) leading to onset of preterm labour (Goldenberg et al., 2000). Ascending bacteria from the vagina can infect the decidua, membranes and the foetus (Figure 1.4) (Romero and Mazor, 1988) leading to infection resulting in a systemic inflammatory response syndrome (Athayde et al., 1998).

The association of intrauterine infection and neonatal morbidity have been the focus of several studies. The incidence of neonatal sepsis was found to be higher in neonates with evidence of foetal inflammation (Yoon et al., 2000). Studies have also shown the increased incidence of neurological and gastrointestinal morbidity in such neonates (Viscardi et al., 2004, Goepfert et al., 2004). Watterberg and colleagues (Watterberg et al., 1996) showed that foeti exposed to the effects of chorioamnionitis had a decreased incidence of RDS but an increased incidence of CLD with increased levels of inflammatory markers (interleukin-1β, thromboxane B2, leukotriene B4, prostaglandin E2) in the lung. Matsuda and colleagues (Matsuda et al., 1997) demonstrated positive association of necrotising funisitis (stage four in intrauterine infection sequence) with CLD. These two studies collectively suggested that inflammation initiated in the uterus can affect respiratory outcome in preterm infants.

Clinical trials in human neonates since Watterberg have confirmed the above association (Been et al., 2009). A case control study by Van Marter and colleagues (Van Marter et al., 2002) demonstrated the effect of postnatal factors that modify respiratory outcome in preterm infants who have been exposed to chorioamnionitis. They found intra-uterine infection to decrease the risk of BPD if babies were ventilated for less than 7 days postnatally. However, if the duration of ventilation was more than 7 days, or there was postnatal sepsis, then the risk increased. This suggests that post-natal modifying factors affect respiratory outcome started by inflammation in the uterus and introduced the concept of "multiple hits" on the lung having additive effects (Inatomi et al., 2012).

Subsequent observational studies on the effect of chorioamnionitis on respiratory morbidity after birth have not reported significant association (Laughon et al., 2009, Soraisham et al., 2009). Histologic chorioamnionitis of maternal side (by placental pathology) decreases odds ratio of RDS (Been et al., 2009, Kaukola et al., 2009); however, foetal involvement seemed to increase odds ratio for RDS (Been et al., 2009). No effect on the incidence of CLD was observed.

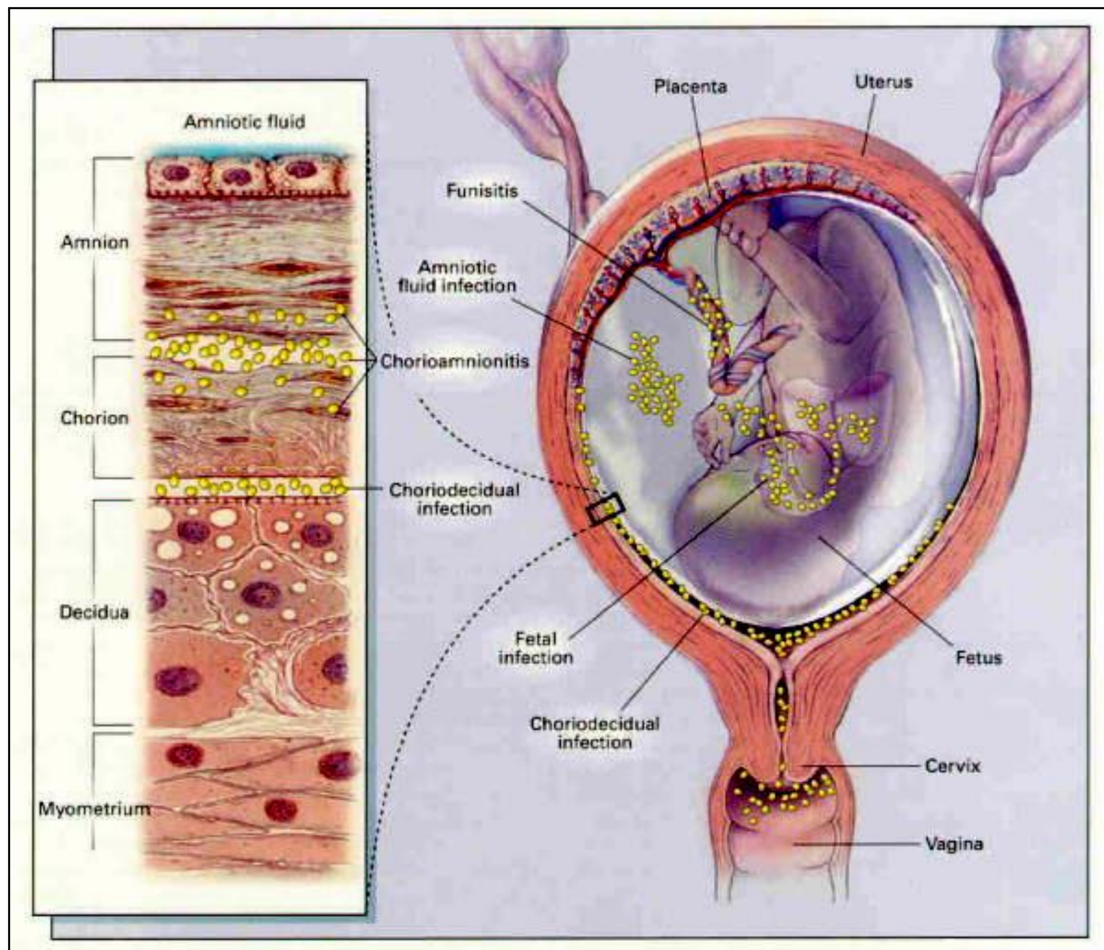


Figure 1-4: Potential sites of bacterial infection in the uterus. Reproduced with permission from (Goldenberg et al., 2000).

Animal models of intrauterine infection/inflammation have helped to understand the effects of intra-uterine inflammation. Introduction of *Ureaplasma urealyticum* into the amniotic fluid of pregnant mice induces lung inflammation in the newborn pups (Normann et al., 2009). Intra-amniotic injection of endotoxin in such a model resulted in a selective inflammatory response in the lung (Kramer et al., 2002, Willet et al., 2000). The overall effect has been to accelerate maturation of the existing lung structure but at the cost of simplification of the alveoli and growth arrest, the histological pattern that characterises the "new" BPD (Kramer, 2008, Kramer et al., 2009). An increased expression of transforming growth factor-β1 (TGF-β1), a cytokine involved in lung growth and remodelling, and suppression of connective tissue growth

factor (CTGF), which modulates fibrosis and promotes vascular development, highlights the potential link between inflammation and altered lung development (Kunzmann et al., 2007). Increased expression of cytokines which inhibit vascular development (interferon γ inducible protein 10, TGF-β1) and reduced expression of vascular markers (vascular endothelial growth factor [VEGF], vascular endothelial growth factor receptor-2 [VEGFR-2], platelet endothelial cell adhesion molecule-1 [PECAM-1]) support the model of inflammation-induced alteration of lung development (Kramer et al., 2009). In pregnant baboons, experimental introduction of intrauterine *Ureaplasma* results in lung inflammation in newborn preterm baboons. This inflammation resolves in baboons who clear the airway *Ureaplasma* but persists in the animals who are unable to clear the microbe (Afshar et al., 2003); TGF- β1 expression is increased in the lungs of such animals, indicating a pro-fibrotic response (Viscardi et al., 2006a).

Upper-vaginal bacteria are the commonest cause of intra-uterine infection. Mollicutes have been identified as the single most common class of bacteria infecting the uterus ranging from 6-12% in amniotic fluid in mid trimester collected for genetic tests (Kramer et al., 2009) to 23% from cord blood cultures of preterm neonates delivered between 23-32 weeks (Goldenberg et al., 2008). These bacteria can cause inflammation of the uterus and preterm labour occurs more commonly in women with a positive polymerase chain reaction (PCR) for bacterial genes (Schelonka and Waites, 2007). Mollicutes have also been implicated in neonatal respiratory morbidity such as pneumonia (Schelonka and Waites, 2007) and BPD (Schelonka and Waites, 2007, Maxwell et al., 2009, Colaizy et al., 2007).

In summary, intra-amniotic inflammation may decrease the incidence of RDS by maturing the lung. Its effect on CLD is debatable with conflicting data from studies. Early studies suggest the association of chorioamnionitis with arrest of lung-growth resulting in a BPD-like pathology. This was enhanced by post-natal injurious factors, such as ventilation and infection, which perpetuate inflammation. However, the exact mechanism of these effects has not yet been elucidated.

Glucocorticoids

As with intra-uterine infection which matures the preterm lung structure, glucocorticoids also have similar effects. The use of a single course of steroids to women with threatened preterm labour has been one of the most effective therapies so far to decrease respiratory morbidity in babies born extremely preterm (Roberts and Dalziel, 2006). Use of the drug has reduced the incidence of RDS significantly but has had little effect on CLD, probably due to increased survival of extremely premature infants who are most at risk of BPD (Crowley, 1995). The mechanism of action of glucocorticoids given antenatally seems to be brought about by the maturational effect of the drugs on the developing lung (Figure 1.5) (Bolt et al., 2001). The increase in the secretion of surfactant proteins and lipids seems to be the key effect which has decreased the incidence of RDS in preterm infants. However, similar to the effect of chorioamnionitis on the developing lung, it leads to global growth arrest and lung “simplification” (Bolt et al., 2001, Vyas and Kotecha, 1997). Studies conducted on animal models have confirmed the effect of a course of antenatal steroids on alveolar development (Jobe, 2003). This has been shown to be reversible, if preterm delivery is avoided.

Currently, the benefits of using steroids for lung maturation in preterm infants outweighs its negative effects on lung growth and development, and has been recommended in routine care.

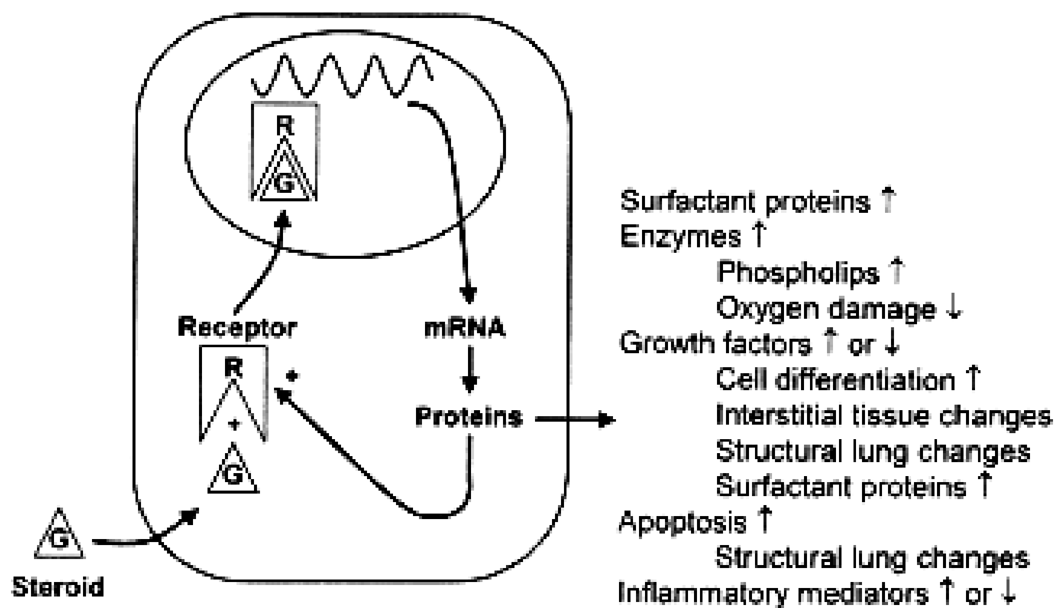


Figure 1-5: Possible effects of glucocorticoids on the developing lung in utero. Reproduced with permission from (Bolt et al., 2001)]

Genetic Factors

Complex diseases like diabetes, heart disease and atherosclerosis, to name a few, have been reported to be caused by an interaction of genetic and environmental factors (Bhandari et al., 2006). Recent studies on twin populations have suggested that genetic factors could affect the susceptibility to BPD (Lavoie et al., 2008, Bhandari et al., 2006, Parker et al., 1996). Several small studies have looked at gene polymorphisms and their effect on the outcome of BPD (Bhandari et al., 2006) with mixed results. Studies which have looked at difference in outcome between male and female babies showed that being female is advantageous especially after preterm birth (Ingemarsson, 2003).

The main approach in these studies has been to look at “candidate genes” which could affect the course or outcome of BPD. However, with the potential of modern genetic techniques using a genome-wide approach, possible associations of genes with BPD may become more apparent. Recently, such a study, looking at genome-wide associations with BPD, did not identify any single nucleotide polymorphisms associated

with BPD (at a pre-specified significance level). Additionally, the study could not replicate significance levels of some polymorphisms identified previously in the literature to be associated with BPD (Wang et al., 2013a). In contrast, other genome-wide association studies have found increased expression of mast-cell (Bhattacharya et al., 2012) and susceptibility (Hadchouel et al., 2011) genes in BPD.

The main pathology noted in the "new" CLD is a dysregulation of alveolar growth and development (Coalson, 2006). Several genetic signals work in close concert for alveolar development to take place. Thus, this suggests that preterm birth and subsequent events alter the genetic signalling pathways controlling alveolar development in infants who develop CLD. Currently, pulmonary inflammation is strongly implicated in the pathogenesis of CLD (Ryan et al., 2008). The link between inflammation and alteration of genetic signals needs further elucidation (Ahlfeld and Conway, 2012).

1.6.2. Post-Natal Causes

Ventilator Induced Lung Injury

Preterm infants often need assistance to initiate and establish breathing. The target is to establish a functional residual capacity in the lungs. Residual fluid present in the lungs at birth impedes this process. The deficiency of surfactant in these infants increases surface tension in the airspaces, which prevents them from being "opened" up at birth, and makes them more prone to collapse. Ventilation of such lungs causes shear stress and has been termed atelectrauma (Attar and Donn, 2002). On the pressure-volume hysteresis curve (Figure 1.6), this has been termed the "low-volume injury zone" (Jobe and Ikegami, 1998). The use of high tidal volumes during resuscitation to open up alveoli can cause damage leading to long-term sequelae (Bjorklund et al., 1997). This is termed volutrauma and occurs in the "high-volume injury zone" (Jobe and Ikegami, 1998). Preterm babies are more prone to volutrauma because of their compliant chest wall, which allows uncontrolled expansion. If chest-wall expansion is restricted, then lung injury can be limited even at high pressures (Hernandez et al., 1989). Similar stresses can occur during prolonged ventilation.

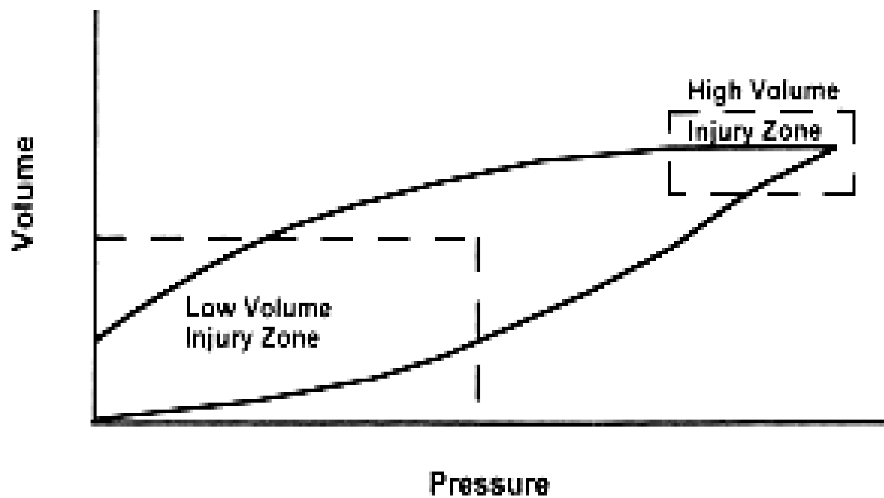


Figure 1-6: Pressure-volume curve showing low and high volume injury zones. Reproduced with permission from (Jobe and Ikegami, 1998)].

The use of animal models has been invaluable to study the effects of conventional ventilation on the lung. It is widely accepted that mechanical ventilation can actually worsen lung conditions, especially if they have been injured before (Dreyfuss and Saumon, 1998, Dreyfuss et al., 1995), by altering fluid balance, increasing endothelial and epithelial permeability and causing severe tissue damage. Pulmonary oedema produced by mechanical ventilation (Webb and Tierney, 1974) is thought to be mainly due to alterations in alveolar-capillary permeability resulting in increased efflux of solutes through the epithelium (Marks et al., 1985, Nolop et al., 1986) and increased epithelial pore radius (Egan et al., 1976). Changes in permeability have been confirmed by electron-microscopic studies of ventilated rabbits with discontinuities in alveolar type I cells (John et al., 1982) and alterations of endothelial and epithelial barriers (Dreyfuss et al., 1985). Increased pressures during mechanical ventilation were traditionally considered the cause of such changes (barotrauma). However, it has been shown that an increase in lung volume (volutrauma) due to the high pressures was the main factor causing damage (Dreyfuss et al., 1988). Repeated collapse of alveoli at the end of expiration and re-expansion at the beginning of inspiratory breaths (as happens the absence of surfactant) can cause severe structural

and functional damage (Taskar et al., 1997). The use of positive end expiratory pressure (PEEP) reduced oedema formation and cell damage (Dreyfuss et al., 1988).

Ventilation of preterm lungs causes pulmonary inflammation resulting in influx of leucocytes and production of mediators of inflammation (Chakraborty et al., 2010), that results in lung damage and is termed biotrauma (Tremblay and Slutsky, 1998). Studies on rabbits and rats have shown that the use of PEEP can help to reduce inflammatory cell recruitment into the lungs during prolonged ventilation (Markos et al., 1993). Similar benefits were seen with high frequency oscillatory ventilation (HFOV) (Matsuoka et al., 1994). High volume ventilation with no PEEP resulted in increased cytokine concentrations in the lung (Tremblay et al., 1997). Similar patterns of cytokine expression have been observed in human preterm infants after ventilation, with higher concentrations observed in those who develop CLD later than in those who resolve their inflammation (Kotecha et al., 1995, Munshi et al., 1997). This suggests that cytokines and chemokines, along with the inflammatory cells they attract, play a role in lung injury and damage. Indeed, ventilation at physiological settings for a short period of time has been shown to be able to produce changes in lung morphology in foetal sheep that resemble CLD (Allison et al., 2008).

Clinicians have realised that mechanical ventilation of preterm infants through an endo-tracheal tube has adverse effects on the lungs mediated by multiple factors, including ventilation itself (by mechanisms as above) and infection, which adds to lung inflammation. Prolonged ventilation along with post-natal infection, in preterm infants born on a background of chorioamnionitis has been shown to make them more prone to develop CLD (Van Marter et al., 2002). Several non-invasive ventilatory strategies, using continuous positive airways pressure (CPAP), have had encouraging results on respiratory outcomes of these preterm infants (Jobe, 2011).

Surfactant Deficiency

Surfactant is a lipid-rich complex which is produced in the lung by type II alveolar epithelial cells. It lowers surface-tension in the alveoli and thus make them less likely to collapse at the end of expiration. In newborn babies, this is of immense importance

as it allows them to establish an effective functional residual capacity (FRC) and helps to transform a fluid filled space to an air-filled one. Attempts to ventilate a surfactant deficient lung, resulting in repeated opening and closing of the airspace, causes severe functional impairment of the lung as well as histological damage with formation of hyaline membrane (Taskar et al., 1997). Use of positive end expiratory pressure (PEEP) prevents collapse of the surface film of surfactant as well as loss of surfactant into the airways (Dreyfuss and Saumon, 1998), thus preserving its function.

Preterm infants who are at high risk of lung damage (≤ 28 weeks gestation) produce inadequate quantities of surfactant due to an immature stage of lung development. This has been identified as the primary cause of RDS (also sometimes known as surfactant deficiency lung disease), and provides the rationale for exogenous replacement of surfactant. Since the mid 1980s, several well designed clinical trials and their recent meta-analysis have established the effectiveness of this therapy in neonatology (Sweet and Halliday, 2009). Evidence supports the early use of surfactant in babies with or at risk of RDS with extubation to CPAP rather than a selective later administration of surfactant (Stevens et al., 2007). This approach results in the decreased need for mechanical ventilation later, decreased pulmonary air-leaks and the decreased need for supplementary oxygen at 28 days. However, no change in incidence of CLD (at 36 weeks corrected gestational age) was reported, suggesting that long-term lung damage occurred with either strategy. When used as a prophylactic measure in infants at risk of RDS, there was a decreased incidence of air leaks and mortality was lowered, compared to rescue therapy with surfactant (Soll and Morley, 2001). However lung damage, as evidenced from the incidence of oxygen requirement at 28 days, remained unaffected.

In summary, replacement of surfactant has resulted in significant short-term benefits for preterm infants with RDS. However, its use has not been able to prevent long-term lung injury in this population of babies. It is likely that this is due to the increased survival of a population of extremely preterm infants who are at maximum risk of developing CLD. The effects of surfactant on survival seem to be more than its effect on preventing lung-injury in small preterm babies. Though RDS and its related treatment strategies (mechanical ventilation and oxygen) are risk factors for lung

damage, other factors which contribute to it (see sections 1.6.2.1-.5) may be equally important in causing injury. Thus, prevention or effective treatment of RDS with exogenous surfactant has not been able to prevent lung damage completely.

Oxygen Toxicity

The use of oxygen in neonatology has a long history, as reviewed by Sola *et al* (Sola et al., 2007). In their report from the NICHD workshop in 2005, the authors noted that oxygen was administered to premature infants as a stimulant in the late 19th century (Higgins et al., 2007), and administration of intragastric oxygen was practiced in the first half of the 20th century (Sola et al., 2007). In the initial description of BPD in 1967 (Northway et al., 1967), the authors proposed that oxygen-induced damage to the lung was one of the key causative factors in the pathogenesis of the disease. Gerschman and colleagues (Gerschman et al., 1954) suggested that the injurious products of oxygen are the reactive oxygen species (ROS). These compounds can interact with and alter cell components including protein, lipid, carbohydrates and DNA (Saugstad, 2003). Uncoupling of respiration from ATP synthesis in the mitochondria by free-radicals can lead to cell death (Ciencewicki et al., 2008).

The body contains both extracellular and intracellular defences against ROS. A number of substances including vitamin C, urate and bilirubin make up the extracellular defences. The major intracellular antioxidant is glutathione. These systems maintain a balance between pro- and anti-oxidant effects in the body. The term oxidative stress is used to describe the state when the anti-oxidant effects become ineffective or overwhelmed leading to local tissue damage.

Preterm infants are at increased risk of oxidative stress for several reasons. They are often exposed to high concentrations of oxygen as part of their respiratory support. The normal defences against oxidant stress in preterm babies are also less efficient. Vitamin C levels decline in the first few days of life and intracellular defences undergo developmental changes in the later part of pregnancy (Saugstad, 2003). Preterm animals are unable to efficiently upregulate their anti-oxidant enzymes like superoxide dismutase, catalase and glutathione peroxidise making them susceptible to oxidative stress (Frank and Sosenko, 1991).

Evidence has accumulated over the last few years for the role of oxidative stress in CLD. Markers of peroxidation have been found in tracheal lavage fluid and urine of babies who went on to develop BPD (Saugstad, 2003). Lipid peroxidation (Pitkanen et al., 1990, Inder et al., 1994) and surfactant oxidation have been implicated in BPD. ROS have the ability to oxidise proteins (Saugstad, 2003) which has been detected at higher concentrations in infants who develop BPD (Inder et al., 1994, Gladstone and Levine, 1994, Varsila et al., 1995). Animal studies seem to show that hyperoxia may be the most important trigger for the pathologic changes seen in BPD and it possibly potentiates the effects of other contributory factors (Weinberger et al., 2002).

In summary, oxygen is potentially toxic to cells due to its free radicals. It has pro-inflammatory effects in the lung and this has been associated with the development of CLD in preterm infants, who are at increased risk of oxidative stress and have limited antioxidant mechanisms.

PDA and Fluids

The majority of body water in very low birth weight (VLBW) infants is contained in the extracellular fluid compartment (Friis-Hansen, 1957, Friis-Hansen, 1961). Excessive extracellular fluid volume can lead to increased interstitial fluid in the lung causing pulmonary oedema, which can interfere with gas exchange necessitating respiratory support. Excessive fluid can also lead to persistent patency of the ductus arteriosus with similar implications due to increased pulmonary blood flow.

Several studies have looked at respiratory outcomes in these small babies in relation to their fluid intake in the first few days of life with mixed results. Van Marter and colleagues (Van Marter et al., 1990) found that infants who later developed CLD had consistently higher fluid intake (crystalloids, colloids and total) in the first few days of life which resulted in a net weight gain compared to babies who did not develop CLD who had lower intakes and a net weight loss. Tammela and Koivisto (Tammela and Koivisto, 1992) randomised babies to a “dry” group with fluid restriction between 20%-47% in the first week and 25% thereafter compared to a control group with normal fluid regimen as practiced in the hospital. No significant difference was found in the number of babies with CLD in either group. However, babies in this study were more

mature (mean gestational age 31 weeks) than the population of infants who develop CLD nowadays.

More recently, Marshall and colleagues (Marshall et al., 1999) found an increased risk of CLD with increased fluid intake and lower weight loss in their study population. The cohort study of Oh and colleagues (Oh et al., 2005) found that the group of infants who went on to develop CLD had a higher fluid intake and lost less weight in the first few days of life. However, a randomised trial of fluid (crystalloids and colloids) restriction of at least 20% between two groups of matched preterm infants did not find a difference in the incidence of CLD between the groups (Kavvadia et al., 2000). In their meta-analysis of all trials of fluid restriction, Bell and Accaregui (Bell and Acarregui, 2008) recommended a careful restriction of fluids in preterm infants which improves weight loss and reduces the risk of patent ductus arteriosus (PDA) and necrotising enterocolitis (NEC). They also noted a trend towards reduction of CLD, which was not statistically significant. However, this analysis was based on randomised trials only, most of which were conducted in the pre surfactant and prenatal steroids era, and thus were on more mature infants than we see today. As a result, direct extrapolation of this data on to preterm infants seen currently may not be appropriate. A large retrospective observational study (Wadhawan et al., 2007) concluded that the incidence of CLD and/or death (treated as a competing variable for CLD) was significantly lower in extremely preterm infants if they lost weight (below their birth weight) in the first ten days of life.

In summary, although infants who lose weight early seem to have a better outcome in observational studies, fluid restriction as an intervention has not had an effect on the incidence of CLD. Further studies would be needed to confirm the role of fluids in the development of CLD.

Physiologically, a PDA can have the same effect on pulmonary mechanics as water overload. There is also a possibility of histopathological changes in the vascular network due to the “abnormal” increase in blood flow (at that gestation) resulting in pulmonary hypertension due to intimal fibrosis and medial hypertrophy (Bancalari et al., 2005). With the use of pulmonary surfactant on preterm infants, the signs and

symptoms of a PDA can appear earlier due to improved oxygenation resulting in decreased pulmonary vascular resistance (Bancalari et al., 2005).

Early reports of the effect of PDA on respiratory status were provided by Cotton and colleagues (Cotton et al., 1978b) who demonstrated benefits of early surgical closure of PDA in a randomised trial (Cotton et al., 1978a). Benefits of closing the duct with indomethacin was shown in the trial of Merritt and colleagues (Merritt et al., 1981) who were also able to demonstrate a decrease in the incidence of CLD. Several studies have reported the adverse effects of a PDA on respiratory status including the need for prolonged respiratory support (Cotton et al., 1978b), increased risk of pulmonary haemorrhage (Alfaleh et al., 2008), adverse effects on pulmonary mechanics (Clyman et al., 2009) and increased risk of CLD (Brown, 1979). Closure of the duct has been shown to prevent deterioration in pulmonary function (Clyman et al., 2009). Studies on the effects of water and electrolyte balance in preterm neonates on PDA and respiratory function have had mixed results. Bell and colleagues showed an increase in the incidence of PDA with increased volume of fluid intake (Bell et al., 1980) but the same effect was not demonstrated in several studies (Lorenz et al., 1982, Kavvadia et al., 2000, Wadhawan et al., 2007). The effect of fluid management on CLD has also been mixed with some studies showing decreased incidence of BPD with restriction of fluid intake (Marshall et al., 1999, Wadhawan et al., 2007, Oh et al., 2005) while others demonstrating no effect of fluid management on CLD (Lorenz et al., 1982, Bell et al., 1980, Kavvadia et al., 2000). Of note, two important studies reported the synergistic effects of PDA and infection on the incidence of CLD (Rojas et al., 1995, Gonzalez et al., 1996). The latter trial showing an increase in prostaglandins levels with infection which may explain the persistence of the duct in such infants.

Recent authors have argued that there is an association between the presence of a PDA and the incidence of CLD but this is probably not a cause-and-effect relationship (Laughon et al., 2004, Bose and Laughon, 2006). Thus, although the presence of a PDA has been associated with an increase in the incidence of CLD, treatment has not been able to decrease the incidence of CLD (Schmidt et al., 2001, Schmidt et al., 2006, McCurnin et al., 2005c) in human or animal studies. Systematic reviews with meta-analysis of trials reached similar conclusions (Mosalli and Alfaleh, 2008, Cooke et al.,

2003) even when surgical and medical methods of closure were compared (Malviya et al., 2003). In summary, the current prevailing opinion suggests treatment of a PDA when it becomes symptomatic, but its effect on long-term respiratory outcome is still unclear.

Neonatal Infection

Microbial agents stimulate the immune system and cause inflammation at sites of infection. Receptors on the cells of the immune system are activated by recognising patterns of repeating molecular structure on the surface and nuclear material of these organisms (Medzhitov et al., 1997). Activation of Toll-like and other innate immune receptors leads to a sequence of events (see section 1.7) resulting in an influx of inflammatory cells to the site of inflammation. Experimental introduction of intra-uterine infection in animals has resulted in a selective inflammatory response in the lungs of the foetus (Kramer et al., 2002, Willet et al., 2000), priming the lungs to further damage. When such lungs are exposed to post-natal injurious factors like infection, ventilation or PDA, there seems to be an additive effect to the damage produced (Van Marter et al., 2002, Rojas et al., 1995, Gonzalez et al., 1996).

Cohort studies of preterm infants have demonstrated the role of infection in the development of CLD. In a recent cohort of preterm infants, our group have shown that the presence of infection, both early and late, were significantly associated with the development of CLD (Beeton et al., 2011). Inflammatory cytokines and chemokines, which are commonly associated with CLD, were increased in concentration of lung-fluid from infants with evidence of infection, who went on to develop CLD later. Post-natal infection was also associated with the episodic rise of proteases in lung-fluid of infants who develop CLD (Davies et al., 2010).

In recent years, *Ureaplasma Spp*. have gained importance in their association with neonatal lung inflammation. Mollicutes are the single most common class of bacteria cultured from uterine samples (Kramer et al., 2009, Goldenberg et al., 2008). Transmission of *Ureaplasmas* from the mother to the foetus and thus the neonate, as evidenced by its culture from very early endo-tracheal samples, has been demonstrated (Sanchez, 1993). Several studies have looked at the association of

infection with this bacteria with long-term lung damage and the results have been mixed. A recent meta-analysis (Schelonka et al., 2005) demonstrated a positive association of this organism with CLD although there was increased variability between included studies; also, studies with smaller number of subjects demonstrated greater effects. This organism has been shown to affect different steps in the inflammatory cascade (Li and Tullus, 2002) including inflammatory cell infiltration and expression of inflammatory mediators (Schelonka and Waites, 2007). Apoptosis of lung epithelial cells has also been demonstrated *in-vitro* due to this organism (Li et al., 2002).

Experimental intra-uterine *Ureaplasma* introduced in the preterm baboon model of CLD (Yoder et al., 2003b) results in an early rise of pro-inflammatory mediators in the tracheal aspirates of infants. Animals that remained colonised with the bacteria had persistent presence of mediators of inflammation in the lung with worse lung function while those infants who were able to clear their infection resolved their inflammation after 48 hours and had improved lung function. The authors suggested that difference in response of the immune system may determine outcome after colonization with this organism. In a separate study of the same model, TGF-β1 was noted to be increased in infected animals indicating a cytokine expression pattern promoting pro-fibrotic responses (Viscardi et al., 2006a).

In summary, several different insults have been implicated in the pathogenesis of CLD. There is insufficient evidence to suggest that one risk factor is more injurious than the next. It is more likely that CLD is the cumulative effect of multiple risk factors acting on the lungs of preterm infants.

1.7. Inflammation – A Short Summary

Inflammation is defined as the response of living tissue to injury (Ryan and Majno, 1977) by the immune system of the body which consists of two interacting arms, the innate immune system and the adaptive immune system (Janeway et al., 2005). The actions of both of these arms are effected by cells, which originate in the bone marrow from two common progenitor cells, the lymphoid progenitor and the myeloid progenitor. The lymphoid progenitor gives rise to T and B-lymphocytes, which

are part of adaptive immunity, and natural killer (NK) cells, which are considered part of innate immunity. The myeloid progenitor gives rise to granulocytes (innate immunity), monocytes/macrophages (both innate and adaptive immunity) and dendritic cells (adaptive immunity). Dendritic cells can arise from the common lymphoid progenitor as well and has similar antigen presenting functions for the adaptive immune system.

The innate immune system forms the first line of the body's defence against injury (infectious and non-infectious). It produces an immediate response within minutes to hours of an injury and then an early induced response between 4-96 hours before the adaptive immune response becomes active (Janeway et al., 2005). When injury is due to micro-organisms, receptors on the cells of the immune system are activated by recognising patterns of repeating molecular structure on the surface and nuclear material of these organisms (Medzhitov et al., 1997). Macrophages residing in tissues are the cells initially activated by pattern-recognition receptors (macrophage mannose receptor, Toll-like receptors). Activation of these receptors (mainly Toll-like receptors in case of microorganisms) leads to a sequence of events resulting in an influx of neutrophils to the site of inflammation (Kolaczkowska and Kubes, 2013). The first step in this process involves production of cytokines and chemokines, which have local and systemic effects. Cytokines like IL-1β and TNF-α activate endothelial cells of blood vessels to express cell adhesion molecules (selectins) and intercellular adhesion molecules (ICAM-1 and -2). Adhesion molecules arrest the flow of neutrophils in the blood vessel and firmly attach them to the endothelial cells. The neutrophils then pass through in-between two endothelial cells (diapedesis) and migrate down the concentration gradient of the chemokines to the site of infection. Chemokines like interleukin-8 create a concentration gradient to guide inflammatory cells to the site of inflammation. Both cytokines and chemokines "prime" neutrophils for activation on reaching sites of inflammation.

Neutrophils function as phagocytic cells at sites of inflammation. They recognise microorganisms by the same process as described for macrophages earlier, i.e. by pattern recognition receptors on the cell surface (toll-like receptors). Phagocytosis is also facilitated by another arm of the innate immune system, the

complement system, which promotes the uptake and destruction of pathogens (Kobayashi et al., 2005). This also involves the production of reactive oxygen species, which are part of the mechanism involved in killing pathogens. The other major function of these cells is the production of cytokines and chemokines as part of the acute phase reaction. Activated neutrophils trigger cytoplasmic granules, containing several enzymes and antimicrobial peptides, to fuse with the phagocytosed pathogen and result in their killing.

The receptors of the cells in the innate immune system are germline encoded and can recognise molecular patterns in microorganisms, which are common to many pathogens. Thus, they have low specificity. Pathogens have also evolved strategies to evade recognition by cells of the innate immune system by various methods, and this has created the need for another family of cells, which can respond to them in a specific manner. The adaptive immune system is formed by T- and B-lymphocytes and their related antigen presenting cells. This system has a memory and can learn to respond to most challenges to the body, which are perceived as injurious. B-cells respond primarily by the production of specific antibodies and T-cells have helper and cytotoxic functions. T helper cells (CD4) produce two types of responses; T_H1 responses, which are directed towards intracellular pathogens and T_H2 responses, which are involved in activating B-cells. Both T- and B-cells need to be exposed to antigens for a period before they can respond with an immune reaction. Newborn infants are born with naïve lymphocytes, which have not been exposed to “non-self” antigens before.

1.8. Resolution of acute Inflammation

Acute inflammation, as summarised above, is characterised by an influx of polymorphonuclear neutrophils (PMN) through sequential and regulated steps. These cells are part of the innate immune system and protect the body against danger e.g. invading microorganisms. However, PMNs themselves and their products can be involved in perpetuation of inflammation if they are not effectively removed (Si-Tahar et al., 2009). Programmed cell-death (apoptosis) of PMNs and their removal from the

site of inflammation by phagocytes are thought to be key steps in the resolution of inflammation (Savill et al., 1989).

A key step at the beginning of resolution is granulocyte apoptosis, a process of programmed cell-death. Apoptosis is considered a non-inflammatory process where the cell maintains its membrane integrity for a period of time. Several pathways are known to affect the survival of PMNs in an inflammatory milieu (Kennedy and DeLeo, 2009, Rossi et al., 2007). Inflammatory cytokines (IL-1β, TNF-α, GM-CSF, IFN-γ), TLR activation and modulation by bacterial pathogens can all lead to increased survival of these cells (Colotta et al., 1992). Among the intracellular pathways involved in inflammation, nuclear factor-κB (NF-κB) is a key regulator of PMN apoptosis along with mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways (Rossi et al., 2007). Ingestion of bacteria by PMNs can lead to apoptosis by phagocytosis-induced cell death (PICD) (Kennedy and DeLeo, 2009) which can override delays due to pro-inflammatory factors (Watson et al., 1996). Thus, inflammation, and the resulting intra-cellular signalling pathways, can lead to increased survival and delayed removal of innate immune cells.

The apoptotic PMNs are able to maintain membrane integrity for a period of time during which they need to be cleared from the site of inflammation. Macrophages are professional phagocytes which are the main cells involved in this clearing process (Savill et al., 1989),. These cells are attracted to sites of apoptosis, preferentially recognise apoptotic cells and phagocytose them to clear sites of inflammation. Apoptotic cells are known to express signals which attract phagocytes to them. Candidate molecules attracting macrophages to sites of apoptotic cells include endothelial monocyte-activating polypeptide II (EMAP II) (Weigert et al., 2009), a heterodimer of thrombospondin and CD36 (Moodley et al., 2003) and chemotactic lipids like lysophosphatidylcholine (LPC) by coupling to G protein coupled receptors (Mueller et al., 2007).

Specific recognition of apoptotic cells by phagocytes occur by recognition of cell-surface changes during apoptosis. Phospatidylserine (PS), which is normally present in the inner cell-membrane is one of the essential molecules which gets externalised on the cell surface during apoptosis (Martin et al., 1995). Receptors on

phagocytic cells interact with PS through bridging molecules leading to specific recognition. Other candidate molecules include calreticulin (Gardai et al., 2005) and CD31 (PECAM-1) which seems to have a dual role (Brown et al., 2002). Thus, ligation of CD31 on viable leucocytes causes their detachment from macrophages while the opposite happens on ligation of CD31 on apoptotic cells. Recognition of apoptotic cells leads to the formation of a phagocytic-synapse on the cell surface with a variety of co-receptors being involved in the process (Vandivier et al., 2006) resulting in internalisation and removal of the apoptotic cell. Phagocytosis of apoptotic cells results in a down-regulation of pro-inflammatory activity of macrophages and causes these cells to express anti-inflammatory cytokines (Fadok et al., 1998a, Fadok et al., 1998b). Thus, apoptosis of innate immune cells and their subsequent phagocytosis are essential steps in the resolution of inflammation.

In the lung, clearance of apoptotic cells by macrophages after pneumonia is seen (Haslett, 1999). The ineffective clearance of PMNs by phagocytes can lead to chronic disease. Increased numbers of apoptotic cells have been found in patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD); high levels of neutrophil elastase in CF patients inhibits efferocytosis (Vandivier et al., 2006). Preterm infants with RDS have apoptotic neutrophils in their lungs which get cleared by macrophage phagocytosis (Grigg et al., 1991). However, infants who develop CLD later seem to have an abundance of viable PMNs in the lung which failed to enter apoptosis (Kotecha et al., 2003, Oei et al., 2003). Persistence of neutrophils in the lungs of preterm infants may contribute to perpetuation of inflammation and continued injury to the lungs.

In summary, apoptosis of PMNs and their subsequent removal are key steps during resolution of inflammation. A complex system exists which ensures efficient working of these steps. Disordered regulation of resolution has the potential to lead to chronic disease. A clear understanding of these processes involved in resolution of lung inflammation in preterm infants could result in new approaches to prevent development of chronic lung disease.

1.9. Inflammation in BPD

A vast body of evidence implicating inflammation in the pathogenesis of BPD has accumulated over the last three decades. Researchers have looked at various stages in the inflammatory cascade as also their temporal profile to link it with the disease process. Presented below is a summary of the evidence, in the sequence it is thought to take place in the inflammatory cascade.

Early Cytokines and Chemokines: Tumour necrosis factor-α (TNF-α) belongs to a family of cytokines of the same name which is produced by alveolar macrophages in response to stimuli by pathogens. It upregulates expression of adhesion molecules on endothelial cells, epithelial cells and on leucocytes. It can also induce production of IL-1β and IL-8 (see below). Preterm neonatal monocytes have decreased capacity (25%) of producing TNF-α (Metinko, 2004). TNF-α is detected in aspirates of epithelial lining fluid in ventilated preterm neonates (Ambalavanan et al., 2009, Jonsson et al., 1997b). However, its bioactivity may be low compared to its immune levels (Bagchi et al., 1994).

Interleukin-1β (IL-1β) is an early response cytokine produced by alveolar macrophages (in response to danger stimuli) and to a lesser extent by endothelial cells and fibroblasts. It up-regulates expression of adhesion molecules on endothelial cells and PMNs and also “primes” them for activation when they reach a site of inflammation (Metinko, 2004). It also induces IL-6 and IL-8 production from cells. IL-1β is detected in increased concentration in BALF from intubated preterm infants in the first day of life who went on to develop BPD (Rozycki, 1994). It is also associated with an increased risk of BPD (or death) when present in TAF of ventilated preterm infants (Ambalavanan et al., 2009). IL-1β expression in mouse lung produces a pathology similar to BPD (Bry et al., 2007) which could be due to suppression of the retinoic acid pathway during alveologenesis (Bry and Lappalainen, 2006). IL-1 receptor antagonist (IL-1ra) is a natural protein synthesized by mononuclear cells, which binds to the IL-1 receptor and inhibits its action. Sub-optimal expression of the antagonist has been

implicated in the pathogenesis of airway inflammation in babies who develop BPD (Kakkera et al., 2005).

IL-8 (CXCL-8) is an early response chemokine, which is a key chemoattractant for PMNs to sites of inflammation. It is produced by a variety of cells including alveolar macrophages, endothelial cells, epithelial cells, fibroblasts etc. on induction by danger stimuli. In-vitro, IL-8 seems to be the dominant CXC chemokine produced by alveolar macrophages and accounts for most of the chemotactic activity on PMNs (Goodman et al., 1998). It has consistently been detected in increased quantities from epithelial lining fluid in lungs of ventilated preterm infants who later develop BPD (Kotecha et al., 1995, Ambalavanan et al., 2009) and has been observed to increase before the peak of inflammatory cell influx (Munshi et al., 1997). High levels of IL-8 in lung at birth correlate well with increased duration of ventilation in small preterm babies (De Dooy et al., 2007).

Arrest of circulating leucocytes: Circulating neutrophils and monocytes express adhesion molecules called integrins (LFA-1 or CD11a/CD18, Mac-1 or CD11b/CD18) and L-selectin (CD62L) which attach to endothelial cell adhesion molecules (ICAM-1 or CD54, ICAM-2 or CD102, VCAM-1 or CD106) for to stop them from flowing away. These molecules are up-regulated by early response cytokines TNF-α and IL-1β. Soluble forms of these adhesion molecules exist which are formed by alternative splicing of the trans-membrane domains. Endothelial cells also contain P-selectin (also in platelets) (CD62P) and E-selectin (CD62E) which attach to ligands on neutrophils to arrest their flow. Arrested cells pass between cells out of the capillaries and travel to sites of inflammation down the concentration gradient of chemokines.

Concentration of soluble L-selectin (Ballabh et al., 2003) and expression of L-selectin by PMNs (Ballabh et al., 2004) was found to be low in peripheral blood in infants who develop BPD. However, the concentration of soluble L-selectin in BALF was reported as increased persistently in the same group of infants in another study (Kotecha et al., 1998) suggesting the preferential retention of PMNs expressing low levels of L-selectin in peripheral circulation. Expression of CD62L on PMNs was further decreased after dexamethasone therapy (Ballabh et al., 2004, Waisman et al., 1998) in

this population of patients. In infants who developed BPD later, soluble E-selectin concentrations was increased in cord blood (Kim et al., 2004a) and in peripheral blood (Ramsay et al., 1998, Ballabh et al., 2003) and concentration was suppressed after dexamethasone therapy (Ballabh et al., 2003). ICAM-1 concentrations was similarly raised in peripheral blood (Ballabh et al., 2003, Ramsay et al., 1998) and in epithelial lining fluid (Kotecha et al., 1995, Kojima et al., 1993) of infants who developed BPD. However, it was not significantly affected by dexamethasone therapy (Ballabh et al., 2003). In summary, these molecules demonstrate dynamic expression on cells, in blood and in epithelial lining fluid, which may be an indicator of inflammatory cell recruitment to sites of inflammation. However, the stimulus for recruitment comes from the inflammatory site and seems to be the initiator of expression of these molecules. Measuring them may be useful as a marker of cell recruitment over time.

Influx of inflammatory cells: An influx of inflammatory cells into the lung epithelial lining fluid recovered by aspiration is seen early in patients of RDS (Merritt et al., 1983, Ogden et al., 1983, Ogden et al., 1984b). This was also noted to be persistently high in infants who went on to develop BPD later. Most of this influx consisted of PMNs (Merritt et al., 1983, Ogden et al., 1983). These studies were done before use of surfactants and prenatal steroids were prevalent; ventilation strategies were different as well. However, similar results have been reported in studies in which infants have benefitted from both surfactant and prenatal steroids (Kotecha et al., 1995, Arnon et al., 1993, Munshi et al., 1997). However, surfactant use seemed to be associated with an increase in MNC numbers (Arnon et al., 1993). Studies in the last decade have confirmed the association between prolonged infiltration of PMNs and CLD (Kotecha et al., 2003).

Cytokine and chemokine expression: Persistence of cytokine and chemokine expression in the lung has been observed in preterm infants who develop BPD (Ryan et al., 2008, Bose et al., 2008, Speer, 2006, Ambalavanan et al., 2009). Inflammatory cells including macrophages and neutrophils are a major source of these cytokines (Cassatella, 1995) along with cells in the lung including airway and alveolar epithelial cells, endothelial

cells and fibroblasts. Cytokines bind to specific receptors on target cell surfaces and mediate intracellular signalling by up regulating or down regulating genes and their transcription factors (NF-κB) (Shimotake et al., 2004). Chemokines are expressed by the same cells in response to cytokine stimulation and they direct cellular influx to the site of inflammation.

Protease and Antiprotease Balance: Proteases are enzymes which hydrolyses peptide bonds and degrade proteins (Papoff, 2000). Inflammatory cells, mainly neutrophils, are the major source of proteases in the lung epithelial lining fluid which potentially can cause damage. The functions of these enzymes are regulated by anti-proteases, which maintain the balance in this system during health. An imbalance between elastase and its inhibitor α_1-proteinase inhibitor (α_1-PI) has been noted in the airways of preterm infants (Speer, 2006). Increased urinary concentrations of desmosine, a degradation product of elastin, has also been identified in such infants and is thought to be a consequence of proteolytic damage. Elastin deposition is an essential step in the formation of secondary septa in the airspaces that develop into mature alveoli, which is not well formed in babies with BPD. Similar imbalances have been observed in cysteine and serine proteases and their inhibitors (Altiok et al., 2006, Yasumatsu et al., 2006) as well as in the matrix metalloproteinases and their inhibitors (Curley et al., 2004, Curley et al., 2003, Fukunaga et al., 2009).

Resolution of Inflammation: Traditionally, the resolution of inflammation was thought to be a passive process related to the phagocytosis of microbes (cessation of stimulus for inflammation) resulting in decreased production of pro-inflammatory molecules and recruitment of neutrophils. Recent evidence suggest that this may be an active process brought about by lipid-derived mediators which are involved in switching the profile of inflammatory response to that of resolution (Serhan, 2008, Serhan et al., 2008, Serhan and Savill, 2005). These mediators act by decreasing further recruitment of PMNs to the site of inflammation/infection and also by increasing uptake of apoptotic PMNs by macrophages to enhance resolution. Phagocytosis of pathogens is

a common inducer of apoptosis of PMNs which overrides anti-apoptotic signals from cytokines or other bacteria derived factors (Kennedy and DeLeo, 2009).

Studies on the resolution of lung inflammation in preterm babies are limited. There is evidence of delayed neutrophil apoptosis in infants who develop CLD later (Kotecha et al., 2003). This suggests that inappropriate suppression of neutrophil apoptosis may contribute to lung inflammation by delaying resolution in this group of patients. The exact mechanism of this delay is unknown. No studies looking at pro-resolving mediators in preterm lungs have been done so far.

Summary: CLD in preterm babies is a multi-factorial disease which is probably starts in utero, initiating lung inflammation and setting it up for further injury after birth. Several factors contribute to lung inflammation after birth and perpetuate the injury. Inflammation seems to be the final common pathway causing lung damage. An imbalance between pro- and anti-inflammatory pathways favouring the former seems to perpetuate this process. There is an arrest of growth of alveoli and vessels as a result of this damage. Further research into the mechanisms of injury is the first step towards understanding this condition better, and may lead to effective treatment strategies.

1.10. Lung Development Disorders in BPD

Human lung development in the foetus occurs in overlapping stages and is summarised in table 1. Most infants who develop BPD are born at a late canalicular or early saccular stage of lung development (24-30 weeks). In this stage, there is functional surface for gas exchange but it is not well developed. Extensive development in the distal airspace structure and microvasculature occurs during this stage of lung development. The major part of development of the gas exchange surface occurs after birth in human infants. The mesenchyme of the developing lung, including cells (fibroblasts), structural proteins (collagen, elastin, fibronectin and laminin) and it's supportive network proteoglycans and glycosaminoglycans), are integral to both early and late lung development (Ahlfeld and Conway, 2012). During

the canalicular stage, the distal airways and it’s related vasculature continue to develop accompanied by thinning of the mesenchyme. Maturity of the lung epithelial cells also takes place including differentiation into distinct cellular types in preparation for gas exchange. These changes continue in the saccular stage with further thinning of the mesenchyme and development of the pulmonary acinus. The primary septa of the acini are primed for development into the secondary septa, a development which occurs during the alveolar stage. The important changes are the thinning of the mesenchyme, maturation of the capillary network from a double layered to a single layer, and deposition of elastin at the tips of the secondary crests. Our limited knowledge of lung pathology suggests that these changes are altered in preterm infant who develop CLD, leading to simplified large airspaces.

Studies in animal models have increased our understanding of lung developmental disorders involved in the pathogenesis of CLD. The primate model of CLD in premature baboons have provided insights in development disorders of the lung (Coalson et al., 1999a). These animals received similar clinical management as current preterm human infants, including prenatal steroids, early postnatal surfactant replacement, gentle ventilation and judicious use of oxygen. Lung pathology in these animals showed an interstitium of variable thickness and hypercellularity, focal deposition of elastin and collagen and dysmorphic microvasculature. Rodent models of CLD have also provided us with valuable insight into the lung developmental disorders resulting from injury before the alveolar stage. A short summary of selected growth factors implicated in the developmental disorders of the preterm lung follows.

The mesenchymal structural protein elastin has a vital role in alveolar development where it is localised to the growing secondary septal tips (Willet et al., 1999, Burri, 2006). Myofibroblasts are the major source of elastin in the mesenchyme, both in animals (Dickie et al., 2008) and humans (Leslie et al., 1990). Platelet derived growth factor A (PDGF-A) acts as an important chemoattractant for fibroblasts, and retinoic acid & sonic hedgehog have been proposed as molecules which establish a morphogen gradient of PDGFA to sites of secondary septation (Bourbon et al., 2005). Humans developing CLD show disorganised elastin deposition in their saccular wall

(Thibeault et al., 2003); however, the mechanisms by which elastin is involved in secondary septal formation is poorly understood.

Fibroblast growth factors (FGF) signalling through their receptors, especially FGFR-3 and -4, also seems critical for alveolar growth and development. Delayed expression of PDGF-A and reduced expression of FGFR-4 have been documented in neonatal rats exposed to hypoxia (Bourbon et al., 2005). FGF-7 (keratinocyte growth factor) is a potent stimulus of adult alveolar type II cells, and its concentration been found to be lower in infants who develop CLD compared to those who did not (Danan et al., 2002). It has also been shown to protect lung epithelium from oxidative stress, mechanical ventilation and infection (Bourbon et al., 2005, Franco-Montoya et al., 2009).

Several molecular signals are involved in this process resulting in a cross-talk between the developing airways and the surrounding mesenchyme. Transforming growth factor-β (TGF-β) is an early modulator of lung growth, which acts by inhibiting or limiting fibroblast growth factor (FGF) signalling. It limits epithelial bud formation during branching morphogenesis (pseudoglandular stage) (Wert, 2004). Overexpression of TGF-β in the late saccular and early alveolar stage also inhibits alveolarisation with significant fibrosis (Gauldie et al., 2003). Elegantly designed experiments of antibody-mediated TGF-β inhibition demonstrated the attenuation of defects in alveologenesis after chronic exposure to 85% oxygen.

Vascular growth and airway morphogenesis run parallel in human lung development and the former process may be important for normal alveolarisation (Thebaud, 2007). Vascular endothelial growth factor (VEGF) with its receptors VEGFR-1 and -2 is a specific mitogen and survival factor for vascular endothelial cells (Thebaud, 2007). VEGF increases cell division in human foetal lung Type II cells and probably plays a key role in the process of alveolarisation (Snyder, 2004). The involvement of VEGF in the pathogenesis of BPD is suggested by the fact that its inhibition blocks alveolarisation and when it is ectopically administered it enhances alveolarisation (Thebaud and Abman, 2007). Levels of VEGF in lung fluid in preterm infants who develop BPD have been found to be lower than in those who recover from RDS (Lassus et al., 2001). Exogenous replacement of VEGF in animal models of lung injury resulted

in improved capillary formation and alveolar development (Thebaud and Abman, 2007).

Retinoic acid (RA) signalling in the lung enhances alveologenesis while vitamin A deficiency delays alveolar development (Bourbon et al., 2005). Levels of vitamin A has been found to be lower in babies who develop CLD (Shenai et al., 1985) and it has been shown to have modest benefits when supplemented in a preterm population (Tyson et al., 1999). More consistent benefits with improvement of alveologenesis has been found in animal models (Bourbon et al., 2005). The post natal formation of alveoli is largely prevented by glucocorticoids, an effect which was partially reversed by RA treatment in an animal model (Bourbon et al., 2005).

In summary, the process of normal lung development involves complex interaction of the growing airways with its surrounding mesenchyme, with each stage being modulated by several molecular mechanisms. Interruption of this process, as by preterm birth, can have implications in the form of alveolar simplification and paucity of vessels as seen in BPD. However, the molecular mechanisms which lead from inflammation in the lungs to dysregulated growth is yet to be determined.

1.11. Modulation of Lung Inflammation in Preterm Infants – Two Potential Pathways

Persistent inflammation has been strongly implicated in the development of CLD in preterm infants. This inflammation is characterised by a poorly resolving neutrophilia, suggesting a prolongation of acute inflammation, and poor recruitment of mononuclear cells suggesting an inadequate resolution phase (see section 1.9). Measurement of several cytokines, chemokines and other mediators of inflammation also support this pattern of cellular influx in infants at risk of developing CLD later. Thus, pathways which can down-regulate the recruitment of neutrophils and up-regulate the influx of mononuclear cells could result in modulating lung inflammation in ventilated preterm infants.

Researchers at Cardiff University have been studying the specific pro-resolution effects of IL-6 signalling. In an experimental animal model of peritonitis, they have

shown that IL-6 *trans*-signalling (see section 1.12.5) can down-regulate the expression of IL-8, one of the key chemokines involved in recruitment of neutrophils to sites of inflammation, and up-regulate the expression of monocyte chemoattractant protein-1 (MCP-1), a chemokine involved in attracting mononuclear cells (Hurst et al., 2001). This changed the pattern of inflammation from a primarily neutrophil mediated acute inflammation to a mononuclear cell mediated resolution. Thus, IL-6 *trans*-signalling could attenuate lung inflammation in CLD, which exhibits dysregulation at both the stages (persistent neutrophil influx and poor recruitment of mononuclear cells), and provides an attractive mechanism to resolve pulmonary inflammation in infants at risk of CLD. Although IL-6 concentration has been measured in the lungs of ventilated preterm infants (see section 1.12.6), detailed studies on the *trans*-signalling pathway has not been conducted yet.

IL-8 is one of the main chemokines implicated in the recruitment and activation of neutrophils at sites of inflammation. Concentration of IL-8 in the lungs of ventilated preterm infants, who later develop CLD, have been consistently shown to be higher than in infants who do not (resolved RDS, see section 1.9). This is in keeping with the persistent influx of neutrophils observed into the lungs of CLD infants. Variations in the numbers of amino acids at the amino-terminal of the IL-8 molecule leads to the formation of several isoforms of IL-8 (see section 1.13.2 and table 1.2). The most common biological isoform involved in inflammation is the 72-amino acid molecule IL-8_{72}, which is functionally much more potent (both in attracting neutrophils and in activating them) than the next common isoform, the 77-amino acid molecule IL-8_{77} (see section 1.13.4). IL-8_{77} is the major isoform found in the circulation of preterm infants (Maheshwari et al., 2009). Although concentration of total IL-8 have been measured in the lungs of preterm ventilated infants, the expression of IL-8_{77} has not been studied. Potentially, modulation of expression of the IL-8 isoforms could result in modulating neutrophil influx into the preterm lung and change the pattern if inflammation.

1.12. Interleukin-6 Biology

Interleukin-6 (IL-6) is a multifunctional cytokine in biology (Kishimoto, 2006). It has important roles in normal physiology (when it is well regulated) as well as in pathology (defective regulation). Before the current name came into use, the molecule has been previously known as B cell stimulatory factor-2 (BSF-2), hybridoma growth factor (HGF) and hepatocyte-stimulating factor (HSF). All of these molecules were found to have the identical cDNA sequence and the name IL-6 was proposed (Akira et al., 1993).

Although IL-6 is commonly regarded as a pro-inflammatory cytokine, recent evidence has brought to light the pro-resolution effects of IL-6 (section 1.11.6). CLD is characterised by a persistent and poorly-resolved neutrophilic inflammation in the lungs of preterm infants. Thus, the pro-resolution effects of IL-6 signalling could be an effective pathway in modulating inflammation in the lungs of preterm infants at risk of CLD. Although the concentration of IL-6 itself has been measured in the lungs of preterm infants (sections 1.9 and 1.11.6), molecules in the IL-6 signalling pathway which are involved in resolution of acute inflammation have not.

1.12.1. Structure of IL-6

The IL-6 molecule is 184 amino acids (AA) in length and is secreted as a 212 AA long precursor by multiple cell types as a 21-28 kDa molecule (Heinrich et al., 1998, Van Snick, 1990). The range of molecular weights is due to post-translational glycosylation and phosphorylation in cells. Human IL-6 gene is located on chromosome 7p21 (Bowcock et al., 1988). There is about 65% homology between human IL-6 and mouse or rat IL-6 at the gene level (42% at protein level), mainly due to the conserved central portion (Akira et al., 1993). Human IL-6 has biologic activity on murine cells but mouse IL-6 does not have biologic activity on human cells (Coulie et al., 1989). IL-6 has a four α-helical structure, A-D, with connecting loops (Xu et al., 1996); receptor recognition epitopes are mainly located in the beginning of the A-helix and the ends of the AB loop and D-helix (Kalai et al., 1997).

1.12.2. Functions of IL-6

A large variety of cells in the body produce IL-6 including cells of the immune system (macrophages, PMNs, lymphocytes), endothelial cells, fibroblasts, bone cells (chondrocytes, osteoblasts) and some tumour cells. Most cells secrete IL-6 after appropriate stimulation which is usually well regulated; tumour cells are able to produce IL-6 constitutively (Akira et al., 1993). Multiple stimuli are known to affect the production of IL-6, by exerting both positive and negative control. Some stimuli are known to be cell specific exerting positive control in some cell types and negative control in others.

IL-6 produced by T-cells act as B-cell differentiation factor. It can augment IL-2 receptor expression resulting in T-cell growth and cytotoxic T-cell differentiation (Kishimoto, 2006). IL-3 and IL-6 have synergistic effects on the formation of multilineage blast cell colonies (Ikebuchi et al., 1987). IL-6 can act as thrombopoietin to control the late stages of megakaryocyte differentiation into platelets (Hill et al., 1990) in times of haematopoietic stress. IL-6 stimulates osteoclast formation & recruitment and bone resorption. Skin keratinocytes produce IL-6 when induced by IL-4 and IL-6 can stimulate proliferation of normal human keratinocytes (Grossman et al., 1989). Ultraviolet light can induce increased production from skin (Kirnbauer et al., 1991) and it may function as a regulator of systemic sunburn reaction. Endothelial cells, when induced by IL-6, produce increased platelet derived growth factor (PDGF) in vitro which in turn proliferation and migration of vascular smooth muscle cells as well as being chemotactic for monocytes and PMNs resulting in priming them (Akira et al., 1993). It can induce endothelial expression of adhesion molecules and production of MCP-1 (Romano et al., 1997). IL-6 is one of the primary cytokines involved in the “acute phase response” resulting in the production of several plasma proteins by the liver including C-reactive protein (CRP), fibrinogen and complement proteins.

1.12.3. The IL-6 Receptor System

IL-6 exerts its biological actions by a low-affinity attachment to a specific cell-surface receptor, the IL-6 receptor α (IL-6Rα, an 80kD ligand-binding subunit, gp80)

which dimerises another cell-surface molecule gp130 (IL-6Rβ, a 130kD signal-transduction subunit, CD130) (Rose-John et al., 2007). Both receptor subunits are transmembrane proteins. The IL-6R precursor is 468 AA in length which matures to a 449 AA protein including a 339 AA extracellular part, a 28 AA transmembrane part and an 82 AA intracellular part (Heinrich et al., 1998, Van Snick, 1990). The extracellular chain contains a cytokine binding domain (CBD) and two fibronectin-type-III-like domains (FNIII). gp130, the signal-transducing subunit, is common to other members of the IL-6 family. Its precursor is secreted as a 918 AA protein which matures to a 896 AA protein including an extracellular part of 597 AA, a transmembrane part of 22 AA and an intracellular part of 277 AA (Van Snick, 1990, Heinrich et al., 1998). The intracellular domain of gp130 is essential for signal transduction. Cytokine binding by gp130 occurs through three membrane-distal domains, D1-D3 while three membrane proximal FNIII domains are involved in receptor activation (Bravo et al., 1998, Chow et al., 2001).

IL-6 has three distinct receptor binding sites (Simpson et al., 1997). Site 1 is formed by the C-terminal residues of helix D and the C-terminal part of the AB loop; this site determines the specificity of binding to IL-6R. Site 2 is formed by residues near the middle of helices A and C and is the universal gp130 binding site which happens only in the presence of IL-6R. There is also a site 3 formed by the end of the CD loop, the N-terminus of the D helix and the N-terminal part of the AB loop; this is a second binding site for gp130 by IL-6. Thus, in the homodimer of the signalling complex, two different binding epitopes of gp130 are involved in ligand recognition.

Site 1 on IL-6 binds to the CBD on IL-6R as the first step in the interaction (Heinrich et al., 1998). Site 2 on IL-6 in the IL-6R/IL-6 complex then engages with D2-D3 on the gp130 molecule to form an intermediate trimolecular nonsignalling complex. This is followed by dimerisation of the complex by interaction of site 3 on IL-6 with D1 (Ig like domain) on the gp130 molecule. The final signal complex is of a hexameric architecture consisting of two molecules each of IL-6, IL-6R and gp130 (figure 1.7) (Boulanger et al., 2003). The complex of IL-6R/IL-6 is essential to recruit gp130 efficiently.

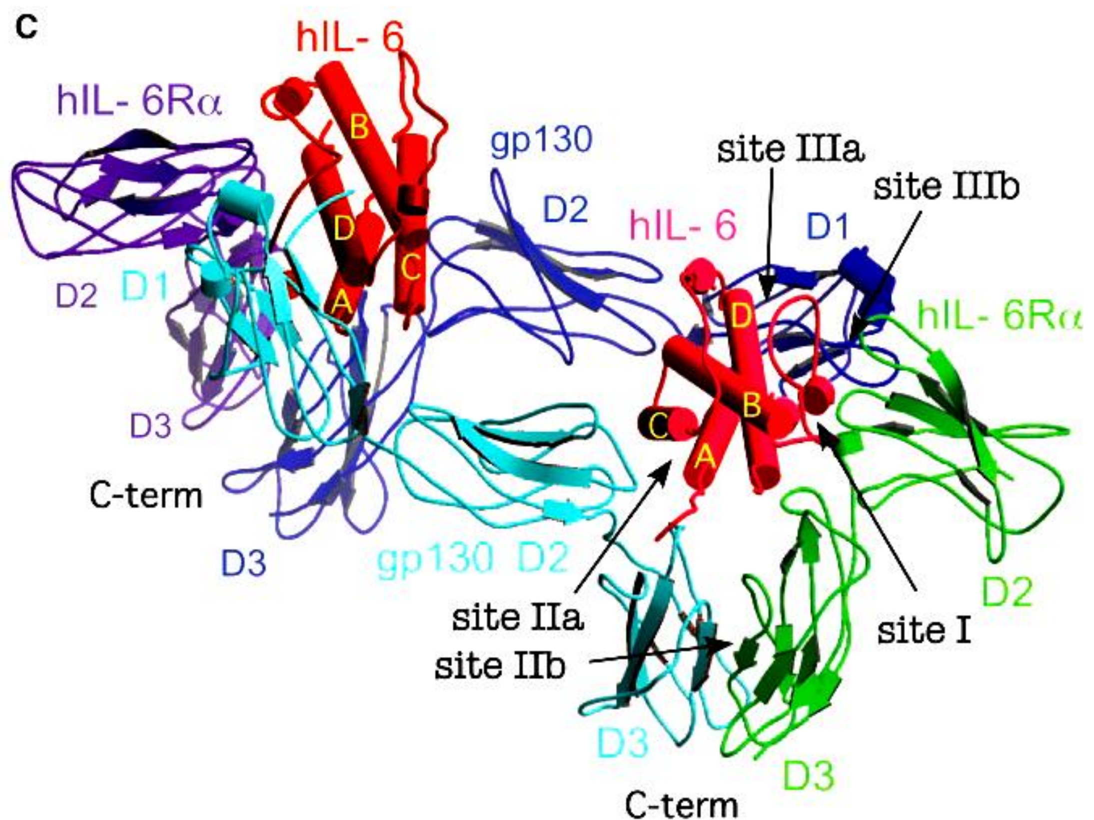


Figure 1-7: Structure of the human Il-6/IL-6R_/gp130 hexameric complex (Boulanger et al., 2003).

1.12.4. Receptor Signalling

Binding of IL-6/IL-6R complex to gp130 results in activation of intra-cytoplasmic kinases, of which the Janus Activated Kinases (Jaks) are the best studied. These tyrosine kinases are associated with the cytoplasmic tail of gp130 and induces tyrosine phosphorylation. IL-6 signalling leads to the activation of Jak1, Jak2 and Tyk2 among which Jak1 seem to be crucial (Guschin et al., 1995). STATs (signal transducers and activators of transcription) are the one of the main transcription factors involved in intracellular signalling via activation of IL-6 receptors. Of the seven STAT genes identified so far, protein products of the STAT1 and STAT3 genes are known to be involved in IL-6 signalling. There are specific docking sites for STATs on the phospho-tyrosine residues on the cytoplasmic tail of gp130 (Stahl et al., 1995). Phospho-STAT1 (Tyr^{701}) and 3 (Tyr^{705}) are able to form *homo*- and *hetero*-dimers which is essential for DNA binding (Heinrich et al., 1998) after active translocation into the nucleus (Heinrich et al., 2003). STATs bind to specific DNA sequences and modulate transcription of

target genes. SOCS (suppressor of cytokine signalling) proteins are cytoplasmic molecules which are involved in a negative feedback loop to control intra-cellular signalling by IL-6. They are activated by IL-6 binding to its receptors and act by inhibiting tyrosine phosphorylation of gp130 (Naka et al., 1997). Other proteins like PIAS (protein inhibitor of activated STAT) are directly involved in STAT inhibition by blocking DNA binding and gene activation (Chung et al., 1997).

Homodimerisation of IL-6 receptors not only activate the JAK/STAT signalling pathways but also can induce the MAPK (mitogen activated protein kinase) cascade. Several members of the MAPK family are known to be activated by IL-6 signalling including ERK1/2 (cell survival effects) as well as p38 and JNK (stress related activation) (Heinrich et al., 2003). Another pathway activated by IL-6 signalling is the PI3K/Akt (phosphoinositide-3-kinase) pathway which is involved in proliferation of myeloma cells in the presence of IL-6 (Hideshima et al., 2001).

1.12.5. Cis- and trans-signalling

The downstream sequence of events when IL-6 binds to its membrane-bound receptor subunits (IL-6R and gp130) resulting in intracellular signal transduction is termed *cis*-signalling. gp130 is ubiquitously expressed on almost all cells of the body (Saito et al., 1992). However, expression of the membrane-bound IL-6R is restricted to mainly hepatocytes and leucocytes (Jones et al., 2001, Jones, 2005). A soluble form of the IL-6R (sIL-6R) was detected in human urine (Novick et al., 1989), in human serum and culture supernatants of human mononuclear cell (Honda et al., 1992). sIL-6R can be generated by limited proteolysis of the membrane-bound receptor (Mullberg et al., 1993b) which could be prevented by a metalloprotease inhibitor (Mullberg et al., 1995). It can also be generated by an alternative splicing mechanism where the transmembrane domain is deleted (Horiuchi et al., 1994a). Both the recombinant (Taga et al., 1989) and the soluble (Mackiewicz et al., 1992) forms of IL-6R interact with IL-6 and the complex was found to be biologically active. Commonly, in biology, the complex of a ligand and its soluble receptor are inhibitory in function. However, the complex of sIL-6R/IL-6 is an exception where they can bind to gp130 on the cell-membrane and initiate intra-cellular signalling. IL-6 signalling via its complex with sIL-

6R through membrane-bound gp130 has been termed *trans*-signalling. Effectively, this vastly widens the repertoire of cells which can respond to IL-6 signalling. Several investigators have since explored the effects of *trans*-signalling on cells and tissues of the body (Jones et al., 2001).

A soluble form of the gp130 subunit (sgp130) is normally present at relatively high levels in body fluids (Narazaki et al., 1993). This was shown to associate preferentially with the complex of sIL-6R/IL-6, but no association was demonstrated in the absence of either sIL-6R or IL-6 (Yasukawa et al., 1992). Further exploration of the role of sgp130 in IL-6 signalling showed it to be a selective inhibitor of the sIL-6R/IL-6 complex, i.e. *trans*-signalling but not of IL-6 via membrane-bound IL-6R (*cis*-signalling). It can competitively bind to the complex and prevent its association with the membrane-bound gp130 (signal-transducer) when present in a molar excess (Jones et al., 2005). It has been proposed that since sgp130 is present in serum at high levels, it acts as the natural inhibitor of IL-6 *trans*-signalling (Jostock et al., 2001a), although an effect on IL-6 *cis*-signalling has also been recently suggested (Garbers et al., 2011).

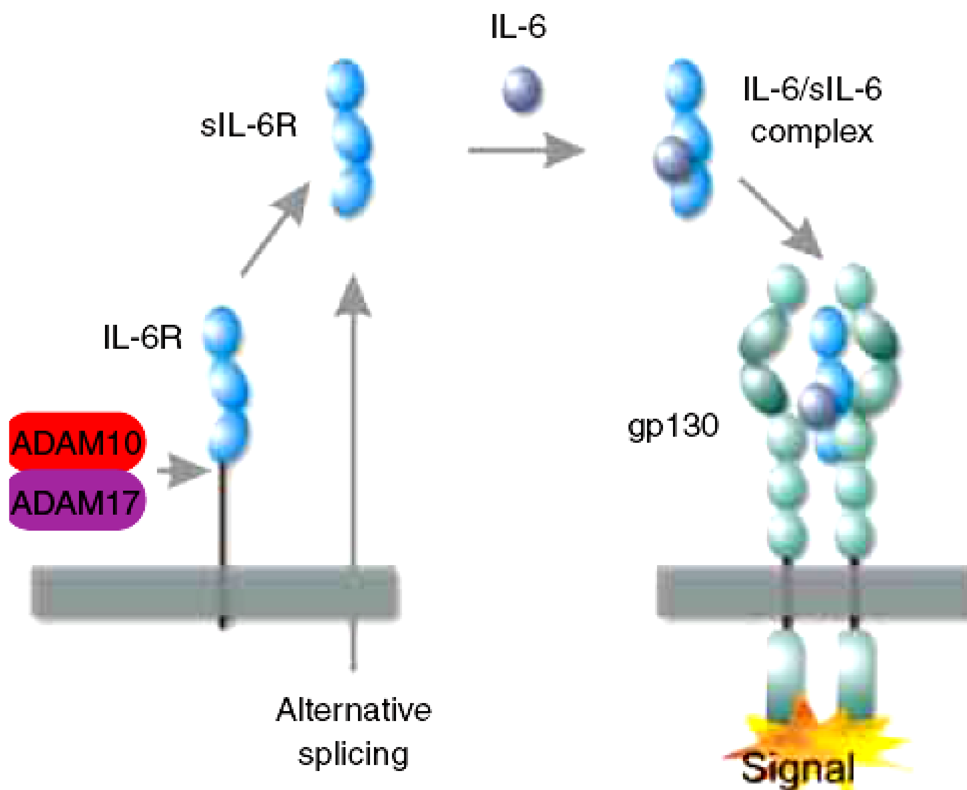


Figure 1-8: IL-6 *trans*-signalling (with permission from Rose-John et.al. 2007)

1.12.6. IL-6 in Disease and Inflammation

The traditional view of IL-6 is as an acute-phase response cytokine which has pro-inflammatory effects, both local and systemic. Several diseases have been shown to be associated with elevated concentrations of IL-6 (Jones et al., 2005). IL-6 signalling has also been implicated in various chronic disease processes like myeloma, rheumatoid arthritis, and Crohn's disease (Jones, 2005). The systemic effects of IL-6 as an acute phase reactant can be explained by signalling through its membrane-bound receptors, i.e. *cis*-signalling. However, since the expression of the ligand-binding subunit (IL-6R) is restricted to only a few cell types, the local effects of IL-6 can only be explained by *trans*-signalling through the soluble receptor (sIL-6R) (Rose-John et al., 2006).

The anti-inflammatory roles of IL-6 are less well known. IL-6 can decrease neutrophil infiltration and production of cytokines in animal models of acute lung inflammation (Ulich et al., 1991, Xing et al., 1998). IL-6 *trans*-signalling is involved in directing leukocyte trafficking to inflammatory sites, regulation of leukocyte apoptosis and directing maturation of T-cell subsets (Jones, 2005). More specifically, IL-6 *trans*-signalling has been shown to effect the transition of inflammatory infiltrate from predominantly neutrophilic to mononuclear cells in an animal model of acute inflammation, thus initiating resolution (Hurst et al., 2001). This is effected by changing the expression of chemokines from those predominantly attracting neutrophils (e.g. IL-8, GRO-α) to those which predominantly attract mononuclear cells (e.g. MCP-1) (Kaplanski et al., 2003). However chronic diseases, which are characterised by a persistent infiltrate of mononuclear cells, can also be adversely controlled by the effects of IL-6 *trans*-signalling.

IL-6 has been studied in the context of CLD in preterm infants. IL-6 has been found in increasing concentrations in lung fluid of preterm infants who later develop CLD (Kotecha et al., 1996b, Jonsson et al., 1997b, Choi et al., 2006b). The increase in concentration of IL-6 was found to precede the influx of neutrophils to the lung (Munshi et al., 1997). As discussed before, CLD is characterised by a persistent neutrophilia (Kotecha et al., 2003) leading to inflammation and lung damage. IL-6

trans-signalling, with its ability to switch leukocyte infiltrate to a mononuclear cell population during acute inflammation may have important implications for CLD. The modifying effects of the soluble receptors (sIL-6R and sgp130) on IL-6 signalling in the lung have not been studied extensively. In a recent report, no differences were found in the concentration of sIL-6R and sgp130 in the lung fluid of preterm infants who developed CLD compared to infants who did not (von Bismarck et al., 2008a). I hypothesised that the pattern of inflammation in the lungs of preterm infants developing CLD was due to a lack of IL-6 *trans*-signalling.

1.13. Interleukin-8 Biology

1.13.1. Early discovery of Interleukin-8 structure and function

Interleukin-8 (IL-8), also known as CXCL8 (2003) was discovered in the laboratory in the late 1980's by researchers while looking for a chemotactic factor and activator of neutrophils (PMN). This was first detected in human mononuclear leukocytes by stimulating them with lipopolysaccharide (LPS) (Yoshimura et al., 1987a) and/or phorbol myristate acetate (PMA) (Schroder et al., 1987) and also by stimulating human lymphocytes with phytoheamagglutinin (PHA)/ Concavalin A/ LPS (Schroder et al., 1988). The estimated molecular weight of the molecule was around 10 kD and attracted PMNs selectively. Amino-terminal sequencing by several groups of investigators revealed an identical protein which was different from other known proteins (Yoshimura et al., 1987b, Walz et al., 1987, Van Damme et al., 1988). The complete sequence of the molecule was finally detected to consist of 72 AA with identical NH_2-terminal sequence as determined previously with a molecular weight of 8 kD (Matsushima et al., 1988). All of these proteins detected had selective activity to attract PMNs but not monocytes.

Researchers soon established that IL-8 is secreted by almost all nucleated cells of the body, along with its various functions including attracting and activating neutrophils (Baggiolini et al., 1989, Remick, 2005). It was suggested that IL-8 acts by binding to specific cell-surface receptors on neutrophils (Baggiolini et al., 1989) and two receptors called CXCR1 and CXCR2 were soon established (Baggiolini, 1993). Since

then, IL-8 has been found to be a key mediator of acute inflammation at all sites in humans.

1.13.2. Isoforms of IL-8

Studies on Staphylococcal enterotoxin A stimulated human peripheral blood leucocytes (Schmid and Weissmann, 1987) had established the upregulation of the IL-8 gene which was predicted to transcribe a 99 AA pre-protein consisting of a 22 AA signal sequence and a 77 AA mature protein (later shown to be identical to IL-8). This upregulated cDNA clone, called 3-10C, was used by several researchers to express the protein in *E.coli* (Lindley et al., 1988a) & 293 (human kidey cell-line) cells (Gimbrone et al., 1989) and to detect mRNA in retinal pigment epithelial cells (Elner et al., 1990) & dermal fibroblasts (Mielke et al., 1990). As predicted earlier (Schmid and Weissmann, 1987), all of these investigators detected primarily the 77 AA protein.

The cDNA for MDNCF (Monocyte Derived Neutrophil Chemotactic Factor, an old name for IL-8) was screened and cloned from stimulated monocytes (Matsushima et al., 1988). This predicted a 99 AA pre-protein with a 27 AA signal sequence resulting in a 72 AA mature protein starting with serine. This cDNA sequence was used to express the protein (72 AA) in *E.coli* (Furuta et al., 1989) and detect mRNA in stimulated endothelial cells (Strieter et al., 1989a) and fibroblasts (Strieter et al., 1989b).

Thus, the two main isoforms of IL-8 were established as the 77 and 72 AA proteins, IL-8_{72} and IL-8_{77} (Figure 1.8). Within the next two years, several investigators discovered different isoforms of IL-8 from various cell-types which differed in the AA sequence at the NH_2-terminal (table 1.2). However, the function of the different isoforms were not known.

Reference	Cell type	Isoforms
(Yoshimura et al., 1989a)	Monocytes	79 a.a. – 6%
		77 a.a. – 38%
		72 a.a – 36%
(Lindley et al., 1988a)	Monocyte	77 a.a. – 17%
		72 a.a. – 70%
		70 a.a. – 8%
		69 a.a. – 5%
(Gregory et al., 1988)	Lymphocytes	77 a.a. – 30%
		72 a.a. – 70%
(Van Damme et al., 1989b)	Mixed mononuclear cells	77 a.a. – 20%
		72 a.a. – 55-70%
		79, 71, 70 & 69 a.a. – rest
(Gimbrone et al., 1989)	Endothelial cells	77 a.a. – 90%
		72 a.a. – rest
(Schroder et al., 1990)	Dermal fibroblasts	77 a.a. – 85-90%
		79 a.a. - rest

Table 1-2: Isoforms of IL-8 from different cellular sources.

A

```
1               5                 10                15
Ser-Ala-Lys-Glu-Leu-Arg-Cys-Gln-Cys-Ile-Lys-Thr-Tyr-Ser-Lys-
<------------------------------------------------------------
                        <----------------- T 7-26 ---------

                20                25                30
Pro-Phe-His-Pro-Lys-Phe-Ile-Lys-Glu-Leu-Arg-Val-Ile-Glu-Ser-
------------------------------------------------------------
------------------------------------------> <--------------

                35                40                45
Gly-Pro-His-Cys-Ala-Asn-Thr-Glu-Ile-Ile-Val-Lys-Leu-Ser-Asp-
------------------------------------------>
----------------------- T 27-47 ---------------------------

                50                55                60
Gly-Arg-Glu-Leu-Cys-Leu-Asp-Pro-Lys-Glu-Asn-Trp-Val-Gln-Arg-
------> <------------------ T 48-60 ---------------------->
                            <------------------------------

                65                70      72
Val-Val-Glu-Lys-Phe-Leu-Lys-Arg-Ala-Glu-Asn-Ser
<---------------- T 61-72 ------------------>
------- A 53-72 ---------------------------->
```

B

```
                  1                               10
                  Ser-Ala-Lys-Glu-Leu-Arg-Cys-Gln-Cys-Ile-   (70%)

-5                                                10
Ala-Val-Leu-Pro-Arg-Ser-Ala-Lys-Glu-Leu-Arg-Cys-Gln-Cys-Ile-   (17%)

                          3                       10
                          Lys-Glu-Leu-Arg-Cys-Gln-Cys-Ile-   ( 8%)

                              4                   10
                              Glu-Leu-Arg-Cys-Gln-Cys-Ile-   ( 5%)
```

Figure 1-9: **(A)** Amino acid sequence of IL-8_{72} and (B) different isoforms of IL-8 resulting from variable a.a. sequence at the NH_2-terminal. From the top in figure (B), the isoforms are IL-8_{72}, IL-8_{77}, IL-8_{70} and IL-8_{69} (Lindley et al., 1988a). (T = peptides obtained by tryptic digestion, A = peptides obtained by cleavage with 75% formic acid)

1.13.3. Conversion of longer isoform of IL-8 to shorter isoforms

The ability of different proteases to convert IL-8_{77} to shorter isoforms was demonstrated by researchers. Conversion to IL-8_{72} was demonstrated in the presence of α-thrombin (Hebert et al., 1990a), plasmin (Nakagawa et al., 1991b), by cathepsin-L (Ohashi et al., 2003) and in the presence of R-gingipain (proteinase from *Porphyromonas gingivalis*, an oral anaerobic rod strongly associated with active periodontitis) (Dias et al., 2008). Matrix metalloproteinase-9 (MMP-9) is another proteinase which converted IL-8_{77} to IL-8_{71} (Van den Steen et al., 2000).

1.13.4. Functional activity of different isoforms of IL-8

Conversion of IL-8_{77} to IL-8_{72} by α-thrombin resulted in difference of potency of the isoforms in functional assays (Hebert et al., 1990a). IL-8_{72} was found to be 10-times more potent in inhibiting adhesion of neutrophils to IL-1 stimulated endothelial-cell monolayers. In the presence of R-gingipains, the chemotactic activity of IL-8_{77} was found to be higher than untreated IL-8_{77}, as it is converted to IL-8_{72} (Dias et al., 2008). After conversion of IL-8_{77} by MMP-9 to IL-8_{71}, a 10-fold lower concentration was able to achieve half-maximal binding to neutrophil receptors. Also, IL-8_{71} was able to mobilise intracellular calcium at 20-times lower concentration compared to IL-8_{77}. Similar differences in functional potency were observed by the authors in release of gelatinase-B from neutrophils and chemotaxis of neutrophils, with the shorter isoform being more potent.

To assess possible functional differences in the two main IL-8 isoforms, Huber *et al.* looked at neutrophil invasion through a blood-vessel wall construct (Huber et al., 1991). Whilst differences in potency were noted between the two isoforms, both caused PMN attachment to endothelial cells and subsequent migration to basal layers. This is in contrast to an earlier report (Gimbrone et al., 1989) suggesting that the 77-aa isoform is a specific inhibitor of PMN-endothelial cell interaction. This potential contrast can be explained by the difference in the two models systems: Huber *et al* created a chemotactic gradient by using conditioned media on one side of the cellular

construct and not stimulating the endothelial cells directly. On the other hand, Gimbrone *et al* stimulated endothelial cells to produce IL-8_{77}, thus abolishing the chemotactic gradient. However, the physiological relevance of either of these observations are not entirely clear; the model used by Gimbrone *et al.* is probably more physiologic as endothelial cells are stimulated by IL-1β and TNF-α during an inflammatory cascade. In an extension of the study, preloaded intravenous IL-8 (both isoforms) inhibited intradermal accumulation of PMNs in rabbits, when challenged with cytokines (Hechtman et al., 1991). No differences in potency were noted between the two isoforms.

In a detailed *in vitro* study of functional activity of the different isoforms (Clark-Lewis et al., 1991), the authors showed a significant difference in potency between IL-8_{77} and IL-8_{72} in their ability to release elastase from neutrophils and in displacing radio-iodine conjugated IL-8 from receptor binding sites. Other important findings from this study was confirming the essential role of the E-L-R sequence at position 69-67 (from the COOH-terminal) for functional activity, as this region is involved in binding to the receptor. A truncated form of IL-8 with missing AA from the COOH terminal was also found to be functionally active, although less potent compared to the full protein, but nonetheless confirming the non-essential nature of the COOH-terminal amino acids for function.

In a direct comparison between the functional activity of IL-8_{72} and IL-8_{77} *in vitro*, the shorter protein was found to have significantly increased potency in elevating cytoplasmic calcium and in displacing bound IL-8 from cell-surface receptors (Nourshargh et al., 1992). However, no significant difference in potency was noted in accumulation of neutrophils at sites of injection *in vivo*. The authors speculated on the possibility of IL-8_{77} being cleaved by proteases *in vivo* to shorter isoforms, resulting in a lack of difference in functional activity.

More recent studies on functional activity of IL-8 isoforms (Mortier et al., 2011b, Proost et al., 2008) confirmed the differences in potency of the main isoforms *in vivo*. Importantly, the authors also demonstrated a higher angiogenic potency of IL-8_{72} in rabbit corneal micropockets (Proost et al., 2008) and accumulation of neutrophils after intra-peritoneal injection compared to IL-8_{77} (Proost et al., 2008,

Mortier et al., 2011b). Thus, the differences in potency between the two major isoforms *in vitro* was reflected by functional differences *in vivo*. The authors proposed a classification of IL-8 isoforms based on their functional activity (figure 1.9)

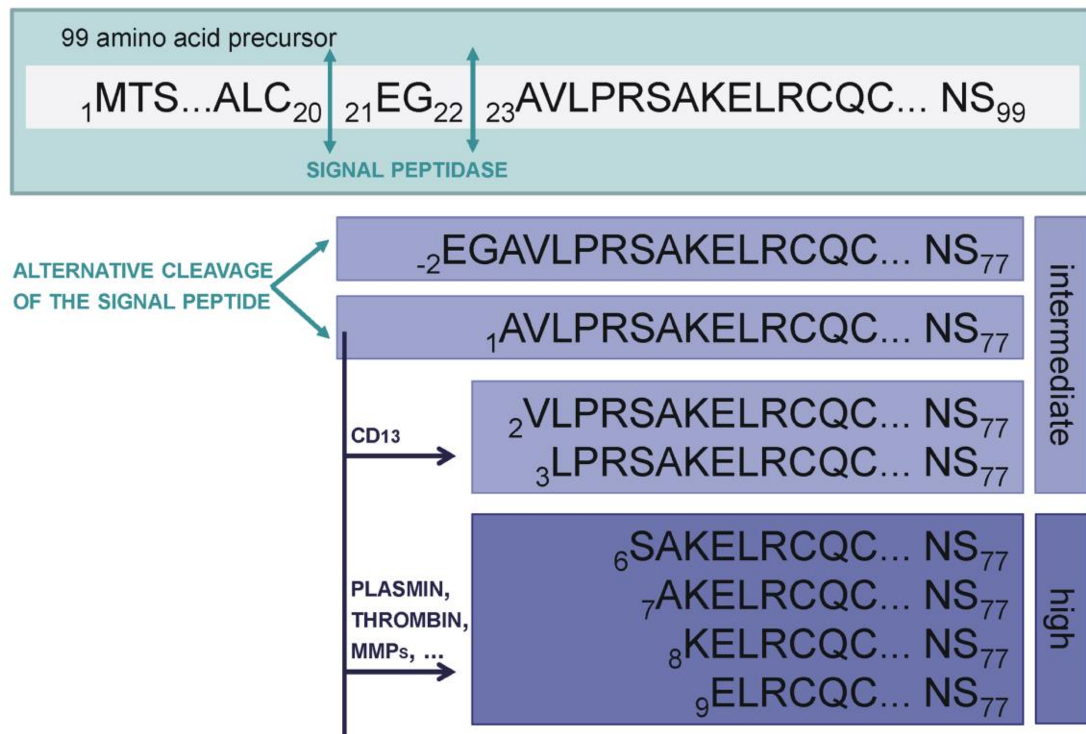


Figure 1-10: Overview of the naturally occurring CXCL8 isoforms. Several NH_2-terminally modified isoforms of CXCL8 have been purified from the conditioned medium of leukocytes, fibroblasts and endothelial cells. The NH_2-terminal and COOH-terminal sequences of these isoforms are depicted in the one-letter code (B = citrulline). Based on the criteria formulated by Von Heijne (von Heijne, 1983), the 99 amino acid precursor molecule may be cleaved by signal peptidases between Cys_{20} and Glu_{21} or between Gly_{22} and Ala_{23}, the latter being more probable. CXCL8(1-77) can be cleaved by a number of proteases, resulting in the listed NH_2-terminally truncated forms. These NH_2-terminal modifications differently affect the ability of CXCL8 to recruit neutrophils. The *in vivo* neutrophil-attracting activity is defined in the boxes at the right (Mortier et al., 2011b).

1.13.5. IL-8 in preterm infants and in CLD/BPD

IL-8 has been extensively studied in lung fluid from preterm ventilated infants (Ryan et al., 2008, Bose et al., 2008). Significantly increased IL-8 in lung fluid from infants who later develop CLD, compared to infants who don't, has been a consistent finding in all studies. However, the isoforms of IL-8 in the preterm lung has never been looked at.

Until the early 2000s, an assay to selectively and easily detect IL-8_{77} did not exist. The earlier ELISAs (Ko et al., 1992), as well as commercially available ELISA were able to detect "total" IL-8. In 2002, a specific monoclonal antibody was developed for IL-8_{77} which was used to detect this isoform in an ELISA (Nashkevich et al., 2002a). Based on the ELISA, a recent study on circulating IL-8 in preterm infants (Maheshwari et al., 2009) suggested that IL-8_{77} may be the dominant isoform compared to term infants and adults. The authors suggested that IL-8 expression is regulated by developmental changes taking place in the foetus, with more mature and active form predominating at later gestations (term) and during adulthood. If this is reflected in the preterm lung, it can have profound effects on the functional activity of IL-8 as the long isoform (IL-8_{77}) is significantly less potent than the more common shorter isoform (IL-8_{72}).

1.13.6. Summary

IL-8 is one of the most potent pro-inflammatory chemokines involved in attracting and activating neutrophils to sites of inflammation. In preterm ventilated infants, the lung is a common site of inflammation and IL-8 concentrations are found to be consistently high in infants who develop CLD. The different isoforms of IL-8 have confirmed differences in functional potency, both *in vitro* and *in vivo*. However, this aspect of inflammation has not been studied in the preterm lungs. Potentially, a better understanding of the expression of different IL-8 isoforms may result in novel pathways of modulation of lung inflammation in preterm infants.

1.14. Summary and Background of the Project

CLD is a disease which is prevalent primarily in survivors after extreme preterm birth. These infants need respiratory support in the short- to medium-term, and have been noted to develop long-term respiratory sequelae. Extreme prematurity and extremely low birth weight are the two main risk factors for development of this condition. However, several clinical risk factors have been implicated to contribute to the development of CLD including chorioamnionitis, mechanical ventilation, post-natal infection and oxygen toxicity among others. Inflammation seems to be the final common pathway which leads to lung injury, and subsequent interruption of lung growth at an immature stage. The pattern of inflammation in CLD shows a persistent influx of acute inflammatory cells, mainly PMNs, and low numbers of mononuclear cells. Thus a persistent and poorly resolved neutrophilia is characteristic in the lungs of preterm infants who develop CLD later. Better understanding of the pathways of resolution of inflammation in the preterm lungs could lead to potential therapeutic targets to modulate the process with possible clinical benefits for these infants.

IL-6 *trans*-signalling has already been shown to have a pro-resolution effect on experimental inflammation, by down-regulating expression of IL-8 (a key chemokine involved in recruiting PMNs to sites of inflammation) and up-regulating MCP-1 (a chemokine involved in recruiting mononuclear cells), which switches the influx of cells from mainly neutrophils to mononuclear cells. Thus, if effective, IL-6 *trans*-signalling could potentially reverse the two key defects in the inflammatory pathway in the preterm lungs, *viz.* persistent influx of PMNs and inadequate recruitment of mononuclear cells. Expression and functional activity of the members of the IL-6 *trans*-signalling pathway have not been explored in detail in the preterm lung.

Interleukin-8 is a key chemokine which attracts neutrophils to sites of inflammation along a chemical gradient. It has been strongly implicated in the pathogenesis of CLD, which shows a pattern of persistent neutrophilic lung inflammation. Modulation of the biological effects of IL-8 could be a key step in limiting inflammation and its effects on lung injury in preterm infants. The long isoform of IL-8, IL-8_{77} is functionally less potent compared to the most common isoform IL-8_{72}.

Since several enzymes expressed at sites of inflammation can convert IL-8_{77} to shorter isoforms, understanding these processes could be provide us with new approaches toward modulating IL-8 activity and inflammation in the preterm lung.

Modelling human diseases in appropriate animals to study mechanisms and gather evidence of efficacy of new interventions is a well established strategy. Rodents have been used as a common animal model for several human diseases, specifically murine models, due to the availability and ease of genetic knockouts. Several researchers have used animal models to study effects of risk factors on lung development in newborn rodents (Auten et al., 2001, Choi et al., 2013, Franco-Montoya et al., 2009) and lambs (Kramer et al., 2002, Willet et al., 2000). However, the most physiologically relevant model of CLD is the primate model in preterm baboons (Coalson et al., 1999b). These animals are delivered preterm at gestational age 2/3rd of term (equivalent to extreme prematurity in human infants) after antenatal steroids, receive exogenous surfactant replacement after birth and are ventilated gently in sufficient oxygen to maintain adequate saturations. After a variable period of time, these animals are sacrificed and their lungs are studied histologically, showing changes similar to human CLD. As this care reflects current clinical practice in human infants closely, in animals that are evolutionally closest to our species, this makes the baboon model of CLD the most relevant for research.

1.15. Hypothesis and Aims of the project

On the above background, the main hypothesis of my project is that modulation of neutrophil infiltration into the preterm lungs may be regulated by

1. the effects of IL-6 *trans*-signalling
2. altering functional effects of IL-8 isoforms.

Since limited information is available about the effects of these two pathways in the preterm lungs, my project was designed to start as an observational study, to proceed to identifying potential therapeutic targets at the molecular level in appropriate laboratory-based *in-vitro* models.

The specific aims of the project are detailed below.

1.15.1. IL-6 *trans*-signalling in the preterm lung

- To determine the expression of the IL-6, sIL-6R, sgp130, IL-8 and MCP-1 in broncho-alveolar lavage fluid (BALF) from ventilated preterm baboons and human infants. To determine the concurrent pattern of influx of inflammatory cells in BALF from preterm ventilated baboons and human infants.
- To analyse possible correlations between expression of the IL-6 *trans*-signalling molecules, the related chemokines and influx of inflammatory cells.
- To compare expression in the human infants between those who develop CLD and those who do not.
- To develop a sensitive and specific bioassay to measure IL-6 functional activity (*cis*- and *trans*-signalling) in BALF from preterm ventilated infants.
- To compare functional activity between infants who develop CLD and those who do not.

1.15.2. IL-8 isoforms in the preterm lung

- To determine the expression of total IL-8 and IL-8_{77} in BALF from preterm ventilated infants.
- To compare the expression of total IL-8 and IL-8_{77} in infants who develop CLD with those who do not.
- To determine the functional potential of BALF to convert IL-8_{77} to shorter isoforms and identify possible proteases involved in the mechanisms of conversion.
- To determine the effect of proteases, potentially involved in the above conversion, on IL-8_{77} *in-vitro* and determine the functional relevance of this conversion on PMNs.

2. Materials and Methods

2.1. Buffers and Solutions

2.1.1.

2.1.2. Phosphate Buffered Saline (PBS)

10 x concentrated stock solution (Fisher Scientific, Loughborough, UK) was used to prepare a working solution of 1 x concentration by diluting the stock 1 in 10 in de-ionised water (dH_2O). Freshly prepared before use.

2.1.3. Assay diluent for total IL-8 ELISA (PBS + 10% foetal calf serum)

PBS (1 x) was mixed with 10% (v/v) of heat inactivated (see later) foetal calf serum (FCS, Sigma-Aldrich Company Ltd., Gillingham, UK) and stored frozen at -20°C (henceforth called PBS FCS).

2.1.4. Protease-free bovine serum albumin (BSA) stock solution

10% BSA (w/v) (Sigma-Aldrich Company Ltd., Gillingham, UK) was diluted in PBS (1x) and stored frozen at -20°C (henceforth called BSA).

2.1.5. ELISA wash buffer

PBS (1 x) was supplemented with 0.05% (v/v) Tween-20 solution (Fisher Scientific, Loughborough, UK) and stored at 4°C.

2.1.6. ELISA wash buffer for IL-8_{77} ELISA

ELISA wash buffer as before with 300 mM sodium chloride (NaCl) (Fisher Scientific, Loughborough, UK).

2.1.7. Tris buffer for all protease experiments (TBS BSA)

50 mM tris(hydroxymethyl)aminomethane (Tris) (Fisher Scientific, Loughborough, UK) with 154 mM NaCl, pH 7.4, and 0.05% (w/v) BSA. Freshly prepared before use.

2.1.8. Sodium citrate solution

3.8% (w/v) sodium citrate (citrate) (Fisher Scientific, Loughborough, UK) was dissolved in 0.9% NaCl solution (Fresenius Kabi, Runcorn, UK). Solution was sterile filtered by passing through a low protein-binding polyethersulfone (PES) membrane with a pore-size of 0.22 µm (Elkay Labs, Basingstoke, UK) and stored at room temperature.

2.1.9. Dextran solution

6% Dextran (w/v) from Leuconostoc mesenteroides (dextran), average mol wt 425,000 to 575,000 D (Sigma Aldrich Company Ltd., Gillingham, UK) was dissolved in 0.9% NaCl solution. Solution was sterile filtered by passing through a low protein-binding polyethersulfone (PES) membrane with a pore-size of 0.22 µm and stored at 4°C.

2.1.10. Percoll solution

90% working solution (v/v) was prepared from Percoll stock solution (100%), density 1.125 – 1.135 g/ml (Sigma Aldrich Company Ltd., Gillingham, UK) by diluting with 0.9% NaCl solution. Solution was prepared sterile and stored at 4°C. Henceforth, Percoll refers to the 90% solution.

2.1.11. Purified human neutrophil elastase and cathepsin G buffer solution (for diluting lyophilised powder)

50 mM sodium acetate (Fisher Scientific, Loughborough, UK) pH 5.5 with 150 mM NaCl.

2.2. Cytokines, Chemokines and Inflammatory Stimuli

Molecule	Company	Typical stock concentration
Recombinant human interleukin-6 (rh IL-6)	R & D Systems, Abingdon, UK	30 µg/ml
Recombinant human soluble interleukin-6 receptor (rh sIL-6R)	R & D Systems, Abingdon, UK	30 µg/ml
Hyper interleukin-6 (Hyper IL-6)	Generous gift from Prof Stefan Rose-John	30 µg/ml
Recombinant human soluble gp130 (rh sgp130)	R & D Systems, Abingdon, UK	120 µg/ml
Recombinant human soluble gp130 Fc chimera (rh sgp130Fc)	Generous gift from Prof Stefan Rose-John	120 µg/ml
Recombinant human interleukin-1β (rh IL-1β)	Peprotech EC, London, UK	100 µg/ml
Recombinant human interleukin-8 (72 a.a.) (rh IL-8_{72})	Peprotech EC, London, UK	100 µg/ml
Recombinant human interleukin-8 (77 a.a.) (rh IL-8_{77})	Peprotech EC, London, UK	100 µg/ml
Lipopolysaccharide (LPS) from Escherichia Coli 0127:B8	Sigma Aldrich Company Ltd., Gillingham, UK	10 mg/ml
N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)	Sigma Aldrich Company Ltd., Gillingham, UK	10 µM

Table 2-1: List of cytokines, chemokines and other inflammatory stimuli

2.3. Antibodies

Antigen	Clone	Isotype	Conjugate	Company	Stock conc.	Dilution factor
Human IL-6	MQ2-13A5	Rat IgG1, κ		BioLegend, London, UK	1 mg/ml	
Isotype control	RTK2071	Rat IgG1, κ		BioLegend, London, UK	0.5 mg/ml	
Mouse sIL-6R	D7715A7	Rat IgG2b, κ		BioLegend, London, UK	1 mg/ml	
Isotype control	RTK4530	Rat IgG2b, κ		BioLegend, London, UK	0.5 mg/ml	
Mouse sIL-6R	2B10			NovImmune, Switzerland	6 mg/ml	
Isotype control	Y13			NovImmune, Switzerland	4 mg/ml	
Human CD14	M5E2	Mouse IgG2a, κ	FITC	BioLegend, London, UK		1/100
Isotype control	MOPC-173	Mouse IgG2a, κ	FITC	BioLegend, London, UK		1/100
Human IL-8_{77}	N11	Mouse IgG1, κ		Generous gift from Prof N Voitenok, Moscow	1.9 mg/ml	1/360
Human IL-8_{77}	H6	Mouse IgG1, κ		Generous gift from Prof N Voitenok, Moscow		

Table 2-2: List of antibodies. FITC = Fluorescein Isothiocyanate

2.4. Other Reagents

Molecule/ Reagent	Company	Typical Stock Solution
Purified human neutrophil elastase (HNE)	Athens Research & Technology, Athens, USA	500 µg/ml (16.9 µM)
Purified human neutrophil cathepsin G (CG)	Athens Research & Technology, Athens, USA	500 µg/ml (16.9 µM)
Purified human neutrophil proteinase-3 (Pr-3)	Athens Research & Technology, Athens, USA	1000 µg/ml (33.8 µM)
Purified human plasma alpha-1 antitrypsin (AAT)	Talecris Plasma Resources, NC, USA	50 mg/ml (1.0 mM)
Purified human plasma antithrombin III (ATIII)	Merck Chemicals Limited, Nottingham, UK	1.0 mg/ml
Phenylmethanesulfonyl fluoride (PMSF)	Sigma Aldrich Company Ltd., Gillingham, UK	200 mM
1, 10 phenanthroline	Sigma Aldrich Company Ltd., Gillingham, UK	500 mM
[3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES)	Promega UK, Southampton, UK	Ready to use solution
Cytochalasin B	Sigma Aldrich Company Ltd., Gillingham, UK	5 mg/ml
2-mercaptoethanol (2ME)	Sigma Aldrich Company Ltd., Gillingham, UK	Ready to use solution at 14.3M

Table 2-3: List of other reagents used.

2.5. Enzyme Linked Immunosorbent Assay (ELISA)

2.5.1. Human IL-6 ELISA

Human IL-6 ELISA (DuoSet, R&D Systems, Abingdon, UK) was performed according to the manufacturer's guidelines. Briefly, NUNC Maxisorp 96-well plates (Fisher Scientific, Loughborough, UK) were coated with 100 µl of 2 µg/ml capture antibody (mouse anti-human IL-6, stock 360 µg/ml) diluted in PBS and incubated overnight at room temperature. Wells were washed with 200 µl wash buffer (x 3) and blocked with 200 µl of assay diluent (Reagent Diluent, 1% BSA in PBS, R&D Systems, Abingdon, UK) for 1 hour at room temperature (RT). The wash step was repeated as before prior to adding 100 µl of standard, human BALF or baboon tracheal aspirate fluid (TAF), all in duplicate. Top standard of 600 pg/ml was prepared from recombinant human IL-6 (35 ng/ml) and a seven point standard curve using 2-fold serial dilution was prepared using assay diluent. BALF samples were initially diluted between 1/5 and 1/10; if values were beyond the range of the curve, then higher dilutions or "neat" samples were used as appropriate. Baboon TAF samples were diluted between 1/10 and 1/200 in the manufacturer's recommended buffer. Control wells contained assay diluent. Following 2 hour incubation at RT, wells were washed as before, 100 µl of 200 ng/ml of biotinylated detection antibody (goat anti-human IL-6; stock 36 µg/ml) was added and incubated at RT for 2 hours. Wash step was repeated and 100 µl Streptavidin conjugated horseradish-peroxidase (HRPO) enzyme (1 in 200 dilution from stock, R&D Systems, Abingdon, UK) was added for 20 minutes at RT in the dark. Following the wash step (x 5), 100 µl of tetramethylbenzidine (TMB, substrate solution, eBioscience, Hatfield, UK) was added. Once the standards had developed, 50 µl of 2 N sulphuric acid was added to stop the reaction. Optical density of each well was determined using a microplate reader (Dynex Corporation, Worthing, UK) at 450 nm with wavelength correction at 570 nm. Range of detection was between 9.38 – 600 pg/ml.

2.5.2. Human sIL-6R ELISA

Human sIL-6R ELISA (DuoSet, R&D Systems, Abingdon, UK) was performed according to the manufacturer's guidelines. Briefly, NUNC Maxisorp 96-well plates were coated with 100 µl of 2 µg/ml capture antibody (mouse anti-human IL-6R, stock 360 µg/ml) diluted in PBS and incubated overnight at room temperature. Wells were washed with 200 µl wash buffer (x 3) and blocked with 200 µl of assay diluent (Reagent Diluent, 1% BSA in PBS, R&D Systems) for 1 hour at RT. The wash step was repeated as before prior to adding 100 µl of standard, human BALF, human plasma or baboon TAF in duplicate. Top standard of 1000 pg/ml was prepared from recombinant human sIL-6R (70 ng/ml) and a seven point standard curve using 2-fold serial dilution was prepared using assay diluent. BALF samples were initially diluted between 1/5 and 1/10; if values were beyond the range of the curve, then higher dilutions were used as appropriate. Plasma samples were diluted 1/50 or as appropriate. Baboon TAF samples were diluted between 1/10 and 1/200 in the manufacturer's recommended buffer. Control wells contained assay diluent. Following 2 hour incubation at RT, wells were washed as before, 100 µl of 100 ng/ml of biotinylated detection antibody (goat anti-human IL-6R; stock 18 µg/ml) was added and incubated at RT for 2 hours. Wash step was repeated and 100 µl Streptavidin conjugated HRPO enzyme (1 in 200 dilution from stock, R&D Systems) was added for 20 minutes at RT in the dark. Following the wash step (x 5), 100 µl of TMB was added. Once the standards had developed, 50 µl of 2 N sulphuric acid was added to stop the reaction. Optical density of each well was determined using a microplate reader at 450 nm with wavelength correction at 570 nm. Range of detection was between 15.6 – 1000 pg/ml.

2.5.3. Human Differentially Spliced (DS) sIL-6R ELISA

Human DS sIL-6R was measured as described previously (Horiuchi et al., 1998). Briefly, NUNC Maxisorp 96-well plates were coated with 100 µl of 3 ug/ml anti-human DS-sIL-6R monoclonal antibody (2F3, stock 3 mg/ml) in PBS and incubated overnight at 4ºC. Wells were washed with 200 µl wash buffer (x 3) and blocked with 200 µl of assay diluent (5% BSA w/v in PBS) for 1 hour at RT. The wash step was repeated as before

prior to adding 100 µl of standard, BALF or plasma in duplicate. Top standard of 1000 pg/ml was prepared from recombinant human DS sIL-6R (2 µg/ml) and a seven point standard curve using 2-fold serial dilution was prepared using assay diluent. All BALF & term cord-blood plasma samples were diluted 1/2 and adult plasma samples were diluted 1/5 in assay diluent. Control wells contained assay diluent. Following 2 hour incubation at RT, wells were washed as before, 100 µl of 100 ng/ml of biotinylated detection antibody (anti-human IL-6R, baf227; stock 100 µg/ml) was added and incubated at RT for 2 hours. Wash step was repeated and 100 µl Streptavidin conjugated HRPO enzyme (1/200 dilution from stock, R&D Systems) was added for 20 minutes at RT in the dark. Following the wash step (x 5), 100 µl of TMB was added. Once the standards had developed, 50 µl of 2 N sulphuric acid was added to stop the reaction. Optical density of each well was determined using a microplate reader at 450 nm with wavelength correction at 570 nm. Range of detection was between 15.6 – 1000 pg/ml.

2.5.4. Human sgp130 ELISA

Human sgp130 ELISA (DuoSet, R&D Systems, Abingdon, UK) was performed according to the manufacturer's guidelines. Briefly, NUNC Maxisorp 96-well plates were coated with 100 µl of 2 µg/ml capture antibody (mouse anti-human gp130, stock 720 µg/ml) diluted in PBS and incubated overnight at room temperature. Wells were washed with 200 µl wash buffer (x 3) and blocked with 200 µl of assay diluent (Reagent Diluent, 1% BSA in PBS, R&D Systems) for 1 hour at RT. The wash step was repeated as before prior to adding 100 µl of standard, human BALF or baboon TAF in duplicate. Top standard of 10,000 pg/ml was prepared from recombinant human sgp130 (150 ng/ml) and a seven point standard curve using 2-fold serial dilution was prepared using assay diluent. BALF samples were initially diluted between 1/10 and 1/20; if values were beyond the range of the curve, then higher dilutions were used as appropriate. Baboon TAF samples were diluted between 1/10 and 1/200 in the manufacturer's recommended buffer. Control wells contained assay diluent. Following 2 hour incubation at RT, wells were washed as before, 100 µl of 400 ng/ml of biotinylated detection antibody (goat anti-human gp130; stock 72 µg/ml) diluted in Reagent Diluent

with 2% heat inactivated normal goat serum was added and incubated at RT for 2 hours. Wash step was repeated and 100 µl Streptavidin conjugated HRPO enzyme (1/200 dilution from stock, R&D Systems) was added for 20 minutes at RT in the dark. Following the wash step (x 5), 100 µl of TMB was added. Once the standards had developed, 50 µl of 2 N sulphuric acid was added to stop the reaction. Optical density of each well was determined using a microplate reader at 450 nm with wavelength correction at 570 nm. Range of detection was between 156 – 10000 pg/ml.

2.5.5. Human IL-8 ELISA

Total human IL-8 ELISA (BD Biosciences, Oxford, UK) was performed according to the manufacturer's guidelines. Briefly, NUNC Maxisorp 96-well plates were coated with 100 µl/well capture antibody diluted 1/250 in coating buffer (0.1 M sodium carbonate, pH 9.5: 7.13 g sodium bicarbonate, 1.59 g sodium carbonate; q.s. to 1.0 L; pH to 9.5 with 10 N sodium hydroxide) and incubated overnight at 4ºC. Wells were washed with 200 µl wash buffer (x 3) and blocked with 200 µl of assay diluent (PBS FCS) for 1 hour at RT. The wash step was repeated as before prior to adding 100 µl of standard, human BALF, human cell-culture supernatant or baboon TAF in duplicate. Human BALF and supernatants were diluted between 1/100 and 1/200, or as appropriate. Baboon TAF samples were diluted between 1/10 and 1/200 in the manufacturer's recommended buffer. Top standard of 200 pg/ml was prepared from lyophilized recombinant human IL-8 (95 ng/ml, Peprotech EC, London, UK) and a seven point standard curve using 2-fold serial dilution was prepared. PBS FCS was used as the control. Following a 2 hour incubation and wash step (x 5), 100 µl of the working detector (detection antibody and streptavidin-HRPO, each diluted 1/250 in PBS FCS) was added to each well. After a 1 hour incubation, the wells were washed (x 7) and 100 µl of TMB was added. Once the standards had developed, 50 µl of 2 N sulphuric acid was added to stop the reaction. Optical density of each well was determined using a microplate reader at 450 nm with wavelength correction at 570 nm. Range of detection was between 3.125 – 200 pg/ml.

2.5.6. Human IL-8_{77} ELISA

Human IL-8_{77} was detected as described previously (Nashkevich et al., 2002a) with modifications. NUNC Maxisorp 96-well plates were coated with 100 µl/well of 5 µg/ml capture antibody N11 (stock 1.3 mg/ml) and incubated overnight at 4°C. Wells were washed with 200 µl wash buffer (x 3) for IL-8_{77} ELISA (PBS + 0.05% Tween-20 + 300mM NaCl) and blocked with 200 µl of assay diluent (IL-8_{77} wash buffer + 0.5% protease-free BSA) for 1 hour at room temperature. The wash step was repeated as before prior to adding 100 µl of standard, BALF or cell-culture supernatant in duplicate. Top standard of 200 pg/ml was prepared from recombinant human IL-8_{77} (10 µg/ml) and a seven point standard curve using 2-fold serial dilution was prepared. Assay diluent was used as the control. Following a 2 hour incubation and wash step (x 3), 100 µl of the working detector (detection antibody from BD OptEIA human IL-8 ELISA kit and streptavidin-HRPO, each diluted 1/250 in assay diluent) was added to each well. After a 1 hour incubation, the wells were washed (x 5) and 100 µl of TMB was added. Once the standards had developed, 50 µl of 2 N sulphuric acid was added to stop the reaction. Optical density of each well was determined using a microplate reader at 450 nm with wavelength correction at 570 nm. Range of detection was between 3.125 – 200 pg/ml.

2.5.7. Human monocyte chemotactic protein-1 (MCP-1) ELISA

Human MCP-1 ELISA (Human CCL-2 ELISA Ready-SET-Go! eBioscience,) was performed according to the manufacturer's guidelines. Briefly, NUNC Maxisorp 96-well plates were coated with 100 µl/well capture antibody (purified anti-human CCL2; clone 5D3-F7) diluted 1/250 in coating buffer (coating buffer powder reconstituted in 1.0 L deionised water) and incubated overnight at 4°C. Wells were washed with 250 µl wash buffer (x 5) and blocked with 200 µl of assay diluent (supplied as 5 x; diluted to 1 x in sterile water) for 1 hour at room temperature. The wash step was repeated as before prior to adding 100 µl of standard, human BALF, cell-culture supernatant or baboon TAF in duplicate. Human BALF and supernatants were diluted between 1/100 and 1/200, or as appropriate. Baboon TAF samples were diluted between 1/10 and 1/200

in the manufacturer's recommended buffer. Top standard of 1000 pg/ml was prepared from stock recombinant human MCP-1 solution at 1.0 µg/ml and a seven point standard curve using 2-fold serial dilution was prepared. Assay Diluent was used a s negative control. Following a 2-hour incubation at RT and a wash step, the detection antibody (biotin-conjugate anti-human CCL2; clone 2H5) was diluted 1/250 and 100 µl/well was added and incubated for 1 hour. Wells were washes as before and 100 µl of enzyme (Avidin-HRPO) was added for 30 minute incubation at RT. Wash step (x 7) was followed by the addition of 100 µl/well of TMB. Once the standards had developed, 50 µl of 2 N sulphuric acid was added to stop the reaction. Optical density of each well was determined using a microplate reader (Dynex Corporation) at 450 nm with wavelength correction at 570 nm. Range of detection was between 15.625 – 1000 pg/ml.

2.5.8. Human total matrix-metalloprotease-9 (MMP-9) ELISA

Human MMP-9 ELISA (R&D Systems DuoSet) was performed according to the manufacturer's guidelines. Briefly, NUNC Maxisorp 96-well plates were coated with 100 µl of 1 µg/ml capture antibody (mouse anti-human MMP-9; stock 180 µg/ml) diluted in PBS and incubated overnight at RT. Wells were washed with 200 µl wash buffer (x 3) and blocked with 200 µl of assay diluent (Reagent Diluent, 1% BSA in PBS, R&D Systems) for 1 hour at RT. The wash step was repeated as before prior to adding 100 µl of standard or supernatant in duplicate. Top standard of 2000 pg/ml was prepared from recombinant human MMP-9 (50 ng/ml) and a seven point standard curve using 2-fold serial dilution was prepared using assay diluent. Samples were diluted between 1/100 and 1/200. Control wells contained assay diluent. Following 2 hour incubation at RT, wells were washed as before, 100 µl of 100 ng/ml biotinylated detection antibody (goat anti-human MMP-9; stock 36 µg/ml) diluted in Reagent Diluent with 2% heat inactivated normal goat serum was added and incubated at RT for 2 hours. Wash step was repeated and 100 µl Streptavidin conjugated HRPO enzyme (1/200 dilution from stock, R&D Systems) was added for 20 minutes at RT in the dark. Following the wash step (x 5), 100 µl of TMB was added. Once the standards had

developed, 50 µl of 2 N sulphuric acid was added to stop the reaction. Optical density of each well was determined using a microplate reader (Dynex Corporation) at 450 nm with wavelength correction at 570 nm. Range of detection was between 31.25 – 2000 pg/ml.

2.5.9. Proteinase-3 ELISA

[Proteinase-3 ELISA was designed by Dr Brad Spiller in the department, using commercial reagents. BALF samples from preterm infants were performed and analysed by Dr Salima Abdulla]

Mouse monoclonal anti-proteinase 3 antibody (Hycult Biotechnology, Netherlands) was immobilised onto 96-well plates prior to blocking with 5% BSA in PBS containing 0.5% Tween-20 (blocking buffer). Samples and standards were diluted in blocking buffer and incubated with the coated plates for 1 h. Purified proteinase-3 (Athens Research and Technology, GA, USA) was diluted from 0 - 250ng/ml for the standard curve. Unbound protein was washed away with PBS prior to detection with rabbit polyclonal anti-proteinase-3 antiserum (Eurogentec, Belgium) and peroxidase-conjugated donkey anti-rabbit immunoglobulin (Jackson Immuno Research Laboratories, Newmarket, UK). Plates were developed with OPD-EASY (Acros organics, Belgium), and the reaction stopped with 2N H_2SO_4 before reading at 490 nm.

2.6. Primate (baboon) model of CLD

[All animal procedures were by Drs JJ Coalson and V Winter in San Antonio, Texas. ELISAs were performed by Dr E McGreal on site at San Antonio, Texas. Differential counts from digital images of cytospins and data analysis were undertaken in Cardiff by me.]

2.6.1. Animal Management

All animal husbandry, handling and procedures were as previously described (Coalson et al., 1999a). These were performed at the Southwest Foundation for Biomedical Research (San Antonio, Texas, USA), approved by the Institutional Animal

Care and Use Committee and conformed to American Association for Accreditation of Laboratory Animal Care Guidelines. Briefly, preterm baboons *(Papio cynocephalus)* were delivered by elective caesarean section under general anaesthesia at 125 ± 2 or 140 ± 2 days (term: 185 days) from pregnant dams. Antenatal steroids were given 48 and 24 h before planned delivery. All newborns were intubated at birth, received a single bolus (100 mg/kg) of exogenous surfactant (Survanta; donated by Ross Laboratories, Columbus, Ohio, USA) prior to ventilation with a pressure-limited, time-cycled infant ventilator (InfantStar; Infrasonics, San Diego, Calif., USA) for 14 days. Ventilation was initiated at a rate of 40 breaths/min with peak inspiratory pressure (PIP) sufficient to move the chest, positive end-expiratory pressure (PEEP) at 5 cm H_2O and fraction of inspired oxygen (FiO_2) at 0.4. A volume-sparing ventilatory strategy was followed to maintain tidal volumes at 4–6 ml/kg. Briefly, the ventilation rate was adjusted as required to regulate $PaCO_2$ between 45 and 55 mm Hg to a maximum rate of 60. If a PIP > 40 cm H_2O was needed to achieve target tidal volumes and $PaCO_2$, the acceptable limit for $PaCO_2$ was increased to 65 mm Hg. Target goals for PaO_2 were 55–70 mm Hg. Oxygenation was primarily manipulated through changes in PEEP and its effect on mean airway pressure (P_{aw}) and FiO_2. All animals survived to necropsy following administration of sodium pentobarbital at day 14 (336 h). None of the study animals exhibited any evidence of pneumonia histopathologically or by chest radiograph, nor was sepsis evident clinically or microscopically.

2.6.2. Tracheal Aspirate and Lavage Fluids and Cell Counts

Tracheal aspirate fluid (TAF) was obtained at the indicated time-points (day 1 = 24 h, day 3, day 6-7, day 10-11 and day 14) from 10 animals undergoing ventilation PRN by disconnecting the ventilator and instilling 1 ml sterile saline via the endo-tracheal tube. The ventilator was reconnected and 4–5 breaths were allowed before disconnecting once more and aspirating the fluid. This was repeated once and finally the catheter lumen was cleared with 1 ml of sterile saline suctioned into the Luken's trap. In the case of five 125-day and five 140-day gestational control animals, necropsy lavage was performed by instilling the lower left lobe with sterile saline via the bronchus until the lobe was completely filled; saline was withdrawn and the procedure

repeated four more times. Total cell counts were performed on TAF and necropsy lavage specimens using a hemocytometer. TAF and lavage specimens were centrifuged at 1,500 rpm for 10 min in a refrigerated centrifuge, supernatants were aliquoted and stored at –70 ° C.

2.6.3. Differential Cell Counts of Baboon Cytospins

Digital images of four different fields of Wright-Giemsa stained cytospin slides from baboon TAFs were taken. Differential counts were obtained by counting at least 300 events from each slide (field) manually (total 1200 events from 4 slides) and total cell numbers (polymorphonuclear leucocytes, mononuclear cells, epithelial cells & others) and percentages calculated from the differential count.

2.6.4. ELISA

As described before in section 2.5. IL-6, IL-8 and MCP-1 have previously been measured in this species using human antibodies (Coalson et al., 1999a, Viscardi et al., 2006b). Commercial human ELISA kit were used, as described above.

2.7. CLD in Preterm Ventilated Infants

Ethical approval for project was obtained by Prof S Kotecha. Consent and collection of BALF was undertaken by Drs PL Davies and Dr N. Maxwell. IL-6, sIL-6R and sgp130 ELISAs were performed by Dr. Mallinath Chakraborty, with some data also contributed by Dr Eamon McGreal

2.7.1. Patient Groups

Ventilated preterm infants born before 32 weeks gestation were recruited, after obtaining written parental consent, from the Regional Neonatal Unit at University Hospital of Wales in Cardiff, UK. Ethical approval was obtained from the local Research Ethics Committee. Infants with significant hypoxia, known congenital abnormalities, abnormal morphology or who were extremely unwell (not expected to survive) were

excluded from the study. All infants in the study were categorised as: CLD group who required respiratory support or supplemental oxygen at 36 weeks corrected gestational age and RDS group who were free of respiratory support at 36 weeks corrected gestational age.

2.7.2. BALF Collection and Processing

Non-bronchoscopic broncho-alveolar lavage (BAL henceforth) was performed on ventilated infants (daily in the first week and twice-weekly thereafter) as previously described (de Blic et al., 2000). Briefly, infants were disconnected from the ventilator and, with their head turned to the left, an appropriate size suction catheter was passed down the endo-tracheal tube until resistance was felt. 1 ml/kg of warmed 0.9% saline was instilled down the tube and was immediately suctioned out by connecting the upper end of the suction tube to a water trap using wall suction pressure of 8-12 kPa. This was repeated once more and the total volume of fluid instilled and recovered was recorded. The recovered fluid was pooled and placed on ice before being transported to the lab. In the lab, a haemocytometer count of total cells was done from the pooled fluid before it was centrifuged at 4°C at 1000 g for 10 minutes. The supernatant was removed, aliquoted out to smaller volumes (25µl) and frozen at -80ºC until further use. The pellet was re-suspended in PBS with 5 mM of EDTA to a density of 0.5 X 10^6 cells/ml. 50 µl of suspension was used to prepare cytospins by spinning at 300 rpm for 3 minutes followed by air-drying and fixing in methanol. Diff-Quik (Medion Diagnostics AG, Duedingen, Switzerland) stained cytospins were used to enumerate differential cell counts by counting at least 300 cells per sample under direct vision with a high powered light microscope.

2.8. B9 cell assay

2.8.1. Introduction

The B9 cells are an IL-6 dependent mouse B cell hybridoma derived from spleen tissue and are lymphoblastoid in morphology. They were developed as a sub-clone of

the B13.29 cell-line (Lansdorp et al., 1986), which were chosen for their IL-6 dependent proliferation (Aarden et al., 1987b). Since their isolation, these cells have been used as an IL-6 bioassay due to their sensitivity to human IL-6 (Nordan et al., 2001b).

2.8.2. B9 cells and initial thawing

Cells were bought from European Collection of Cell Cultures (ECACC, Health Protection Agency, Porton Down, Salisbury, UK). Media used was RPMI 1640 containing 2 mM L-glutamine (Lonza, Slough, UK) with 5% HI-FCS and 50 µM of 2-ME. For cell cultures, 25 pg/ml of rhIL-6 was freshly added to media. All incubations were in a humidified incubator at 37°C and 5% CO_2.

A frozen vial was thawed, cells re-suspended in 5 ml of fresh warm media with IL-6 and incubated. Condition of cells was monitored by observation under microscope. After initial thawing, cells needed 2-3 media changes before they started to grow.

2.8.3. Subcultures

Cells in suspension were poured into a universal container and centrifuged at 150 g for 5 minutes. The pellet was re-suspended in 1 ml of fresh media (no IL-6) and counted for viable cells. 10 µl of the cell suspension was withdrawn and mixed with 10 µl of 0.4% Trypan blue (Sigma). Cells were counted in a haemocytometer by light microscopy at 10X magnification. For subcultures, 3×10^4 viable cells were re-suspended per ml of fresh media with IL-6 (4.5×10^5 cells/15 ml for a 75 cm^2 flask). Flasks were incubated for 72-96 hours before cells were harvested for experiments or subculture.

2.8.4. Assays

All assays were performed between passage numbers 10-25 (Nordan et al., 2001a). For assays, 5000 cells were seeded per well in 96-well plates with or without rhIL-6 (as appropriate) and with other cytokines or BALF and incubated under the same conditions as above in a total volume of 100µl. All BALF samples were appropriately

diluted to an estimated IL-6 concentration of 2 pg/ml (based on the immune values) to keep the bioactivity within the range of the assay. A colorimetric assay was used at the end of the incubation to estimate the number of viable cells, using a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate; PMS). MTS is reduced by dehydrogenases in metabolically active (viable) cells to a yellow water-soluble dye, formazan, which is freely soluble in the culture medium (Slater et al., 1963). The amount of formazan in solution is directly proportional to the number of viable cells, and can be measured by the absorbance at 490 nm. At 68 hours, 20 μl of a solution of MTS was added to each well and absorbance was read at 490 nm in a spectrophotometer. Functional activity was assessed by comparing with an IL-6 standard curve. For controls, a dose of 8 pg/ml of IL-6 was chosen to give a more visible effect in relation to the standard curve.

For specific *trans*-signalling assays, cell-surface IL-6R was blocked by using a monoclonal anti-mouse IL-6R antibody 2B10 (Greenhill et al., 2011, Lissilaa et al., 2010) which was incubated with cells for 30 minutes at 37°C and 5% CO2 before stimulation with cytokines or BALF. 2B10 antibody was a generous gift from NovImmune SA, Geneva, Switzerland. For assays using combinations of cytokines or cytokines with specific antibodies, all proteins (or BALF) were premixed at room temperature for at least 30 minutes before stimulating cells.

2.9. Rheumatoid Arthritis Synovial Fibroblast (RASF) culture and stimulation

Sterile Human Synovial Fibroblasts were isolated from synovial tissue obtained after synovectomy from Rheumatoid Arthritis patients at the time of total knee joint replacement (Dayer et al., 1976). RASF cells do not express IL-6R on the cell-membrane but can respond to IL-6 *trans*-signalling (*via* cell-surface gp130) to selectively express MCP-1 (Nowell et al., 2003). Thus, a culture of these cells was used as a bioassay to test the effects of IL-6 *trans*-signalling *in vitro.*

RASF, growing in Dulbecco's Minimum Enrichment Media (DMEM) with 10% (v/v) HI-FCS, were obtained from Dr Mari Nowell (Department of Biochemistry, Cardiff University). Cells were incubated in a humidified incubator at 37°C and 5% CO_2 and allowed to grow to confluency. For subcultures, cells were harvested with Trypsin EDTA for 10 minutes in the incubator and then neutralised with serum-supplemented media. Cell-suspension was centrifuged at 500 g for 6 minutes and the pellet was re-suspended in 2.0 ml of fresh media. 1 ml was re-suspended in 15 ml of fresh media and incubated.

1 ml of the suspension was re-suspended in 10 ml of fresh media and 400 µl was pipetted into each well of a 24-well plate. Cells were allowed to adhere and grow to 70-80% confluency. Then media was aspirated and cells were washed x 2 with 500 µl of 0.9% NaCl per well. 400 µl of fresh DMEM (no FCS) was added to the wells and incubated overnight. Cells were washed again as before and then stimulated with media only, IL-6 (50 ng/ml), HIL-6 (50 ng/ml) or IL-6 (50 ng/ml) in the presence of sIL-6R (50, 100 and 500 ng/ml), both with or without 2B10 antibody (2.5 µg/ml) for 18 hours. All combination of cytokines and antibodies were pre-incubated at room temperature for 30 minutes before adding to cells. Supernatants were collected at the end of the incubation period and stored at -80°C until further analysis. Supernatants were assayed for MCP-1 as described before.

2.10. Cord-blood collection from term infants

[Ethical approval for project was obtained by Prof S Kotecha.]

2.10.1. Subjects and Consenting

For *in vitro* studies on newborn PMNs and monocytes, blood was collected from the placental cord after delivery of term infants by elective caesarean section. Mothers with any medical complications were excluded from the study. The two most common indications for elective section were previous section and breech presentation. For the current study, it was considered unethical to collect venous blood from healthy term infants. Also, infants delivered by normal labour were considered inappropriate as there is evidence of cell activation during the process of labour (Shen et al., 2009).

There is increasing evidence that preterm delivery is associated with chorioamnionitis and foetal inflammation. Thus, cells from preterm infants were also considered to be inappropriate as there is significant possibility of activation.

Cord blood was collected from term infants delivered by elective caesarean section after obtaining written parental consent. Indications for elective caesarean section included previous caesarean section or breech presentation. Mothers with any medical conditions potentially complicating pregnancy were excluded from the study. Prior ethical approval was obtained from the local Research Ethics Committee before sample collection.

2.10.2. Cord blood collection and initial processing

After the baby was delivered and cord clamped & cut, the placenta was slowly delivered into a bag. Blood was collected from the vessels in the cord attached to the placenta. 17 ml of blood was collected into a syringe containing 3 ml of 3.8% sodium citrate and immediately processed. Anti-coagulated blood was centrifuged at 450 g for 20 minutes to separate cells from the plasma. The plasma was centrifuged at 1300 g for 20 minutes to obtain platelet poor plasma (PPP), part of which was aliquoted and frozen at -80°C for future use. The cell layer was further processed as below.

2.11. Peripheral venous blood collection from healthy adult volunteers

[Ethical approval for project was obtained by Prof S Kotecha]

Control blood was collected from healthy adult volunteers after informed consent. Prior ethical approval was obtained from the local Research Ethics Committee before sample collection. 18 ml of blood was collected into a syringe containing 2 ml of 3.8% sodium citrate and immediately processed similar to cord blood above.

2.12. Purification of polymorphonuclear leucocytes and mononuclear cells from cord and adult blood

2.12.1. Cell purification by discontinuous Percoll gradient

Purification of cells has been described previously (Haslett et al., 1985). The cell layer (from 2.10.2) after separation from plasma was further processed. 3 ml of 6% Dextran solution was added per 20 ml of blood and was reconstituted to the original volume of blood (20 ml) with warmed 0.9% NaCl. Cells were mixed well with dextran by manual rolling and incubated in a water bath at 37°C for 30-45 minutes. Two layers of cells were visible after this step: an upper leukocyte-rich layer and a lower RBC layer with a clear border of separation. The leukocyte layer was carefully aspirated and centrifuged at 200 g for 6 minutes; the pellet at the end of this step was re-suspended in 2 ml of autologous PPP (as before).

Two Percoll gradients were prepared as below:

51% layer – 1.02 ml 90% Percoll + 0.98 ml PPP

42% layer – 0.84 ml 90% Percoll + 1.16 ml PPP

The 51% layer was added to the bottom of a 15ml conical tube and carefully overlaid with the 42% layer followed by the leukocyte layer, so as to avoid mixing. The 15 ml tube with the three separate layers was centrifuged at 350 g for 13 minutes without braking. At the end of this step, three layers, two bands and a pellet were visible, as detailed in figure 2.1 below.

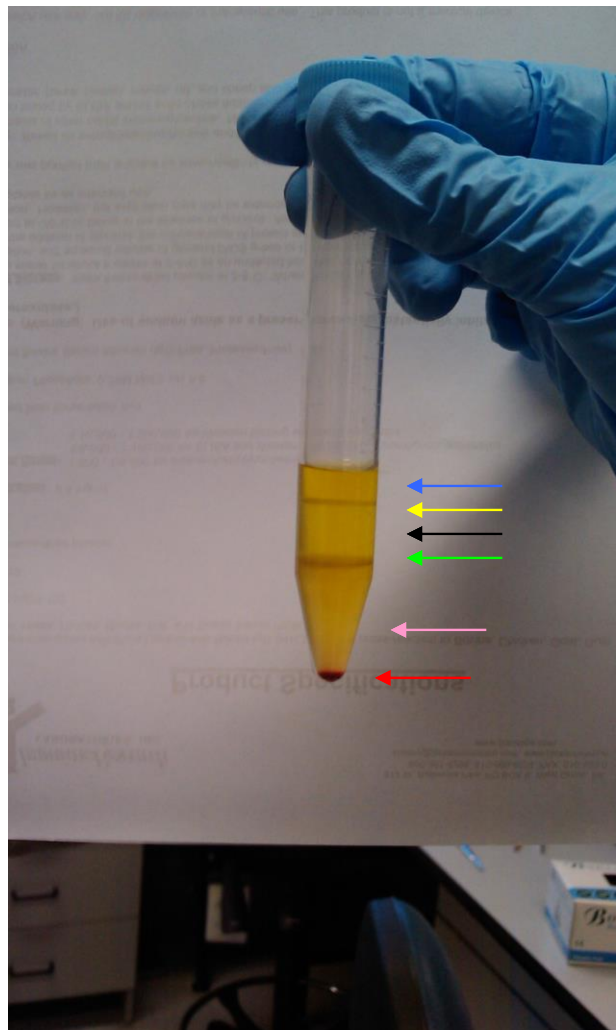


Figure 2-1: Representative layer and bands after purification of leukocytes through a discontinuous Percoll gradient. Arrows point to layers from top to bottom as described above: blue = top layer of plasma, yellow = band of mononuclear cells (MNC), black = 42% Percoll layer, green = band of polymorphonuclear leukocytes (PMN), pink = 51% Percoll layer, red = RBC pellet at the bottom.

The MNC and PMN bands were carefully and separately aspirated with a 1 ml pipette and re-suspended in 10 ml of Hank's Balanced Salt Solution (HBSS) without calcium (Ca), magnesium (Mg) or phenol red.

2.12.2. Processing of PMNs

PMNs were washed twice in 10ml HBSS without Ca and Mg using centrifugation at 200 g for 5 minutes. Cells were counted in a haemocytometer and finally re-suspended in RPMI 1640 (for cell stimulation experiments) or HBSS with Ca and Mg (for degranulation experiments) to a final concentration of 5 x 10^6 cells per ml.

2.12.3. Processing of MNCs

MNCs were washed twice in 10ml HBSS without Ca and Mg using centrifugation at 200 g for 5 minutes. Cells were counted in a haemocytometer and finally re-suspended in RPMI 1640 (for cell stimulation experiments) to a final concentration of 2.5 x 10^6 cells per ml.

2.12.4. Preparation of PMN cytospins

50 µl of cell suspension at a concentration of 5 x 10^6 cells/ml were pipetted into a cytofuge funnel and cytospins prepared using a 'Cytospin 3' (Shandon) by centrifuging at 300 rpm for 3 minutes onto polysine slides (Thermo Scientific). Slides were air-dried and fixed with 100% methanol before staining with Diff-Quick for a differential count.

2.13. Stimulation of purified PMNs and MNCs

PMNs and MNCs were re-suspended in RPMI 1640 with 10% heat inactivated (HI) foetal calf serum (FCS, Invitrogen) at a concentration of 5 X 10^6 cells/ml. 200 µl of cell suspension (1 x 10^6 cells) were pipetted per well into a 24-well plate and stimulated overnight with media or LPS (diluted in media) at a concentration of 10 ng/ml. Supernatants were collected after 18 hours of incubation and frozen at -80°C until further analysis.

Total IL-8 and $IL\text{-}8_{77}$ were measured in supernatants by specific ELISA as described before.

2.14. Processing of IL-8_{77}

2.14.1. Conversion of IL-8_{77} by BALF

Equal volumes of recombinant human (rh) IL-8_{77} (10 ng/ml) was incubated overnight with TBS-BSA, BALF or BALF with AAT (10 µM), PMSF (10 mM), 1-10 phenanthroline (10 mM) or ATIII (150 µg/ml). Table 2.4 provides details of all experimental conditions. Conditions 1, 2 and 3 were added together in equal volumes to create the reaction. BALF was pre-incubated with the protease inhibitors for 30 minutes at room temperature before adding IL-8_{77}.

Condition 1	Condition 2	Condition 3	Sample time-point (hours)
TBS+BSA	TBS+BSA	TBS+BSA	0, 18
TBS+BSA	TBS+BSA	IL-8_{77} (10 ng/ml)	0, 18
TBS+BSA	AAT (10 µM)	IL-8_{77} (10 ng/ml)	18
TBS+BSA	PMSF (10 mM)	IL-8_{77} (10 ng/ml)	18
TBS+BSA	1-10 phenanthroline (10 mM)	IL-8_{77} (10 ng/ml)	18
TBS+BSA	ATIII (150 µg/ml)	IL-8_{77} (10 ng/ml)	18
TBS+BSA	Ethanol	IL-8_{77} (10 ng/ml)	18
TBS+BSA	Serum (1 in 3)	IL-8_{77} (10 ng/ml)	0, 18
BALF	TBS+BSA	IL-8_{77} (10 ng/ml)	0, 18
BALF	AAT (10 µM)	IL-8_{77} (10 ng/ml)	18
BALF	PMSF (10 mM)	IL-8_{77} (10 ng/ml)	18
BALF	1-10 phenanthroline (10 mM)	IL-8_{77} (10 ng/ml)	18
BALF	ATIII (150 µg/ml)	IL-8_{77} (10 ng/ml)	18

Table 2-4: Details of all experimental conditions in conversion of IL-8_{77} by BALF. Concentrations (in parenthesis) are all final. Samples were collected at 0 hour & 18 hours and frozen at -80°C until further analysis.

2.14.2. Conversion of IL-8_{77} by purified neutrophil serine proteases

Purified human neutrophil elastase (HNE), cathepsin-G (CG) & proteinase-3 (Pr-3) were incubated with rhIL-8_{77} at 37°C in TBS BSA buffer. For time-course experiments, 300 nM of IL-8_{77} was incubated with buffer or 3 µM of each of the three proteases (enzyme:substrate = 10:1). Samples (20 µl) were collected at 0 hour, 1 hour,

2 hours, 4 hours, 6 hours, 12 hours and 24 hours and the reaction was stopped by adding 280 µl of AAT at 2.14 µM concentration (final concentration of IL-8 = 20 nM, protease = 0.2 µM and AAT 2 µM) and then frozen at -80°C until further analysis.

For dose-response experiments, 300 nM of IL-8_{77} was incubated with buffer or proteases at a concentration of 3 µM, (enzyme:substrate = 10:1), 1.5 µM, (enzyme:substrate = 5:1), 300 nM (enzyme:substrate = 1:1), 60 nM (enzyme:substrate = 1:5) and 30 nM (enzyme:substrate = 1:10). Samples (10 µl) were collected at 0 hour and 18 hours and the reaction was stopped by adding 10 µl of AAT at 30 µM. Samples were frozen at -80°C until further analysis.

2.14.3. Neutrophil Degranulation Assay

PMNs were purified from healthy adult human volunteers as described above. Cells were re-suspended in HBSS with C and Mg at a concentration of 5 x 10^6 cells/ml and 200 µl of this suspension (containing 1 x 10^6 cells) was pipetted into individual test tubes. Cells were then exposed to 5 µg/ml of cytochalasin B for 15 minutes at 37°C in a water-bath before being stimulated by fMLP (2 µM), rhIL-8_{72} or rhIL-8_{77} controls (100 & 10 nM each), or samples collected from conversion experiments with purified proteases (final estimated IL-8 concentration in samples 10 nM). After incubation for 30 mins in a water-bath at 37°C, tubes were centrifuged at 200 g for 2 minutes and supernatants stored at -80°C until further analysis. Total MMP-9 was measured in samples by specific ELISA as described above.

2.15. Airway Epithelial Cell Culture and Stimulation

All airway epithelial cell cultures were undertaken by Dr Wendy Powell. I have used supernatants stored by her at -80°C.

2.15.1. Culture of transformed epithelial cells

Transformed epithelial cells (table 2.5) were cultured in 75cm^2 flasks containing media supplemented with L-glutamine and 5% HI-FCS. Cells were grown to 80%

confluency in a humidified incubator at 37ºC and 5% CO_2. The cells were detached with 0.25% (w/v) trypsin-Ethylenediaminetetraacetic (EDTA) solution. The cells were subcultured at splitting ratios of 1:3 to 1:6 depending on individual experiments. The medium was changed every other day.

Epithelial cells were seeded at the density appropriate to individual experiments, into 25cm^2 flasks, 6, 24 or 96 well plates. Cells were left to adhere prior to washing in saline and serum starving. Prior to cytokine treatments, cells were washed again in saline and fresh serum-free media added. Transformed epithelial cells were starved in DMEM media with no HI-FCS or additives.

Epithelial cells	Medium required	Supplier
A549	DMEM	Hyclone
BEAS2B	F12-K	Hyclone

Table 2-5: List of transformed airway epithelial cells with culture conditions

2.15.2. Small airway epithelial cells (SAEC)

SAEC (Lonza CC-2547) were cultured in T-25cm2 flasks in SAEC basal media containing growth supplements (Clonetics SAGM BulletKit CC-3118). The vials contained 5 x 10^6 cells/ml. Cells were seeded at an appropriate density specific to individual experiments. The recommended seeding density is 2,500 cells/cm^2. An appropriate volume of media at 1ml/5cm2 was added to the flasks and allowed to equilibrate at 37ºC, 5% CO_2 humidified incubator for 30 minutes. The cryo vial was wiped with ethanol. The cap was loosened to relieve pressure and was retightened, before rapidly thawing the cryo vial in a 37ºC water bath. The vial was removed once the cells had thawed. The cells were resuspended and added directly to the flasks containing the warmed up media. The flask was gently rocked to ensure an even distribution of cells. The flask containing the cells was returned to the 37ºC, 5% CO_2 incubator.

The cells were subcultured when they had reached 60-80% confluency. For a 25cm^2 flask, 2ml of Trypsin/EDTA , 7-10ml of HEPES buffered saline solution (HEPES-BSS) 4ml Trypsin Neutralising Solution (TNS) and growth media were brought to room temperature. New culture flasks were prepared with 4ml growth media and placed in the incubator for 30 minutes. The media in each flask of cells was aspirated and discarded. The cells were rinsed with 5ml of room temperature HEPES-BSS. After aspirating the HEPES-BSS, the cells were covered in Trypsin/EDTA solution. The cell layer was examined microscopically. Trypsinization takes 2-6 minutes and was allowed to continue until 90% of the cells were of a rounded appearance. The flask was given a firm tap to remove the remainder of the cells from the flask surface. If the cells did not detach, the flask was returned to the incubator for 30 seconds before firmly tapping the flask again. Once the cells had released, the Trypsin was neutralised with 4ml of room temperature TNS. The detached cells were transferred to a sterile 15 ml tube. The flask was rinsed with 2ml HEPES-BSS to collect residual cells and added to the 15ml tube. The cells were centrifuged at 220 x g for 5 minutes to obtain a pellet. The supernatant was removed, leaving 100-200µl around the pellet. The tube was flicked to resuspend the cells. The cells were diluted in 2-3ml growth medium and counted. The cells were re-suspended and the appropriate density of cells were added to the new flasks containing the warmed up media. To each flask, 1ml of growth media was added for every 5cm^2 surface area of the flask. The new flasks were returned to the incubator.

The growth media was changed the day after seeding and then every other day. More media was added as the cells became more confluent. At 25% confluency, the cells were given 1ml/5cm^2. When confluency reached 25-45%, 1.5ml per 5cm2 was added. When the cells were more than 45% confluent, the cells received 2ml/5cm^2 of growth media. Fresh media was warmed in a sterile container in the incubator. The medium was removed from the cells and replaced with the warmed, fresh medium. The flask was returned to the incubator.

2.15.3. Airway Epithelial cell stimulations

Epithelial cells were seeded into 6-well plates at a density of 3 x 10^5 cells/ml and allowed to adhere for more than 12 hours prior to washing with saline and further culture in serum-free media (A549 and BEAS-2B) for six hours (SAECs were grown in defined serum-free medium). Cells were washed again in saline prior to stimulation with IL-1β (5 ng/ml for A549 and BEAS-2B cells and 1 ng/ml for SAEC) for 18 hours at 37ºC and 5% CO_2. Supernatants were collected after incubation were and immediately frozen at -80°C until further analysis. Total IL-8 and $IL\text{-}8_{77}$ were measured in supernatants by ELISA as described before.

2.16. Thrombin functional assay

Thrombin functional assay was performed by Mr John Hogwood and supervised by Dr Elaine Gray from the National Institute of Biological Standards and Controls, as per established methods using commercial reagents.

Thrombin activity was measured by a single point estimation against a calibration curve using the 2nd International Standard for Thrombin (01/580, NIBSC, South Mimms, UK) in a microtitre plate, with colour developed from a thrombin substrate, S2238 (Instrumentation Laboratories, Warrington, UK). To exclude the activity of any additional material present in the samples that could cleave the substrate, a highly specific inhibitor hirudin (Refludan, Pharmion, UK) to thrombin was used to provide a background level of activity. Thrombin levels were calculated with this background activity excluded.

2.17. Statistical analysis

All statistical analysis was performed using GraphPad Prism v 5.03 (GraphPad Software Incorporated, La Jolla, USA).

2.17.1. Primate (baboon) model of CLD

Data are reported as medians with interquartile ranges (IQR). Comparisons between individual days were made using a one-way ANOVA for repeated measures (Friedman's test) with Dunn's multiple comparison post-test (comparing all pairs of columns). Comparisons between groups were made using one-way ANOVA for non-parametric data (Kruskal-Wallis test) with Dunn's multiple comparison post-test. Correlations were assessed using Spearman's rank correlation test for non-parametric data. Significance was set at $p < 0.05$.

2.17.2. CLD in ventilated preterm infants

Data is reported as medians of peak-values from each infant in the first week of life. I chose to analyse peak-values because of incomplete serial data. Numerical data was compared using Mann-Whitney U test and categorical data by using Fisher's exact test. Correlation was tested by calculating Spearman correlation coefficient. Significance was set at $p<0.05$.

2.17.3. B9 cell assay for IL-6

Data from BALF experiments are expressed as a fold change in proliferation of cells compared to rhIL-6 at 2 pg/ml (as all BALF samples were diluted to an estimated concentration of 2 pg/ml to maintain cell proliferation within the range of the assay). Data is reported as mean with standard error (SEM). Data between the groups was compared by using two-tailed Student's unpaired t-test. Correlation was tested by calculating Spearman correlation coefficient. Significance was set at $p<0.05$.

2.17.4. Processing of IL-8_{77}

Observational data from clinical samples are reported as medians; data from all other experiments are reported as means with standard errors (SEM). Data from time-course experiments with IL-8_{77} and purified proteases were normalised to starting values and expressed as fold change from 0 hour. Data from dose-response experiments are expressed as percentage of IL-8_{77} remaining after incubation. Comparison of data between two groups was done by Mann-Whitney U test (observational data from clinical groups) or two-tailed unpaired Student's t-test (all other experimental data). Data between multiple groups was compared by one-way ANOVA with post-hoc multiple-comparison tests as appropriate (details mentioned in figure legends). Data from time-course and dose-response experiments were compared by two-way ANOVA with Bonferroni post test. Categorical data was compared by using Fisher's exact test. Correlations were assessed by using Spearman's rank correlation test. Significance was set at $p < 0.05$.

3. Expression of IL-6, sIL-6R and sgp130 in the preterm lung

3.1. Overview

Interleukin-6 (IL-6) is a key pro-inflammatory cytokine which has been detected at increased concentration in the preterm lung in infants who develop CLD (Kotecha et al., 1996b). However, in several animal models, IL-6 has been found to have an anti-inflammatory and protective effect. This could be explained by the effects of IL-6 *trans*-signalling which can have a profound influence on the resolution of the neutrophilic phase of acute inflammation (Hurst et al., 2001). Functional activity of IL-6 *trans*-signalling is modulated by the soluble form of its two receptor subunits, sIL-6R and sgp130. Although expression of IL-6 is well described in preterm infants, little is known about expression of sIL-6R or sgp130.

The primate model of CLD in preterm baboons (Coalson et al., 1999a) has been an invaluable tool to further our understanding of the disease. These animals, who receive similar clinical care as human preterm infants, have been studied in several trials, both observational and intervention. However, expression of the members of the IL-6 *trans*-signalling family the preterm baboon lung has not been looked at. As a first step towards my hypothesis, I collected tracheal aspirate fluid from preterm baboons and looked at the expression of IL-6, sIL-6R &sgp130, their interrelationships and their association with cellular influx into the lungs.

3.2. Aims of the Project (Expression in the Preterm Baboon Lung)

- To determine the expression of the IL-6, sIL-6R, sgp130, IL-8 and MCP-1 in broncho-alveolar lavage fluid (BALF) from ventilated preterm baboons. To determine the concurrent pattern of influx of inflammatory cells in BALF from preterm ventilated baboons.
- To analyse possible correlations between expression of the IL-6 *trans*-signalling molecules, the related chemokines and influx of inflammatory cells into the lungs.

3.3. Effect of Ventilation on Lung Inflammation in Preterm Baboons

This model is designed to replicate ventilation conditions currently practiced on human preterm neonates ("gentle ventilation"). Total cells counts were significantly increased in animals that were born at 125 days and ventilated in this manner (as described before) for 14 days (125d + 14d *pro re nata* [PRN]) compared to gestational controls (GC) born at 125 days (125d GC *vs* 125d + 14d PRN $p < 0.01$) or at 140 days (140d GC *vs* 125d + 14d PRN $p < 0.05$, figure 3.1). This suggests that even gentle ventilation, after receiving prenatal steroids and postnatal surfactant replacement, results in significant pulmonary inflammation in the lungs of premature baboons.

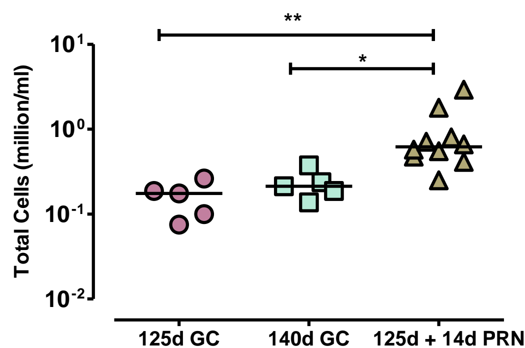


Figure 3-1: Pulmonary inflammation due to gentle ventilation. Total cell counts in necropsy lavage of animals born at 125 days gestation (125d GC; n=5), 140 days gestation (140d GC; n=5) and animals born at 125 days gestation and ventilated for 14 days before being euthanised (125d + 14d PRN; n=10). Groups are represented on the x-axis while cell counts in millions/ml is represented on the y-axis. Each datapoint represents an individual animal and solid bars at each timepoint represent medians. (** = $p < 0.01$, * = $p < 0.05$).

3.4. Expression of IL-6, sIL-6R and sgp130

A total of 50 tracheal aspirate fluid (TAF) samples (10 animals at 5 time-points) and 20 necropsy lavage samples (five 125 day gestational controls, five 140 day gestational controls and ten 14 day post ventilation samples) were analysed. IL-6 was detected from 49 TAF samples (98%) but from only 2 necropsy lavage samples (10%); sIL-6R and sgp130 were detected in all samples.

The concentration of IL-6 (figure 3.2a and table 3.1) significantly increased from day 3 to day 6-7 ($p < 0.001$) before reaching its peak at day 10-11 ($p < 0.01$ compared to day 3); this rise decreased to levels comparable to day 1 and day 3 by day 14 ($p < 0.05$ compared to day 6-7). In contrast, expression of sIL-6R (figure 3.2b and table 3.1) did not change significantly between the time-points in the study period. Expression of sgp130 (figure 3.2c and table 3.1) increased significantly from day 1 to day 6-7 ($p < 0.01$) to reach a peak at day 10-11 ($p < 0.05$ compared to day1). There was a trend towards decreasing concentration at day 14, although this was not statistically significant from earlier time-points.

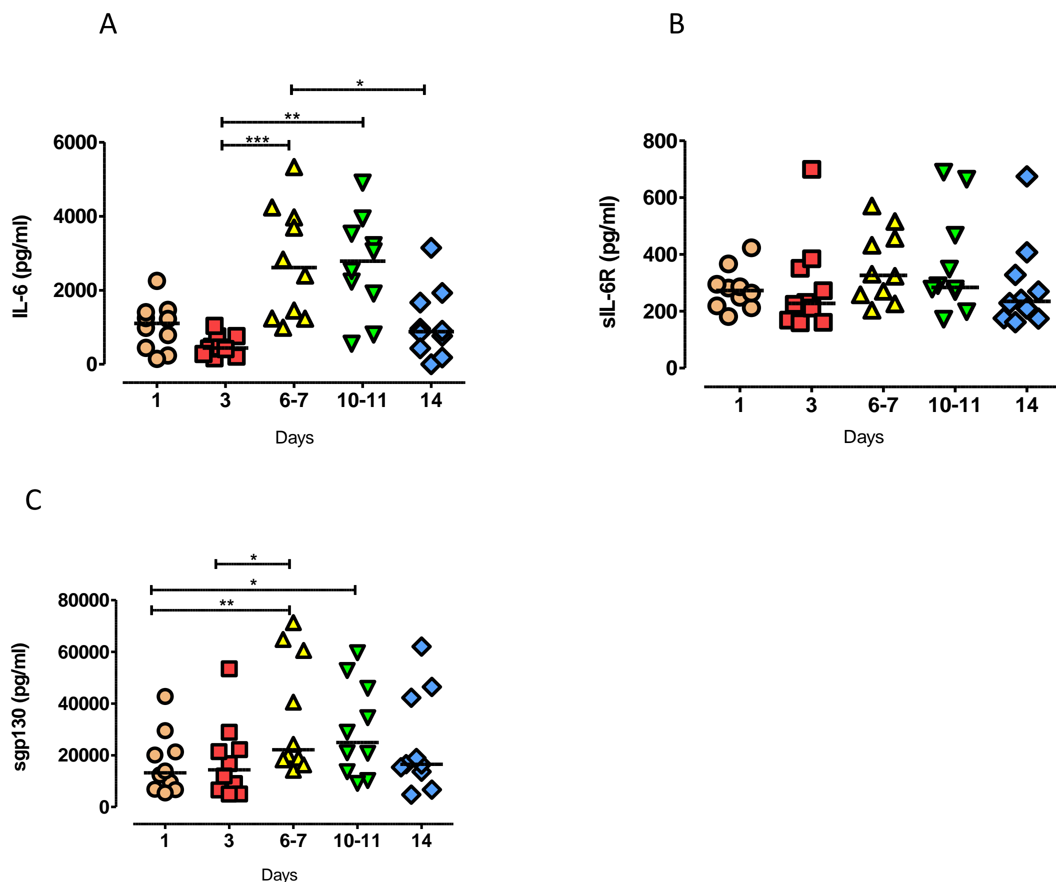


Figure 3-2: Expression of *trans*-signalling molecules. Concentrations of (a) IL-6, (b) sIL-6R and (c) sgp130 in the ventilated baboon lung. Individual time-points (days) are represented on the x-axis while concentrations (pg/ml) are on the y-axis. Each datapoint represents an individual animal and solid bars at each timepoint represent medians. (*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$).

3.5. Ratios of trans-signalling molecules

For *trans*-signalling, an excess of sIL-6R is needed to form complexes with IL-6 (sIL-6R/IL-6) which dimerises membrane gp130 and initiates intra-cellular signalling; an excess of sgp130 binds to this complex and inhibits its interaction with membrane gp130. Thus, a potential for *trans*-signalling is better represented by ratios of these molecules to show relative excess. To represent pro *trans*-signalling complexes, I calculated the molar (human equivalent) ratio of sIL-6R/IL-6 and for anti *trans*-signalling complexes, the molar ratio of sgp130/sIL-6R.

In this cohort of 10 animals, concentrations of IL-6 were almost always in excess of sIL-6R (except in two animals on day 14); concentrations of sgp130 were always in excess of both IL-6 and sIL-6R. IL-6 was below the range of detection in one sample; for the purposes of statistical analysis of sIL-6R/IL-6 ratio using a rank test, the IL-6 concentration was transposed to the detection limit of the ELISA (9.375 pg/ml). The ratio of sIL-6R/IL-6 (figure 3.3a and table 3.1) decreased significantly from day 3 to its nadir on day 6-7 ($p < 0.01$) which was sustained until day 10-11 ($p < 0.01$ compared to day 3). There was a trend towards recovery of this ratio on day 14 but this was not statistically significant. In contrast, the ratio of sgp130/sIL-6R (figure 3.3b and table 3.1) reached a peak on day 6-7 ($p < 0.05$ compared to day1).

Comparison of the two ratios on individual days shows that the anti *trans*-signalling ratio (sgp130/sIL-6R) was always in excess of the pro *trans*-signalling ratio (sIL-6R/IL-6) (table 3.1).

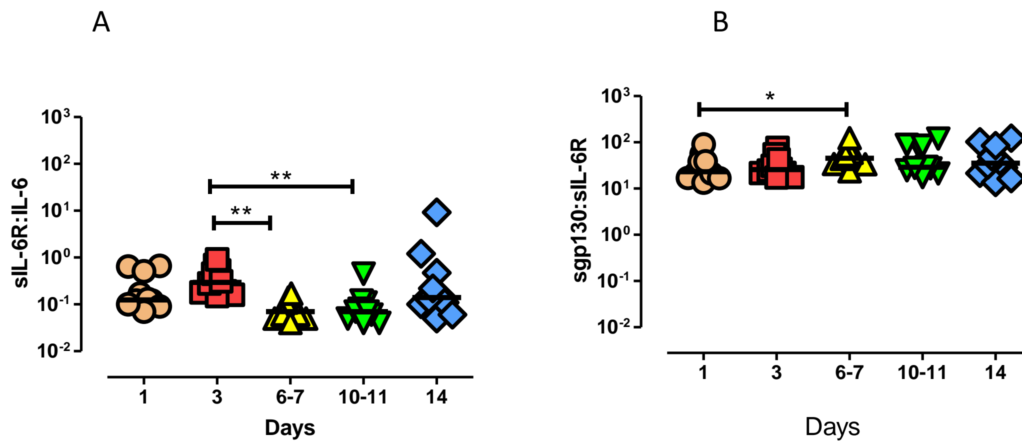


Figure 3-3: Molar ratio of *trans*-signalling molecules. Ratios of (a) sIL-6R:IL-6 and (b) sgp130:sIL-6R in the ventilated baboon lung. Individual time-points (days) are represented on the x-axis while the molar ratios are represented on the y-axis (log scale). Molar ratios are calculated as human equivalents. Each datapoint represents an individual animal and solid bars at each timepoint represent medians. (** = $p < 0.01$, * = $p < 0.05$).

	Day 1	Day 3	Day 6 -7	Day 10 -11	Day 14
IL-6	1107 (392.3 – 1421.0)	440.5 (261.4 – 756.5)	2613 (1238.0 – 4036.0)	2787 (1635.0 – 3628.0)	881 (370.8 – 1732.0)
sIL-6R	273.1 (217.2 – 312.9)	227.8 (167.0 – 359.5)	326.7 (250.3 – 471.4)	284.3 (251.6 – 516.9)	234.8 (175.1 – 347.7)
sgp130	13175 (6793 – 23341)	14373 (6194 – 23840)	22133 (17710 – 61597)	24945 (12795 – 47603)	16481 (11897 – 43297)
sIL-6R/IL-6	0.125 (0.0975 – 0.5425)	0.295 (0.175 – 0.57)	0.07 (0.05 – 0.125)	0.07 (0.0475 – 0.1125)	0.14 (0.09 – 0.6525)
sgp130/sIL-6R	23.05 (16.96 – 42.19)	25.64 (20.72 – 45.78)	45.27 (33.84 – 64.28)	28.78 (23.88 – 85.53)	35.33 (20.18 – 89.57)

Table 3-1: Concentrations of IL-6, sIL-6R & sgp130 in pg/ml and the molar ratios (human equivalents) of sIL-6R/IL-6 & sgp130/sIL-6R represented as medians (IQR) at each time-point.

3.6. Cell counts

To study the effect of ventilation on influx of inflammatory cells, I analysed total and differential cell counts in TAF. Total cell counts in the lungs of ventilated animals (figure 3.4a and table 3.2) were not significantly different between time-points during the length of the study. Differential cell counts could not be calculated for two animals on two days (one on day 3 and the other on day 14) due to poor quality of the slides; these were excluded from the analysis. Similar to total cells, the number of PMNs (figure 3.4b and table 3.2) and mononuclear cells (figure 3.4c and table 3.2) did not significantly differ between the days. To understand the pattern of shift of the predominant inflammatory cell population, I calculated the ratio of PMN/MNCs. Analysis of this ratio (figure 3.4d and table 3.2) showed a gradual shift of inflammatory cell influx from a 1.8-fold excess of PMNs on day 1 to a 1.5-fold excess of MNCs on day 14 (day 1 *vs* day 14 $p < 0.01$).

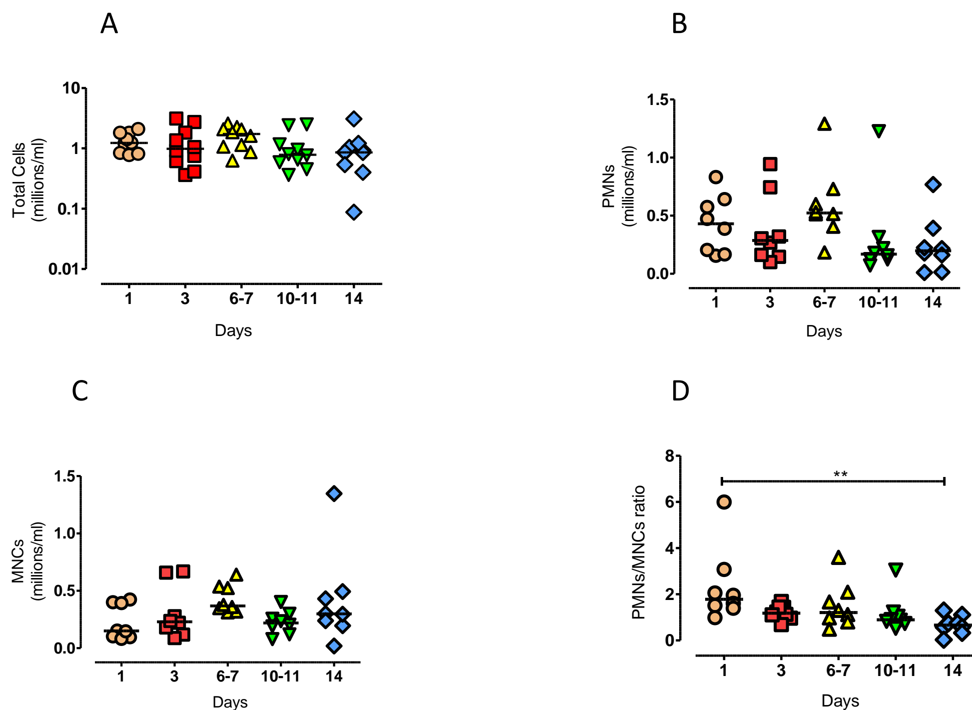


Figure 3-4: Cell counts in TAF. Numbers of (a) total cells, (b) PMNs and (c) MNCs recovered in TAF from ventilated preterm baboons. Individual time-points (days) are represented on the x-axis while cell numbers in millions/ml are represented on the y-

axis. (d) Ratio of numbers of PMN/MNC. Each datapoint represents an individual animal and solid bars at each timepoint represent medians. (** = $p < 0.01$).

	Day 1	Day 3	Day 6-7	Day 10-11	Day 14
Total cells	1.225 (.8313 – 1.813)	0.9813 (0.5625 – 2.05)	1.731 (1.013 – 2.112)	0.7813 (0.5531 – 1.475)	0.8563 (0.5031 – 1.081)
PMNs	0.4306 (0.1763 – 0.6251)	0.2870 (0.1502 – 0.6385)	0.5228 (0.4323 – 0.6971)	0.1692 (0.1239 – 0.2927)	0.1977 (0.05108 – 0.3502)
MNCs	0.1502 (0.09748 - 0.3983)	0.2297 (0.134 – 0.5633)	0.368 (0.3263 – 0.5335)	0.2199 (0.1376 – 0.2895)	0.2987 (0.2066 – 0.4775)
PMN/MNC	1.783 (1.425 – 2.824)	1.183 (1.001 – 1.442)	1.211 (0.8461 – 1.984)	0.8926 (0.7577 – 1.188)	0.6625 (0.3884 – 1.021)

Table 3-2: Cell counts (millions/ml) and ratios of PMN/MNC in TAF represented as medians (IQR) at each timepoint.

3.7. Expression of Chemokines

As IL-6 *trans*-signalling has been shown to have a profound effect on cell trafficking in an acute inflammatory model by switching the expression of key chemokines from a pro-inflammatory to pro-resolution phenotype, I analysed the concentrations of IL-8 and MCP-1 in TAF from ventilated preterm baboons. IL-8 (figure 3.5a) concentration significantly increased on day 6-7 (day 1 *vs* day 6-7 $p < 0.01$, day 3 *vs* day 6-7 $p < 0.05$) and persisted until day 10-11 (day 1 *vs* day 10-11 $p < 0.001$, day 3 *vs* day 10-11 $p < 0.05$) before slowly resolving at day 14 (not statistically significant compared to previous time-points). The pattern of expression of MCP-1 (figure 3.5b) was similar with a significant rise on day 6-7 (day 1 *vs* day 6-7 $p < 0.001$, day 3 *vs* day 6-7 $p < 0.01$) which persisted until day 10-11 (day 1 *vs* day 10-11 $p < 0.01$) before showing a trend towards resolution at day 14.

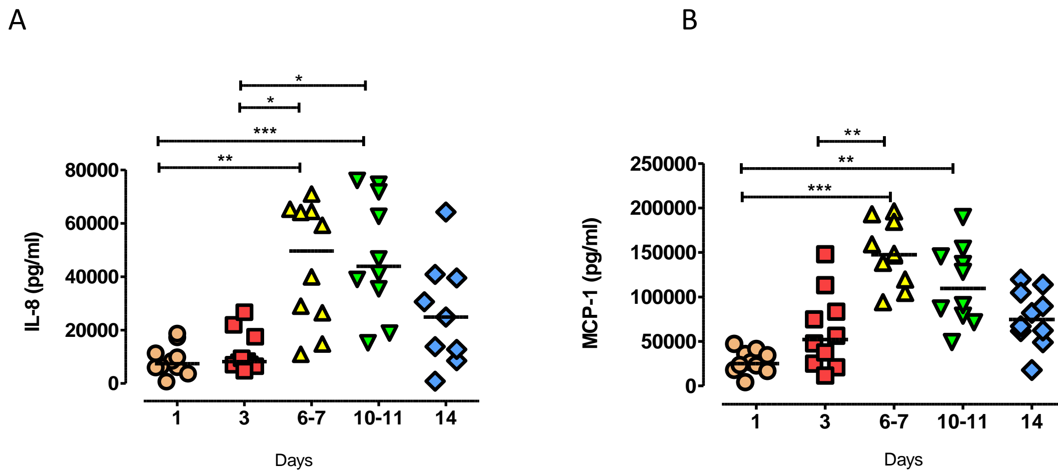


Figure 3-5: Expression of chemokines. Concentrations of (a) IL-8 and (b) MCP-1 in the ventilated baboon lung. Individual time-points (days) are represented on the x-axis while concentrations (pg/ml) are on the y-axis. Each datapoint represents an individual animal and solid bars at each timepoint represent medians. (*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$).

3.8. Summary of results in preterm ventilated baboons

My results in the preterm baboons confirm that IL-6 and its two soluble receptors are expressed in the lung, and that they exhibit dynamic changes during the course of ventilation. Gentle ventilation used in this model, and reflective of current clinical practice (Coalson et al., 1999b), results in significant inflammation in the lung, as measured by the soluble mediators but not reflected in the numbers of the cellular infiltrate. However, there was a change in the pattern of cellular infiltrate over the course of the study period from predominantly PMNs to MNCs. Analysis of the pro (sIL-6R/IL-6) and anti (sgp130/sIL-6R) *trans*-signalling potential suggested a gradual shift in expression of these cytokines towards the former at the end of the study period, and this was reflected in a change in the influx of inflammatory cells resembling a pro-resolution phenotype.

3.9. Expression in Human Preterm Infants: Aims of the Project

- To determine the expression of the IL-6, sIL-6R, sgp130, IL-8 and MCP-1 in broncho-alveolar lavage fluid (BALF) from ventilated preterm human infants. To determine the concurrent pattern of influx of inflammatory cells in BALF from preterm ventilated human infants.
- To determine the proportion of the dsIL-6R isoform in the lungs of preterm infants and compare it between the two groups of infants, CLD and No CLD.
- To analyse possible correlations between expression of the IL-6 *trans*-signalling molecules, the related chemokines and influx of inflammatory cells into the lungs.
- To compare expression of all of the molecules and cells between the two groups of infants, CLD and no CLD.
- To analyse the inter-relationships in the expression of IL-6, sIL-6R and sgp130 by calculating functionally relevant ratios and compare them between the two groups, CLD and no CLD.

3.10. Clinical Subjects

A total of 32 preterm infants were studied, 17 CLD and 15 No CLD. Two infants in the CLD group were excluded from the analysis as they had major congenital abnormalities (sacro-coccygeal teratoma). Thus, a total of 30 infants were included in the final analysis. 104 BALF samples were collected in the first week of life from these infants for analysis (73 CLD and 31 no CLD). The median gestation and birth-weight of CLD infants was lower than No CLD infants, although no significant difference was noted (table 3.3). The duration of ventilation (days) was significantly greater in the CLD infants ($p < 0.01$).

Clinical Characteristic	No CLD	CLD	p
Total infants	15	15	
Gestational age in weeks (IQR)	28^{+3} (27 – 29^{+4})	27^{+5} (25^{+4} – 29^{+2})	0.21
Birth weight (g)	1120 (960 – 1260)	850 (717.3 – 1120)	0.11
Prolonged rupture of membranes (>24 hours)	1 (6.7)	4 (27.7)	0.33
Antenatal steroids (≥24 hours)	12 (80.0)	11 (73.3)	1.00
Caesarean delivery	10 (66.7)	8 (53.3)	0.71
Exogenous surfactant replacement	15 (100)	15 (100)	
Mechanical ventilation (days)	1.5 (1.0 – 2.5)	7.0 (5.0 – 18.0)	<0.01
Patent ductus arteriosus	6 (46.2)	10 (66.7)	0.45

Table 3-3: Clinical characteristics of the study population. Numerical values are expressed as medians (inter-quartile range). Categorical values are expressed as numbers of infants (percentages).

3.11. Inflammatory Markers

To determine if there is a difference in the markers of inflammation in the two groups of patients, I analysed the numbers of infiltrating PMNs and MNCs into the lungs and also the key chemokines IL-8 and MCP-1. The peak number of PMNs (p=0.03, figure 3.6a) and MNCs (p=0.04, figure 3.6b) recovered from BALF were significantly higher in the CLD group compared to the no CLD group (table 3.4). Peak concentrations of IL-8 (p<0.01, figure 3.6c) and MCP-1 (p=0.02, figure 3.6d) were also significantly higher in the CLD group (table 3.4). Thus, infants developing CLD had higher concentrations of the two key chemokines, reflected by an increased cellular infiltrate.

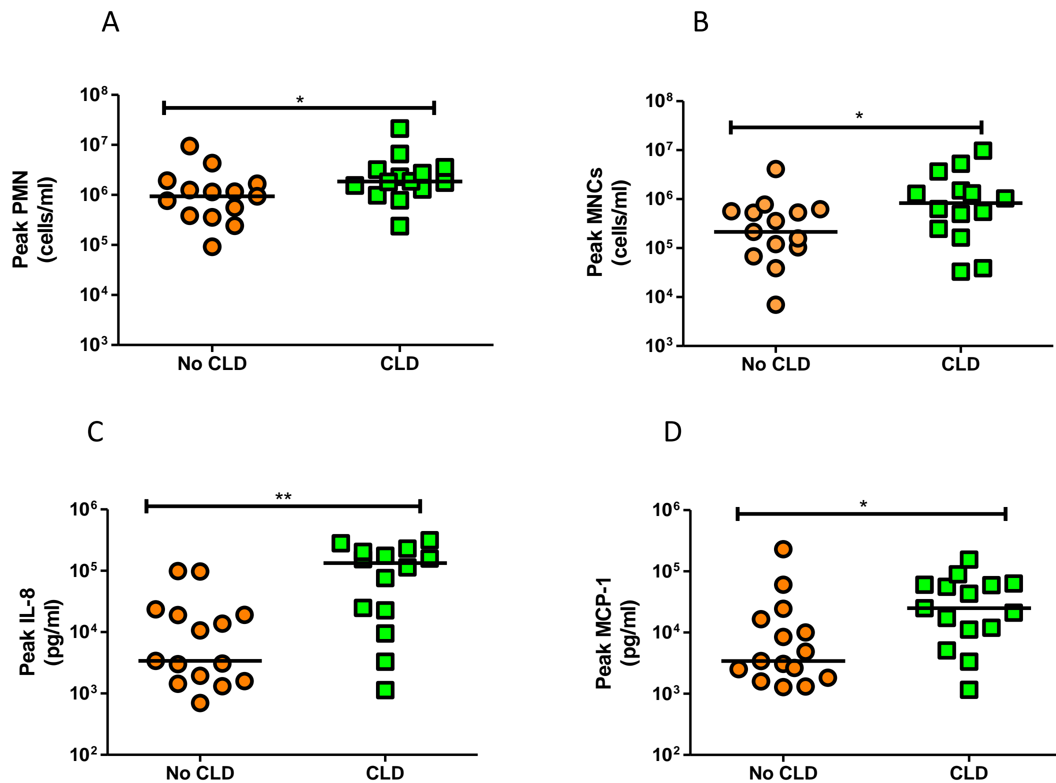


Figure 3-6: Inflammatory markers in the preterm lung. Peak numbers (cells/ml) of (a) PMNs and (b) MNCs and peak concentration (pg/ml) of (c) IL-8 and (d) MCP-1 in the lungs of ventilated preterm infants. The clinical groups are represented on the x-axis while the cells numbers (and a and b) or concentration (in c and d) is represented on the y-axis. Each datapoint represents an individual infant and solid bars are at medians. (** = $p < 0.01$, * = $p < 0.05$)

	No CLD	CLD	p
PMNs	0.94 (0.36 – 1.68)	1.85 (1.21 – 3.29)	0.03
MNCs	0.21 (0.07 – 0.57)	0.83 (0.23 – 2.05)	0.04
IL-8	3401 (1587 – 19080)	133600 (19310 – 209700)	< 0.01
MCP-1	3434 (1821 – 16545)	25080 (11180 – 60670)	0.02

Table 3-4: Inflammatory markers in the lung. Medians (IQR) of peak values of infiltrating PMNs and MNCs (millions/ml) and peak concentration of IL-8 and MCP-1 (pg/ml) in the lungs of ventilated preterm infants.

3.12. Expression of IL-6, sIL-6R and sgp130

I next analysed the concentration of IL-6, sIL-6R and sgp130 in these infants. Although there was a trend towards increased concentrations in the CLD group, both peak IL-6 (p=0.13, figure 3.7a and table 3.5) and peak sIL-6R (p=0.21, figure 3.7b and table 3.5) expression were not statistically different between the two groups. In contrast, peak sgp130 was significantly higher in the CLD group compared to the no CLD infants (p=0.03, figure 3.7c and table 3.5).

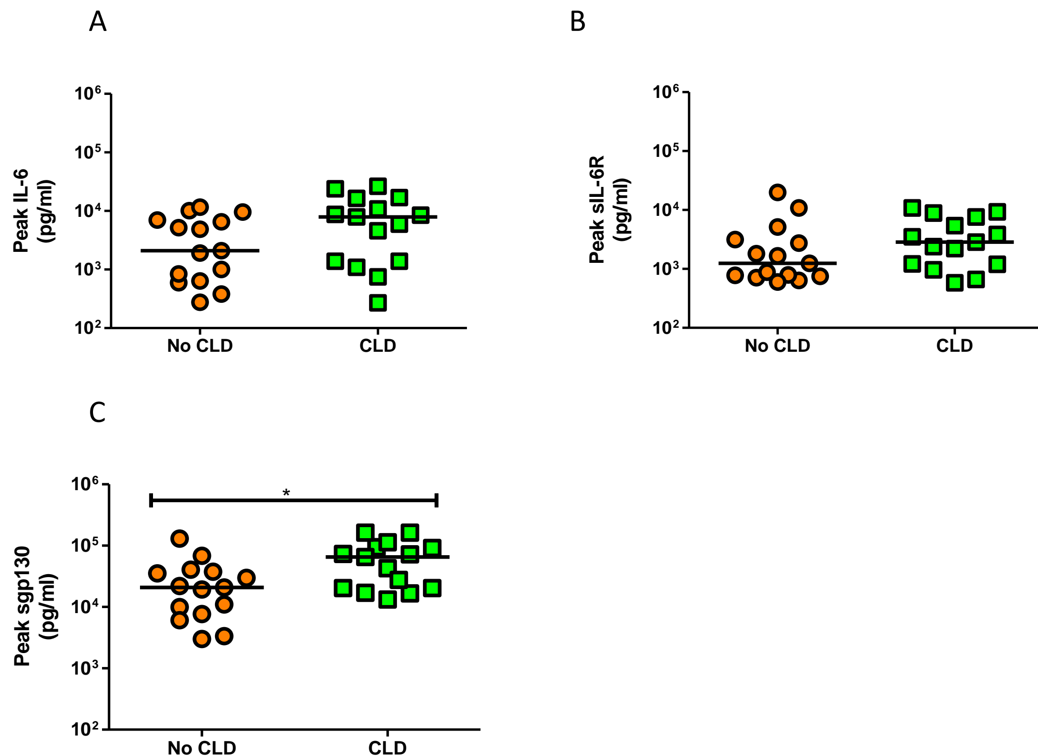


Figure 3-7: Expression of IL-6 *trans*-signalling molecules. Peak concentration (pg/ml) of (a) IL-6, (b) sIL-6R and (c) sgp130 in the lungs of preterm ventilated infants. The clinical groups are represented on the x-axis while the concentration is represented on the y-axis. Each data-point represents an individual infant and solid bars are at medians. (* = $p < 0.05$)

3.13. Molar ratios of IL-6 trans-signalling molecules

As sIL-6R is a key agonist of IL-6 activity whilst sgp130 acts as an antagonist of sIL-6R/IL-6 complexes, to represent relative abundance of pro- and anti- *trans*-signalling potential, I calculated the molar ratios sIL-6R/IL-6 and sgp130/sIL-6R respectively. There was a trend towards increased peak molar ratio in BALF of sIL-6R/IL-6 in the CLD infants compared to the No CLD infants but this was not statistically significant (p=0.06, figure 3.8a). However, the peak molar ratio of sgp130/sIL-6R was significantly higher in the CLD infants compared to the No CLD infants (p=0.02, figure 3.8b, table 3.5).

sgp130 concentration was in excess of sIL-6R concentration in all infants and the median values of the antagonistic ratio (sgp130/sIL-6R) were between six- to ten-fold higher than the agonistic ratio (sIL-6R/IL-6) within each group. Thus, the specific inhibitor of *trans*-signalling, sgp130, and the ratio sgp130/sIL-6R was significantly higher in the CLD group.

My initial hypothesis was that impaired IL-6 *trans*-signalling in the lungs of infants developing CLD could lead to delayed resolution of inflammation and contribute to the pathogenesis of the condition. To test this hypothesis, I looked for any correlation between the anti *trans*-signalling ratio (sgp130/sIL-6R) in the CLD infants and corresponding IL-8 expression or influx of PMNs into the lungs, and the pro *trans*-signalling ratio (sIL-6R/IL-6) in No CLD infants and the corresponding expression of MCP-1 or the influx of MNCs into the lungs. No significant correlations were observed between any of the ratios and the corresponding chemokines or cells in either of the groups. When all preterm infants were combined, there was a weak but statistically significant correlation between the ratio sIL-6R/IL-6 (pro *trans*-signalling) and influx of MNCs into the lungs (r=0.24, p=0.04); no significant correlation was found for the ratio sgp130/sIL-6R (anti *trans*-signalling) and the corresponding IL-8 expression or PMN influx.

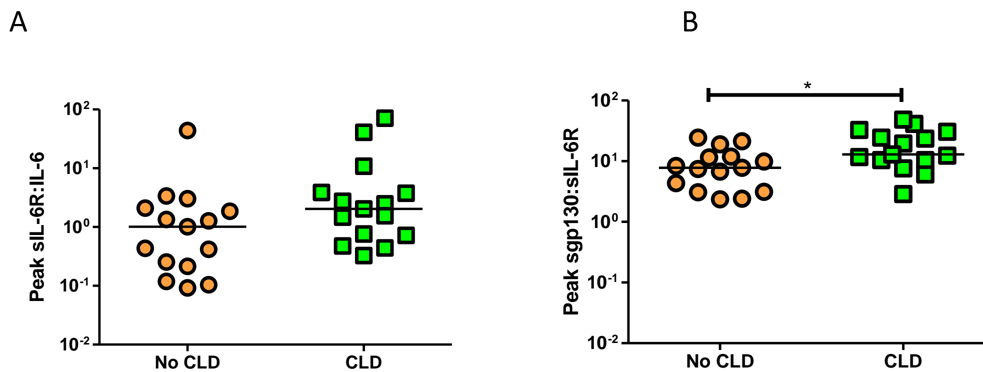


Figure 3-8: Molar ratio of *trans*-signalling molecules. Peak ratio of (a) sIL-6R:IL-6 and (b) sgp130:sIL-6R in preterm ventilated infants. The clinical groups are represented on the x-axis while the ratio is represented on the y-axis. Each datapoint represents an individual infant and solid bars are at medians. (* = $p < 0.05$)

	No CLD	CLD	p
IL-6	2096 (637.1 – 6977)	7900 (1383 – 16440)	0.13
sIL-6R	1244 (750.7 – 3155)	2852 (1195 – 7636)	0.21
sgp130	20788 (7680 – 37440)	65290 (20280 – 94590)	0.03
sIL-6R:IL-6	1.01 (0.21 – 2.09)	2.03 (0.72 – 3.86)	0.06
sgp130:sIL-6R	7.80 (3.13 – 11.92)	12.97 (10.45 – 30.59)	0.02

Table 3-5: Expression of IL-6 *trans*-signalling molecules and their ratios. Medians (IQR) of concentrations of IL-6 *trans*-signalling molecules (pg/ml) and their molar ratios in the lungs of preterm ventilated infants.

3.14. Expression of DS sIL-6R

Two inducible isoforms of sIL-6R can mediate these *trans*-signalling responses; one produced at sites of inflammation by proteolytic cleavage (PC sIL-6R) (Mullberg et al., 1993b) and a second differential (or alternative) mRNA spliced isoform, DS sIL-6R (Horiuchi et al., 1994b). Both lack the membrane-proximal regions of the cognate

receptor but the two isoforms are differentiated by the presence of 10 extra amino acids (a.a.) at the COOH terminal of DS sIL-6R (Jones et al., 2001). Levels of DS sIL-6R seem to be regulated by age (Jones et al., 2001); however, expression of this isoform in the neonatal population has not previously been described. To determine the expression of sIL-6R generated by alternative splicing mechanism, an additional 21 CLD and 11 no CLD samples were tested by ELISA for DS sIL-6R. Due to the limited availability of specimens from the main study population, these samples were from a separate but matched (table 3.6) population collected within one year of the main study population and from the same neonatal unit.

	No CLD	CLD	p
Total infants	4	3	
Gestational age in weeks (IQR)	27^{+2} (25^{+6} – 28^{+3})	28^{+3} (27^{+1} – 29^{+3})	0.4
Birth weight (g)	975 (857.5 – 1243)	1080 (1020 – 1230)	0.63
Prolonged rupture of membranes (>24 hours)	1 (25.0)	0 (0.0)	1.0
Antenatal steroids (≥24 hours)	4 (100)	3 (100)	
Caesarean delivery	2 (50.0)	3 (100)	0.43
Exogenous surfactant replacement	4 (100)	3 (100)	
Mechanical ventilation (days)	12.5 (6.8 – 15.3)	36.0 (12.0 – 44.0)	0.28

Table 3-6: Clinical characteristics of the study population used for measuring DS sIL-6R. Numerical values are expressed as medians (inter-quartile range). Categorical values are expressed as numbers of infants

Both total sIL-6R (p=0.53, fig 3.9a) and DS sIL-6R (p=0.12, fig 3.9a) were comparable between the two groups. However, the majority of the sIL-6R in BALF

seemed to be the proteolytically cleaved isoform (figure 3.9b). Expression of DS sIL-6R in preterm BALF was significantly correlated to the total sIL-6R detected (r=0.82, p<0.0001, figure 3.10).

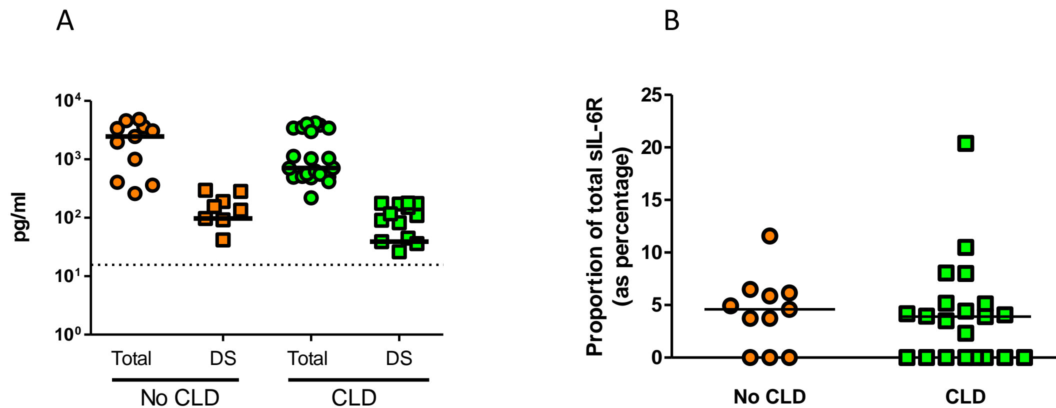


Figure 3-9: Expression of total and DS sIL-6R in preterm BALF. (a) Concentration (pg/ml) of total sIL-6R (orange circles) and DS sIL-6R (orange squares) in RDS and total sIL-6R (green circles) and DS sIL-6R (green squares) in CLD. Interrupted line represents the limit of detection of the ELISA. (b) Proportion (expressed as a percentage) of total sIL-6R expressed as DS sIL-6R in RDS (orange circles) and CLD (green squares).

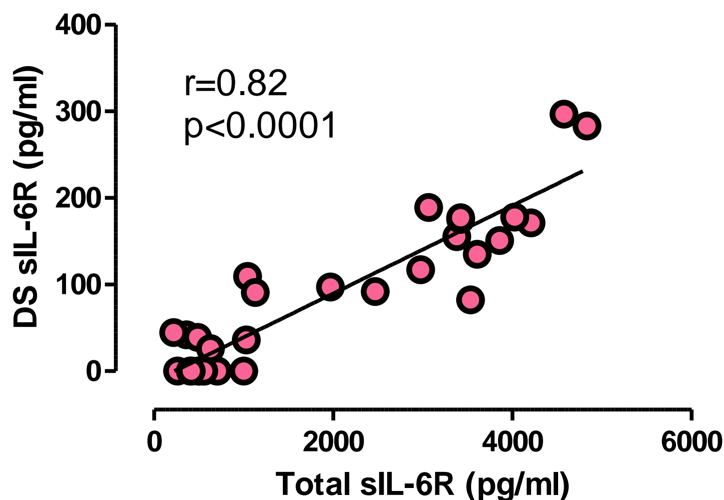


Figure 3-10: Correlation between the expressions of DS sIL-6R (y-axis) with total sIL-6R (x-axis) in preterm BALF. Each datapoint represents a preterm infant and continuous line represents "line of best fit", drawn by linear regression analysis.

It has been previously suggested that the expression of DS sIL-6R in circulation is related to age, with younger subjects expressing higher proportion of total sIL-6R as the DS isoform. To test this hypothesis, I measured total and DS sIL-6R in plasma from newborn infants. As matched plasma was not available from preterm infants, concentration of DS sIL-6R in circulation was measured in samples of cord blood from term infants delivered by elective caesarean section (n=18) and from healthy adult controls (n=14). Plasma levels of total sIL-6R (p 0.051, fig 3.11a) and DS sIL-6R (p 0.052, fig 3.11a) were lower in cord blood compared to adult plasma and the difference approached statistical significance. The proportion of total sIL-6R expressed as DS sIL-6R in plasma was also lower in cord blood (p 0.14, fig 3.11b), but this was not statistically significant. Unlike BALF, there was no significant correlation between the expression of total sIL-6R and DS sIL-6R in newborn plasma (fig 3.12). Thus, DS sIL-6R was expressed as a small proportion of total sIL-6R in both preterm neonatal BALF and in term neonatal circulation.

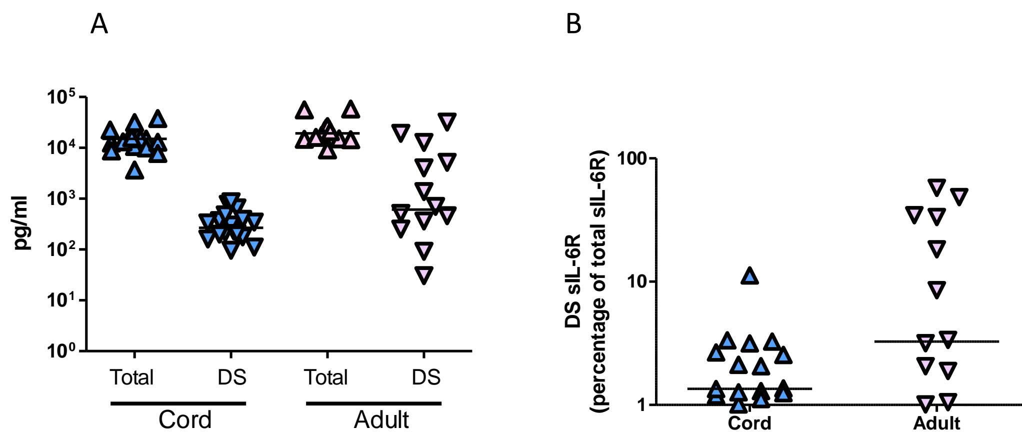


Figure 3-11: Expression of total and DS sIL-6R in newborn circulation. (a) Concentration (pg/ml) of total sIL-6R (blue triangles) and DS sIL-6R (blue inverted triangles) in RDS and total sIL-6R (pink triangles) and DS sIL-6R (pink inverted triangles) in CLD. (b) Proportion (expressed as a percentage) of total sIL-6R expressed as DS sIL-6R in RDS (blue triangles) and CLD (pink inverted triangles).

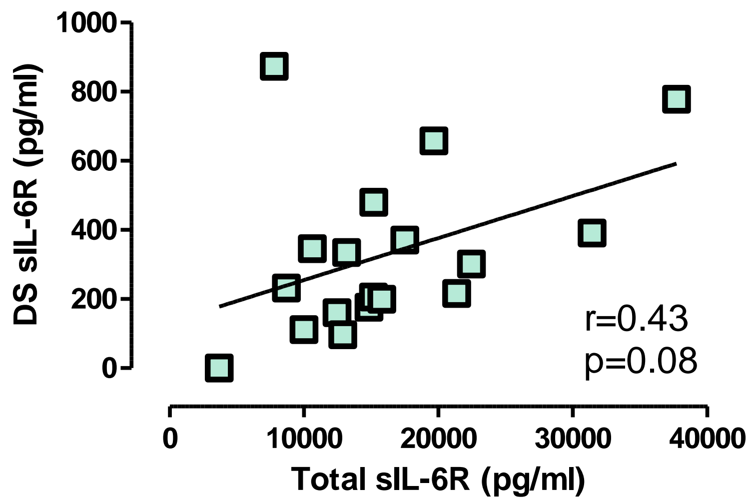


Figure 3-12: Correlation between the expressions of DS sIL-6R (y-axis) with total sIL-6R (x-axis) in term newborn infant circulation. Each datapoint represents a term infant and continuous line represents "line of best fit", drawn by linear regression analysis.

3.15. Discussion

3.15.1. Summary

I have reported expression of IL-6 and its soluble receptors, sIL-6R and sgp130, along with other markers of inflammation, in the lungs of both preterm baboons and human infants.

My analysis in the preterm baboons confirm that IL-6 and its two soluble receptors are expressed in the lung, and that they exhibit dynamic changes during the course of ventilation. Even gentle ventilation used in this model, and reflective of current clinical practice (Coalson et al., 1999b), results in significant inflammation in the lung which shows a change in the pattern of cellular infiltrate over the course of the study period.

In human infants, peak concentrations of the chemokines IL-8 and MCP-1 as well as peak numbers of PMNs and MNCs were all significantly higher in infants who developed CLD. The specific inhibitor of *trans*-signalling, sgp130 was significantly

higher in infants developing CLD later. More importantly, an excess of sgp130 was noted in all infants from both groups, suggesting an abrogation of the IL-6 *trans*-signalling pathway in the preterm lung. I have also described, for the first time, expression of DS sIL-6R in preterm lung and term newborn circulation.

3.15.2. Expression of cytokines and chemokines in the preterm baboon lung

All the cytokines and chemokines measured in our cohort of 10 animals, except sIL-6R, exhibited a similar pattern of expression showing significant increase in concentration at around day 6-7, persisting till day 10-11 and a trend towards resolution on day 14. Expression of sIL-6R and sgp130 has not been described in this model before. IL-8 concentration previously described during development of this model (Coalson et al., 1999b), showed a similar pattern of rise after day 6 which persisted till day 10, and a trend towards resolution after day 10. However, for IL-6, only concentrations on day 10 were significantly higher compared to other time-points (Coalson et al., 1999a). In an interventional trial with high-frequency ventilation in this model, preterm baboons randomised to conventional ventilation (n=12) showed a rise in IL-8 concentrations after day 6 which reached a peak at day 10 before resolving around day 14 (Yoder et al., 2000a). IL-6 concentrations in this cohort did not demonstrate the dynamic changes I have observed. In an extension of this model to include antenatal colonization with Ureaplasma, there was no variability of IL-6 concentrations (n=5) in the lungs of control (not exposed to antenatal Ureaplasma) animals (Yoder et al., 2003a). IL-8 (n=8) showed a rise on day 3 which persisted until day 5 before resolving at around day 10 but increased again to reach a peak concentration on day 14. An important difference in this study was that antenatal corticosteroids were not used in any of the animals. In another interventional study on ductus arteriosus ligation in preterm baboons, both IL-6 and IL-8 in the cohort of control animals (n=13) did not show the pattern of variability that I have observed (McCurnin et al., 2005b). Thus, significant variability in the expression of IL-6 have been noted in these animals, which could partly be explained by differences in interventions used or modes of injury. On the other hand, IL-8 has been consistently shown to increase in concentration after a few days, even with gentle ventilation.

In previous reports from this baboon model, absolute concentrations of IL-6 and IL-8 (pg/ml) were lower than that detected in our cohort. One reason for this could be difference in estimation methods; previously, for IL-6 measurement, a radioimmunoassay (RIA) was used with specific antiserum to human IL-6 and IL-8 was measured by an enzyme immunoassay. Thus, although the pattern of expression of IL-8 in our cohort is in agreement with previous reports, the dynamic changes I have observed in IL-6 concentration in the lung are not. Moreover, I have measured higher absolute values for both IL-6 and IL-8 in our cohort of ventilated animals, possibly due to differences in laboratory methods and sensitivity of ELISA compared to RIA.

In summary, all three members of the IL-6 *trans*-signalling family of molecules were detected in the lungs of preterm baboons after gentle ventilation for 14 days. Dynamic changes were noted in the expression of IL-6 and sgp130, but not sIL-6R. The chemokines IL-8 and MCP-1 also showed dynamic changes similar to IL-6 and sgp130.

3.15.3. Cellular infiltrate in the lungs of preterm ventilated baboons

In our cohort of 10 ventilated baboons, I have not observed significant differences in the numbers of PMNs or MNCs infiltrating into the lung. This agrees with the first report on this model (Coalson et al., 1999a), where no significant differences were noted in the differential cell counts. However, Coalson and colleagues found mononuclear cells to be always in excess of PMNs in tracheal aspirates. In the trial on high-frequency ventilation (Yoder et al., 2000a), PMN numbers increased at around day 9-10 and were higher than MNCs in control animals (standard ventilation, comparable to our cohort). Overall, the authors reported an excess of MNCs at other time-points (no statistics were reported for any variation between the days). I have observed a gradual shift in the population of cells infiltrating into the lungs from PMNs at early time-points to MNCs later on (PMN/MNC); values were significantly different between day 1 and day 14. Thus, my observations are different from previous reports in this model.

A large body of existing literature suggests that CLD in human infants is a disease associated with prolonged PMN and ill-sustained MNC infiltration into the

lungs of infants. The first description of this observation was in the 1980s (Ogden et al., 1983, Merritt et al., 1983, Ogden et al., 1984b) when prenatal steroids and postnatal exogenous surfactant replacement were still experimental. In the '90s, with the advent of both of these interventions, the association of persistent neutrophilia in the lungs with CLD was maintained (Arnon et al., 1993, Groneck et al., 1994, Watterberg et al., 1994, Kotecha et al., 1995, Munshi et al., 1997). However, surfactant use seemed to be associated with an increase in MNC numbers (Arnon et al., 1993). More recent studies in the last decade have confirmed the association between prolonged infiltration of PMNs and CLD (Kotecha et al., 2003). My results of increased influx of PMNs into the lungs of CLD infants is in keeping with previous reports. Increased MNCs in the CLD infants, although previously reported once (Arnon et al., 1993), probably reflects higher intensity and/or duration of inflammation in this group of infants.

3.15.4. Expression of the chemokines IL-8 and MCP-1 in preterm ventilated human infants

Attraction of innate immune cells to sites of inflammation is achieved by induction of a chemotactic gradient. Key chemokines involved in formation of such a gradient are IL-8 for PMNs (Remick, 2005) and MCP-1 for monocytes and mononuclear cells (Yadav et al., 2010). In observational studies, IL-8 has been consistently found to be significantly increased in the lungs of infants developing CLD (Ryan et al., 2008, Bose et al., 2008) compared to infants with No CLD. Almost all cells in the body can secrete IL-8 (Remick, 2005) and it is one of the early chemokines expressed in the preterm lungs which precedes the influx of PMNs (Munshi et al., 1997). Increased concentrations of MCP-1 have also been associated with the development of CLD (Baier et al., 2001, Baier et al., 2004). Thus, my results of increased concentration of both IL-8 and MCP-1 in their lungs of preterm ventilated infants who developed CLD later, is in agreement with previous reports. However, the dynamic pattern of expression of the chemokines IL-8 and MCP-1 in the preterm baboons was not reflected in the cellular infiltrate recovered from the lungs.

I have specifically looked at the expression of IL-8 and MCP-1 as they have been previously shown to be modulated by IL-6 *trans*-signalling (Hurst et al., 2001). The effect of IL-6 trans-signalling on the above chemokines has not been studied in the baboon model or in humans. Evidence on the differential control of neutrophil activating chemokines by IL-6 *trans*-signalling was provided by a rodent model of inflammation (McLoughlin et al., 2004). In a peritoneal inflammatory model in knockout mice, the authors demonstrated up-regulation of keratinocyte-derived chemoattractant (KC, murine homologue of human GRO-α) and MIP-2 (murine homologue of human IL-8) in IL-6$^{-/-}$ mice resulting in an increased influx of PMNs, possibly due to a lack of IL-6 *trans*-signalling (as this pattern was reversed on reconstitution of IL-6 *trans*-signalling). However, a down-regulation of another neutrophil attracting chemokine, LPS-induced CXC chemokine (LIX, murine homologue of human GCP-2) was noted in the same knockout mice. Thus the overall effect of IL-6 *trans*-signalling on chemokines is complex.

Control of expression of chemokines at sites of inflammation and subsequent influx of immune cells is complex and regulated at several different steps. Both IL-8 and MCP-1 are produced by a variety of cells and in response to a wide ranging array of stimuli (Remick, 2005, Yadav et al., 2010). Apart from IL-8, infiltration of PMNs into an inflammatory site is regulated by several other chemokines including growth-related protein-α (GRO-α), GRO-β, GRO-γ, epithelial cell-derived neutrophil activating peptide-78 (ENA-78) and granulocyte chemotactic peptide-2 (GCP-2) (Rollins, 1997). Similarly, several chemokines, apart from MCP-1, are involved in attracting mononuclear cells to a site of inflammation including MCP-2, MCP-3, MCP-4, macrophage inflammatory protein-1α (MIP-1α), MIP-1β (Rollins, 1997).

IL-8 and MCP-1 are the best studied in the preterm lung; however, whether they are the most relevant chemokines involved in cell trafficking in the preterm neonatal lung is unknown. Their increased expression in CLD infants may not represent a “cause and effect” relationship but rather be a sign of increased intensity and duration of inflammation in these infants, probably due to persistent injurious stimuli. Thus, all of the mediators of inflammation (cells and chemokines) I have measured

have complex control mechanisms, and the overall effect is most likely due to a combination of factors rather than due to any one in isolation.

3.15.5. Expression of IL-6, sIL-6R and sgp130 in preterm ventilated human infants

Expression of IL-6 in the preterm ventilated lung in human infants has been extensively studied since the first description of increased concentration in the lungs of infants with prolonged rupture of membranes (PROM) (Grigg et al., 1992). In one of the early studies, IL-6 was found to be significantly higher on day 10 in the lungs of infants developing CLD compared to those who did not (Kotecha et al., 1996b). Significant differences in IL-6 concentration were found earlier in the course of ventilation on days 3 and 5 (Munshi et al., 1997) and days 2 and 3 (Jonsson et al., 1997a). Although elevated IL-6 concentrations in the lungs of preterm infants at birth has been associated with subsequent CLD, (Choi et al., 2006a), this more likely represents intra-uterine inflammation. However, this has not been a consistent finding across other studies where comparable concentrations were recovered from infants who did and did not develop CLD (Kazzi et al., 2001, von Bismarck et al., 2008b). CLD was defined differently in studies as oxygen requirement at 28 days of age (Kotecha et al., 1996b, Jonsson et al., 1997a, von Bismarck et al., 2008b) or at 36 weeks corrected gestational age (Munshi et al., 1997, Kazzi et al., 2001) predictably resulting in variability in the incidence of CLD. There was variation in the clinical characteristics and the care provided to infants in the different studies as well. Overall, IL-6 has been associated with the development of CLD which is in agreement with this cytokine being an important mediator during inflammation, However, similar to the chemokines, raised concentrations of IL-6 by itself is unlikely to be a direct cause of CLD but more likely represents increased duration of inflammation. I have defined CLD as oxygen requirement at 36 weeks corrected gestational. My analysis shows a trend towards higher peak IL-6 concentrations in the lungs of CLD infants which was not statistically

significant. I am not sure whether including more infants in the groups would have made this trend statistically significant.

While there is an extensive literature on IL-6, only one study (which was published as this study was commencing) has reported concentrations of the soluble receptors sIL-6R and sgp130 in the preterm human lung (von Bismarck et al., 2008b). The authors collected two samples of tracheal aspirate fluid (TAF) from ventilated preterm infants ≤ 32 weeks gestation, the first on day 1 and the second between days 4-7. No significant differences in the concentration of mean sIL-6R or mean sgp130 were reported between CLD (defined as oxygen requirement at 28 days of age) and No CLD infants at either of the time-points included in the study (day 1 and day 4-7). However, in the CLD infants, there was a significant increase in both mean sIL-6R and mean sgp130 concentrations from day 1 to day 4-7. The increase in concentration of sgp130 is similar to what I have observed in the cohort of preterm baboons. However, they have reported an increase in sIL-6R concentration in preterm TAF for the first time , although the authors do not report an accompanying increase in innate immune cells. Apart from more number of days on ventilation, the CLD infants were of significantly lower gestation compared to the No CLD infants.

I have noted a trend towards increased peak sIL-6R concentrations in the CLD infants; however, peak sgp130 concentrations were significantly higher in these infants in my cohort (fig 3.7c). Differences in sampling methods (we have collected daily BALF samples from all infants in the first week), group stratification (I defined CLD as oxygen requirement at 36 weeks corrected gestational age) and statistical methods (I have reported medians while von Bismarck and colleagues reported means ± SEM) could be potential explanations for the above observations. However, the significantly higher concentration of sgp130 is an important finding being reported for the first time in preterm infants.

Our current knowledge about the mechanism of production of sIL-6R includes proteolysis (Mullberg et al., 1993c) and also differential mRNA splicing (Horiuchi et al., 1994b). Since the cell-surface expression of IL-6R is limited to hepatocytes and circulating immune cells, these are probably the main sources of sIL-6R. Although innate immune cells seem to be the main source of circulating sIL-6R (McFarland-

Mancini et al., 2010), PMNs seem to be the main source of localised sIL-6R during acute inflammation (Hurst et al., 2001, Chalaris et al., 2007). Apoptosis of PMNs at sites of inflammation has been suggested to be a natural stimulant for the release of sIL-6R from the cell surface, and neutrophil depletion resulted in reduction of sIL-6R concentration (Chalaris et al., 2007). However, MNCs can also contribute to the production of sIL-6R (McLoughlin et al., 2004).

In our cohort of 10 preterm baboons, there were no significant differences in the concentration of sIL-6R between the different time-points. A possible explanation for this could be a lack of variation in the numbers of PMNs or MNCs recovered at the various time-points, which are the cellular sources of this cytokine. In contrast, in the preterm human infants, peak numbers of both PMNs and MNCs were higher in the CLD group (3.6a and 3.6b), although this was not reflected in a significant increase of sIL-6R concentration. Apoptosis of PMNs at sites of inflammation has been suggested to be natural stimulant for the release of sIL-6R from the cell surface (Chalaris et al., 2007). As PMNs in the lungs of CLD infants have been reported to have lower rates of apoptosis (Kotecha et al., 2003, Oei et al., 2003), this could be a possible explanation for the lack of significantly higher sIL-6R concentration in the CLD lungs, in spite of having higher cell counts. Rates of apoptosis of PMNs in the preterm baboon lung are not known.

Expression of membrane gp130 on cells is described to be ubiquitous, and homozygous deletion of *gp130* is lethal (Yoshida et al., 1996), suggesting its vital role during foetal development. Several mechanisms have been described which regulate expression of cell-surface gp130 (Geisterfer et al., 1995, Bhat et al., 1999, Sharkey et al., 1995, Deb et al., 1999, Vallieres and Rivest, 1997, Geisterfer and Gauldie, 1996, Blanchard et al., 2001). At least two isoforms of sgp130 have been detected in human serum (Narazaki et al., 1993) due to alternative splicing of mRNA (Diamant et al., 1997, Tanaka et al., 2000) and limited proteolytic cleavage of cell-surface gp130 (Mullberg et al., 1993a, Montero-Julian et al., 1997). PMNs exhibit time dependent expression of sgp130 when cultured *in vitro* without stimulation (Jablonska and Jablonski, 2002) and inflammatory stimuli have also been shown to upregulate sgp130 expression (Jablonskaca et al., 2003) from PMNs. MNCs have also been proposed as a source if

sgp130 (Memoli et al., 2005). Considering the high levels of sgp130 (390 ± 72 ng/ml) normally detected in human serum (Narazaki et al., 1993), it is not yet clear what the regulatory mechanism involved in its expression are, both normally and during inflammation. Cellular sources of sgp130 have also not been elucidated, although potentially all cells expressing gp130 could be a source.

There is evidence supporting modulation of sgp130 expression in non-inflammatory conditions (Walshe et al., 2010) and during the normal menstrual cycle (Sherwin et al., 2002). Compared to healthy controls, serum levels of sgp130 were found to be significantly higher and cerebrospinal fluid (CSF) levels to be significantly lower in patients with multiple sclerosis (Padberg et al., 1999). Endometrial biopsies from idiopathic infertile women secreted significantly less sgp130 compared to fertile women (Sherwin et al., 2002). The authors of this study (Sherwin et al., 2002) also suggested that proteolytic cleavage of membrane gp130 was the main mechanism of production of sgp130 from the endometrial biopsies as none of the primers encoding the known splice variants of sgp130 had a product after polymerase chain reaction (PCR). This finding has been reported previously from multiple myeloma cell lines (Montero-Julian et al., 1997). Patients with acute ischaemic stroke had significantly lower serum sgp130 levels in the first week compared to healthy controls (Acalo*vs*chi et al., 2003). Modulation of sgp130 expression has also been described in aqueous humour from patients with uveitis (Simon et al., 2008), in plasma from patients with metabolic syndrome (Zuliani et al., 2010) and women with pre-eclampsia (Wang et al., 2011), all compared to matched healthy controls. Together, this data provides ample evidence of modulation of expression of sgp130, both in health and disease, although the mechanism involved in such regulation have not been proposed.

Similar to IL-6, sgp130 concentrations increased significantly on day 6-7 in the preterm baboon, which persisted till day 10-11 before resolving on day 14. In the preterm human infants, significantly higher concentration of sgp130 was found in the lungs of CLD infants compared to those without CLD. Taken together, this data suggests the existence of mechanisms modulating sgp130 expression in the preterm lung during inflammation. It is widely accepted that sgp130 is a specific inhibitor of IL-6 *trans*-signalling and acts to regulate effects of *trans*-signalling *in vivo* (Narazaki et al.,

1993, Jostock et al., 2001b). However, the role of the different isoforms, if any, is yet to be elucidated. As our knowledge regarding expression and regulation of sgp130 is incomplete, the possible mechanisms of its dynamic expression are unclear. Considering the high concentrations detected in the lungs of preterm baboons and human CLD infants, I hypothesise that sgp130 could have a profound regulatory effect on IL-6 *trans*-signalling; however, a firm conclusion cannot be drawn from observational data and further functional studies are needed to clarify the impact of sgp130 in these specimens.

3.15.6. Ratio of IL-6 trans-signalling molecules in preterm ventilated human infants

The effect of IL-6 *trans*-signalling is dependent on the relative presence of IL-6 and its two soluble receptor subunits. Thus, a complex of sIL-6R/IL-6 acts as an agonist on cells lacking the membrane IL-6R. This “cytokine” is specifically inhibited by sgp130, which competes with membrane gp130 to bind the complex. Detailed *in vitro* binding assays (Gaillard et al., 1999) showed that:

In the absence of sgp130, the proportion of free (unbound to any of the soluble receptors) and bound (as part of the sIL-6R/IL-6 complex) IL-6 are constant over a wide range of IL-6 concentrations, free IL-6 being about 30% of the total (free + bound) IL-6. The proportion of free IL-6 is decreased by increasing concentrations of sIL-6R, which is accompanied by a corresponding increase in the sIL-6R/IL-6 complex. The proportion of free IL-6 remains unaltered in the presence of sgp130. However, the proportion of (detectable) sIL-6R/IL-6 complex (bound IL-6) decreases in the presence of sgp130, presumably due to formation of the ternary complex IL-6/sIL-6R/sgp130.

In summary, interaction of these molecules can be predicted by the mathematical model proposed by the authors. They demonstrated that the concentration of the sIL-6R/IL-6 complex is directly proportional to the concentration of sIL-6R (not IL-6) and inversely proportional to the concentration of sgp130. Thus, although IL-6 is the bioactive molecule, *trans*-signalling by the complex is controlled by

the soluble receptor components. The soluble IL-6R seems to be the key molecule, as the complexes of sIL-6R/IL-6 and IL-6/sIL-6R/sgp130 would not form in its absence. To have an impact on concentration, both of the soluble receptors were required in excess of IL-6 concentration.

IL-6 *trans*-signalling has been implicated in several diseases, involving both systemic and localised inflammation. The best characterised among them is arthritis where an excess of sIL-6R, compared to IL-6, is present in synovial fluid of the inflamed joint. Localised synovial sIL-6R/IL-6 (mass) ratio in osteoarthritis was found to be around 50 (Kotake et al., 1996), 320 (Uson et al., 1997) and 10 (Desgeorges et al., 1997) in different studies. For rheumatoid arthritis, the (mass) ratio ranged from 14 (Kotake et al., 1996), 19 (Uson et al., 1997) and 1 (Desgeorges et al., 1997). An excess of sIL-6R was also found in the synovial fluid of the majority of patients with juvenile idiopathic arthritis (Peake et al., 2006). Unfortunately, the corresponding concentration of local sgp130 in the joint was not reported in these studies. Blockade of arthritis by specifically targeting this complex (sIL-6R/IL-6) with sgp130 in an animal model (Nowell et al., 2003) confirmed the role of IL-6 *trans*-signalling in the pathogenesis of this disease.

Compared to IL-6, a large excess of sIL-6R was found in serum of patients with inflammatory bowel disease (Mitsuyama et al., 1995). Circulating sIL-6R levels were increased, in excess of IL-6, in active Crohn's disease compared to patients who were in remission; more interestingly, expression of sgp130 was decreased in patients with active disease compared to patients in remission or healthy controls (Gustot et al., 2005). The authors also found a significant decrease of sgp130 concentrations in patients with active ulcerative colitis. Taken together, these results suggest an alteration of the balance of the IL-6 system towards a pro *trans*-signalling phenotype in inflammatory bowel disease. Delayed apoptosis of T-cells in Crohn's disease was reversed by sgp130, confirming the role of IL-6 *trans*-signalling in the pathogenesis of this disease (Atreya et al., 2000).

In the lungs, IL-6 *trans*-signalling has been reported in the context of asthma. An imbalance in the T-helper 1 (Th1) and Th2 subtypes of $CD4^+$ T-cells has been implicated in the pathogenesis of bronchial asthma (Neurath et al., 2002) and IL-6

seems to shift the balance towards Th2 polarisation of naïve $CD4^+$ T-cells (Diehl and Rincon, 2002). Serum levels of sIL-6R was increased in asymptomatic patients with asthma and even more so during an acute attack, although corresponding IL-6 or sgp130 levels were not reported in this study (Yokoyama et al., 1997). Localised concentration of sIL-6R from BALF samples was significantly higher in asthmatic subjects compared to normal controls at baseline and after allergic challenge (Doganci et al., 2005), although corresponding IL-6 or sgp130 concentrations were not reported by the authors. Historical data of BALF IL-6 levels (measured functionally in a cell-based assay) from a separate study (Broide et al., 1992) would suggest a moderate excess of sIL-6R as measured by Doganci and colleagues (Doganci et al., 2005). However, using a murine model of asthma, the latter authors (Doganci et al., 2005) demonstrated that specific blockade of IL-6 *trans*-signalling by using sgp130 resulted in down-regulation of Th2 type cytokines (IL-4, IL-5 and IL-13) and up-regulation of Th1 phenotype. In an *ex vivo* culture system, IL-6 *trans*-signalling was found to have a profound impact on the airway remodelling phenotype by smooth muscle cells, another long-term consequence of atopic asthma (Ammit et al., 2007).

The other player in this trio, sgp130, was significantly higher in the CLD group of human infants. As noted earlier, few studies have reported concurrent concentrations of all three molecules in a localised site of inflammation. The normal serum level of sgp130 has been reported to be around 390 ng/ml (Narazaki et al., 1993) while that of sIL-6R has been reported around 30 ng/ml (Horiuchi et al., 1998, Keul et al., 1998). This suggest a >10-fold (mass) excess of sgp130 normally exists in human serum. Whether similar values are present during systemic or localised inflammation is not yet clear. In Crohn's disease, where IL-6 *trans*-signalling has been implicated in disease pathogenesis, the (mass) ratio of sgp130/sIL-6R increased from 5.7 in patients in remission to 9.6 in patients active disease, due to both a decrease in sIL-6R and an increase in sgp130 concentrations.

In summary of the previous data, IL-6 *trans*-signalling has been implicated in several localised diseases (with or without systemic involvement), although concurrent measurement of all the three molecules has not been routinely reported. An excess of sIL-6R seems to be necessary for driving the balance towards *trans*-signalling, as

demonstrated in the joints of arthritis patients. IL-6 *trans*-signalling is effectively inhibited by an excess of sgp130.

In our cohort of 10 preterm baboons, I have observed a significant decrease in the agonistic ratio (sIL-6R/IL-6) at day 6-7 which persisted until day 10-11 before increasing again at day 14. This would suggest a decrease in *trans*-signalling potential. During the same time period, the antagonistic ratio (sgp130/sIL-6R) reached a peak on day 6-7, again suggesting a decrease in *trans*-signalling potential. Indeed, a significant increase in IL-8 concentration was observed between day 6-7 and day 10-11, which could be due to a lack of IL-6 *trans*-signalling effects. However, a firm conclusion cannot be drawn as the regulation of chemokines by IL-6 *trans*-signalling is complex, as also many other factors can regulate IL-8 expression. In my cohort of preterm human infants, there were no significant differences noted in the peak IL-6 or sIL-6R levels in BALF between CLD and No CLD infants, although a trend towards higher values in CLD was observed. Analysis of the peak molar ratio of sIL-6R/IL-6 showed an modest excess of sIL-6R compared to IL-6 in CLD infants (median ratio 2.489) but not in No CLD infants (median ratio 1.014).

As the potential for IL-6 *trans*-signalling is determined by the relative expression of the two molecules, I compared the expression of the agonistic ratio (sIL-6R/IL-6) to that of the antagonistic ratio (sgp130/sIL-6R). The medians of the antagonistic ratio in the preterm baboons was in excess of the agonistic ratio at all the measured time-points (184.4 fold on day 1, 86.9 fold on day 3, 646.7 fold on day 6-7, 411.1 fold on day 10-11 and 252.4 fold on day 14), largely due to the excess of sgp130 in the lungs. The ratio of sgp130/sIL-6R in the preterm human infants was about 8-fold more in the No CLD group and 13-fold more in the CLD group. In the study by von Bismarck (von Bismarck et al., 2008b), although sIL-6R was in modest excess of IL-6, there was a 20-fold excess in the relative concentration of sgp130 (sgp130/sIL-6R) in both group of infants at both time-points (these were calculated from reported mean values of individual cytokines). Inferring from the current knowledge, this would seem to be inadequate to favour IL-6 *trans*-signalling in the preterm infant lung.

Since a large excess of sgp130 can completely abrogate the effects of the sIL-6R/IL-6 complex (Gaillard et al., 1999), this supports my hypothesis that there is

potentially a lack of functional IL-6 *trans*-signalling in the preterm ventilated lungs. The strategy of using a large excess of sgp130 to block IL-6 *trans*-signalling has been successful in several disease models (Nowell et al., 2003, Doganci et al., 2005, Atreya et al., 2000). Recently this approach has been found to be useful in a model of lung inflammation by *Chlamydophila pneumoniae* as well (Rodriguez et al., 2010), although the recovery of sgp130 from the lungs was not reported.

In summary, the relative excess of sgp130 in the preterm ventilated lung could have a profound effect on IL-6 *trans*-signalling activity. This excess was evident in both the preterm baboons & human infants, and appropriate functional activity assays would be needed to confirm this hypothesis.

3.15.7. Expression of DS sIL-6R

Two different cDNAs for IL-6R have been generated from cell-lines expressing membrane IL-6R by PCR (Lust et al., 1992, Oh et al., 1996). This finding was confirmed when the protein was detected in cell-culture supernatants (Horiuchi et al., 1994b). A monoclonal antibody was raised to detect the 10 unique amino acids in DS sIL-6R and an ELISA to specifically detect this isoform was described (Horiuchi et al., 1998). The authors also found an inverse relationship of DS sIL-6R and age, with the highest concentrations found in younger age groups. On an average, about 15% of the total sIL-6R in circulation in healthy individuals was found to be the DS isoform. Based on this observation, it has been suggested that the concentration of DS sIL-6R in the neonatal population could be higher that that in adults (Prof Simon Jones, personal communication). Also, the concentration of DS sIL-6R in a localised site of inflammation has not been studied before. Data from my cohort demonstrates that only a minor proportion of the sIL-6R present in the preterm lung was the splice variant, with the majority represented by the proteolytic cleaved form. Also, the splice variant seemed to be a minor proportion of the total sIL-6R in the term newborn circulation. Interestingly, the total sIL-6R in term newborn cord-blood was comparable to levels in the adult control population.

3.16. Summary of Chapter

I have described in detail the expression of IL-6, sIL-6R and sgp130, in the preterm lungs, both in a controlled primate model of preterm ventilation and in preterm human infants, with relation to some key inflammatory markers. The primary finding from this part of the study is the high levels of sgp130 noted in the preterm ventilated lung, and its excess in relation to IL-6 or sIL-6R. This makes me speculate that sgp130 could have a profound influence on IL-6 *trans*-signalling in the preterm lung and leads me on to the next part of my project involving functional activity of IL-6 in the preterm lung.

4. Functional Activity of IL-6 in the Preterm Ventilated Lungs

4.1. Overview

So far, I have analysed the concentration of IL-6 and its soluble receptor isoforms, sIL-6R and sgp130 in the preterm lungs in an animal models and in human infants. My analysis suggested that the concentration of sgp130 in significantly raised during ventilation of the preterm lungs. Since sgp130 is a specific inhibitor of IL-6 *trans*-signalling, raised concentrations of this molecule can have a profound impact on the functional activity of IL-6 in the preterm lung. As antigenic concentration cannot predict functional activity, I proceeded to measure functional activity in a cell based bioassay.

B9 cells are a mouse pre-B cell hybridoma which are IL-6 dependent for proliferation (Aarden et al., 1987a). They have been used by several investigators as a sensitive bioassay for IL-6 (Nordan et al., 2001a). Previously, these cells have been reported to be insensitive to several other cytokines (Helle et al., 1988b) apart from IL-6. The concentration of IL-6 needed to stimulate these cells to proliferate is in the low pg/ml range (Peters et al., 1996, Helle et al., 1988b). This makes the B9 cells an ideal cell-line to measure IL-6 bioactivity from clinical samples. The other major cell-based functional assay for IL-6 *trans*-signalling is the BAF/3 cells which require 100 – 1000 fold more concentration of cytokines for proliferation, making this an unsuitable model for use with clinical samples, particularly low-volume samples like preterm BALF which precludes any possible concentration steps.

To model IL-6 *trans*-signalling in the B9 cells, a synthetic protein, consisting of covalently linked human IL-6 with sIL-6R, was used (Fischer et al., 1997). This protein, called hyper IL-6 (HIL-6), is able to efficiently engage with cell-membrane gp130 and initiate intra-cellular signalling. Importantly, the IL-6R binding site of IL-6 is already occupied in this molecule, making it unable to initiate *cis* signalling (through the membrane-bound IL-6 receptor). This is also a stable molecule and not subject to dissociation as the natural complex is, making it an ideal choice for modelling *trans*-signalling. Thus, HIL-6 was chosen to be used as a positive control of a specific agonist of IL-6 *trans*-signalling.

4.2. Overall hypothesis and aims of the project

In the previous section, I have described observational data on the expression of IL-6 *trans*-signalling molecules in lung fluid collected from preterm ventilated baboons and human infants. My analysis shows a significant increase in the concentration of sgp130, a specific inhibitor of IL-6 *trans*-signalling, in the lungs of preterm baboons and human infants who later develop CLD. This observation leads to the hypothesis that there is possible abrogation of the IL-6 *trans*-signalling pathway in the lungs of these infants, this in turn providing a possible explanation for the poorly-resolved neutrophil-driven inflammation noted in the lungs of these infants.

The specific aims of this part of the project were:

- To establish a sensitive and specific bioassay for IL-6 (using the B9 cells-line).
- To modify the assay appropriately for measuring IL-6 *trans*-signalling activity.
- To measure total IL-6 activity and IL-6 *trans*-signalling activity in BALF samples from ventilated preterm infants.
- To compare differences in activity between the two groups of infants, CLD and No CLD.

4.3. Sensitivity of B9 cells to IL-6 cis-signalling

As reported previously (Nordan et al., 2001a), the B9 cells were highly sensitive to rhIL-6 and started to proliferate at concentrations of 0.1 pg/ml of IL-6 (figure 4.1). Half-maximal proliferation was noted at around 2-4 pg/ml and maximal proliferation at around 10-12 pg/ml.

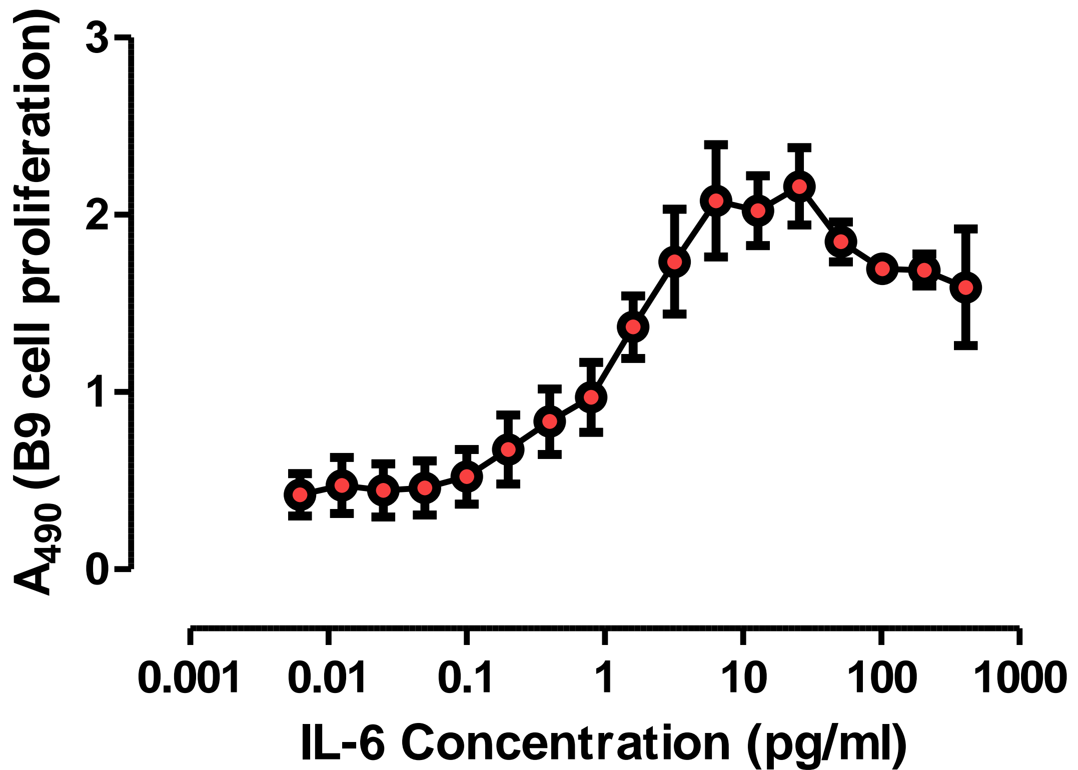


Figure 4-1: Representative dose response curve of B9 cells to rhIL-6. Concentration of IL-6 (pg/ml) is represented on the x-axis while B9 cell proliferation (absorbance at 490 nm) is represented on the y-axis. Points plotted are means (± SEM) of at least three independent experiments.

All of this proliferative activity was completely inhibited in the presence of anti human IL-6 monoclonal antibody (figure 4.2). For all future experiments, a concentration of 10 ng/ml of the antibody was used to ensure complete inhibition of all IL-6 activity.

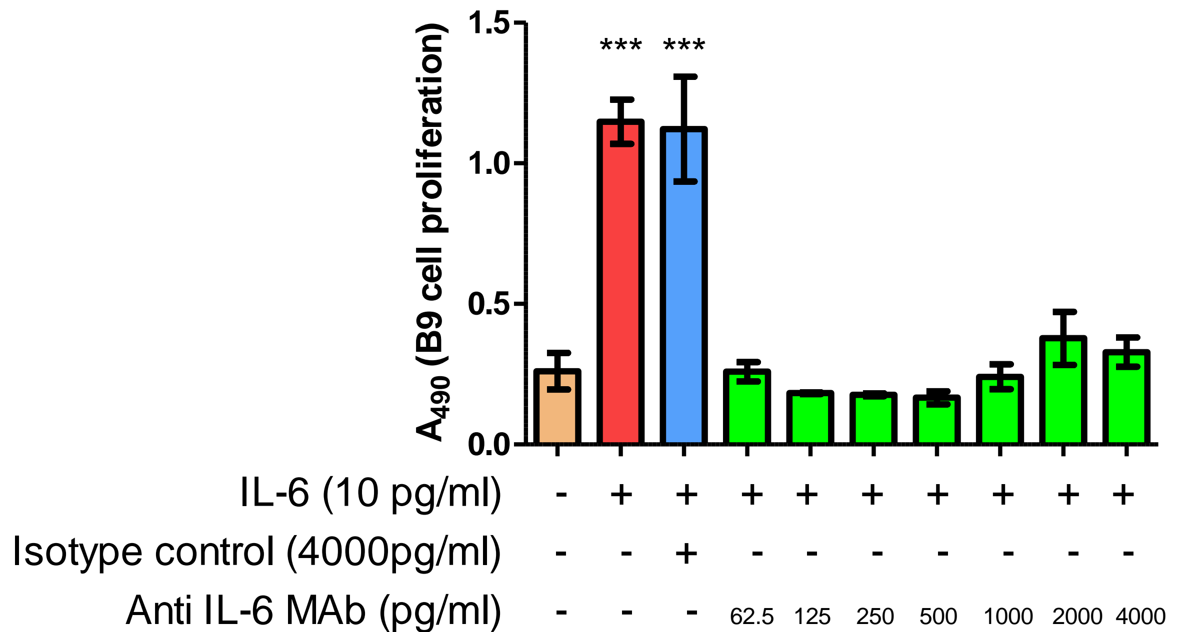


Figure 4-2: Inhibition of B9 cell proliferation in the presence of anti human IL-6 monoclonal antibody. Conditions (with respective concentrations) are represented on the x-axis while B9 cell proliferation (optical density at 490 nm) in represented on the y-axis. Bars represent means (±SEM) of three independent experiments. Significance was tested by one-way ANOVA with Dunnets post-test (comparing with control column – "no IL-6". (MAb = monoclonal antibody, *** = $p < 0.0001$).

4.4. Specificity of B9 cells to IL-6

B9 cells are reported to be unresponsive to a variety of human cytokines, either alone or in combination with human IL-6 (Helle et al., 1988b). In order to confirm this observation, I stimulated the B9 cells with cytokines (IL-1β, TNF-α, IL-4) and phlogistic stimuli of microbial origin (LPS), molecules which are expected to be present at sites of inflammation. Apart from IL-6 (at 5 pg/ml), none of the molecules resulted in any increase in proliferation of cells compared to background (cells without IL-6, fig 4.3).

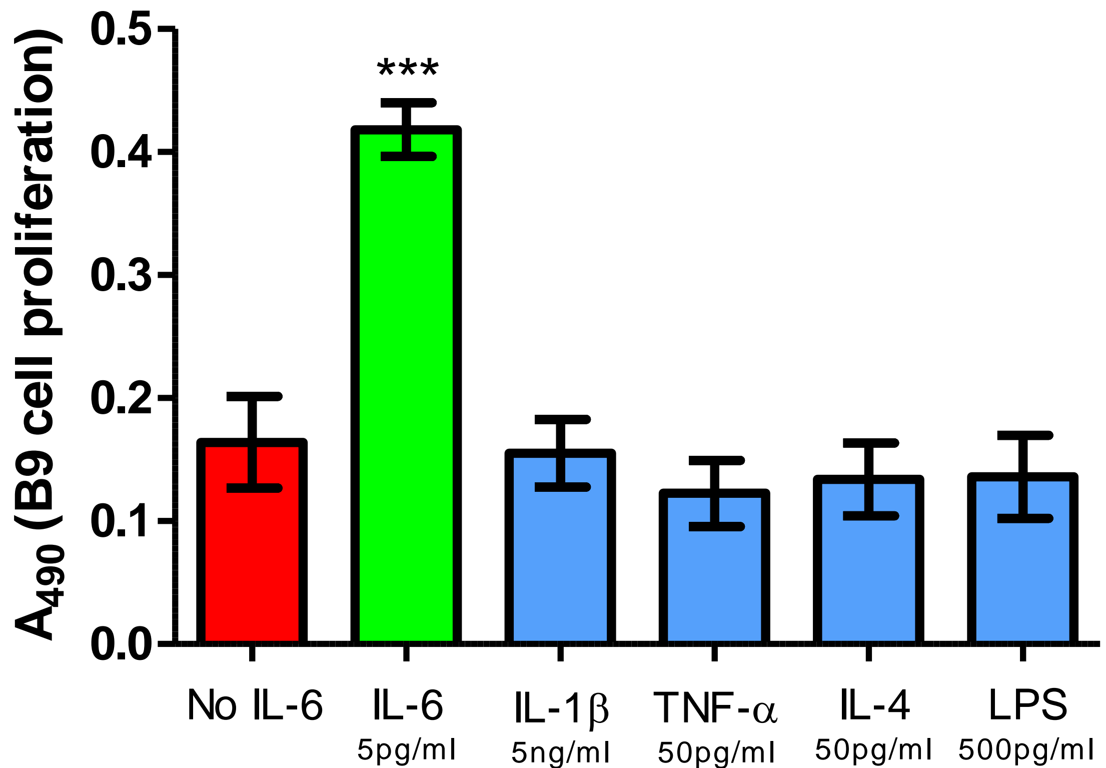


Figure 4-3: Specificity of B-9 cells. Proliferation of B9 cells (represented as absorbance readings on the y-axis) in response to different cytokines and infective agents (with doses, represented on the x-axis). Bars represent means (±SEM) of three independent experiments. Significance was tested by one-way ANOVA with Dunnets post-test (comparing with control column – "no IL-6"). (*** = $p < 0.0001$).

4.5. Sensitivity of B9 cells to IL-6 *trans*-signalling

While the response of B9 cells to IL-6 is well characterised (*cis*-signalling), their response to IL-6 *trans*-signalling is not known. To test this, I used the synthetic molecule hyper IL-6 (Fischer et al., 1997) which is a covalently linked compound of IL-6 and sIL-6R. This molecule is only capable of intra-cellular signalling through the gp130 receptor-subunit. As the IL-6R binding site on HIL-6 is taken up by the sIL-6R molecule,

hyper IL-6 shows no activity towards the membrane-bound IL-6 receptor, thus lacking any IL-6 *cis*-signalling activity.

Stimulation of B9 cells with HIL-6 resulted in significant increase in proliferation of cells in a dose-dependent manner (fig 4.4). Maximum proliferation was observed at a concentration of 4 – 8 pg/ml with half-maximal proliferation at a concentration of around 0.5 – 1.0 pg/ml. Thus, the B9 cells were equally sensitive to IL-6 *trans*-signalling.

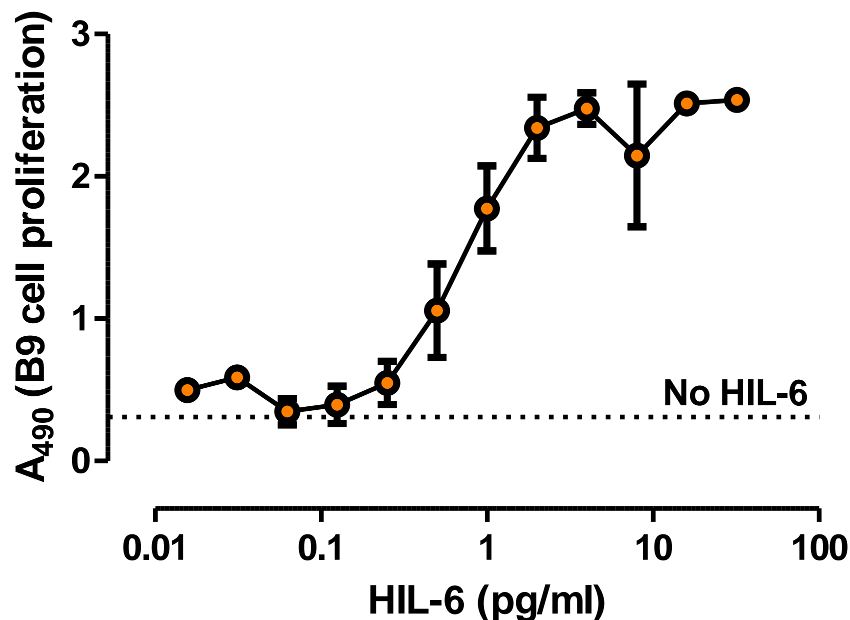


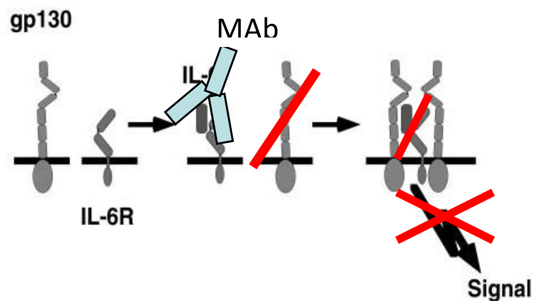
Figure 4-4: Sensitivity of B9 cells to IL-6 *trans*-signalling. Concentration of HIL-6 (pg/ml) is represented on the x-axis (log-scale) while proliferation of cells is represented on the y-axis. Interrupted line represents background proliferation of cells (no HIL-6). Points plotted are means (±SEM) of three independent experiments.

4.6. Modifications of the assay to measure IL-6 *trans*-signalling

To measure specific *trans*-signalling activity in clinical samples, I chose a strategy to selectively inhibit *cis*-signalling. This was achieved by blocking the mouse IL-6R present on B9 cells with monoclonal antibodies. As shown in the schematic below

(fig 4.5), effective blocking of the IL-6R with an appropriate monoclonal antibody (blue) would abrogate intra-cellular responses (marked as red crosses) due to IL-6 *cis*-signalling; however, the gp130 molecule would be free to attach with a complex of sIL-6R/IL-6 to initiate IL-6 *trans*-signalling.

(a)



(b)

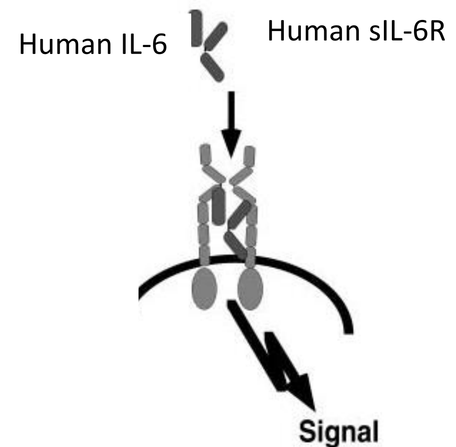


Figure 4-5: Schematic representation of modification of the B9 cell assay to measure IL-6 *trans*-signalling in clinical samples. (a) Anti-mouse monoclonal IL-6R antibody is represented in blue (MAb) which results in blocking of membrane-bound IL-6R, resulting in inhibition of IL-6 *cis*-signalling. (b) The gp130 molecule remains free for IL-6 *trans*-signalling. A complex of sIL-6R/IL-6 can bind to gp130 and initiate intra-cellular signalling. (Modified from Jones SA *et al,* J Interferon Cytokine Res, 2005 May;25(5):241-53)

Initially, a commercial anti-mouse IL-6R monoclonal antibody clone 15A7 (Coulie et al., 1990) was used for the purpose (Biolegend). As shown in fig 4.6 a and b, suppression of cell proliferation by the 15A7 antibody, in the presence of IL-6 at a concentration of 5 pg/ml, started at concentration of antibody above 40 ng/ml and complete suppression (to background levels, “no IL-6”) was achieved at a concentration around 2.5 µg/ml. Thus, by using this strategy, I could specifically block all *cis*-signalling in the B9 cells while leaving the gp130 sub-unit free for *trans*-signalling.

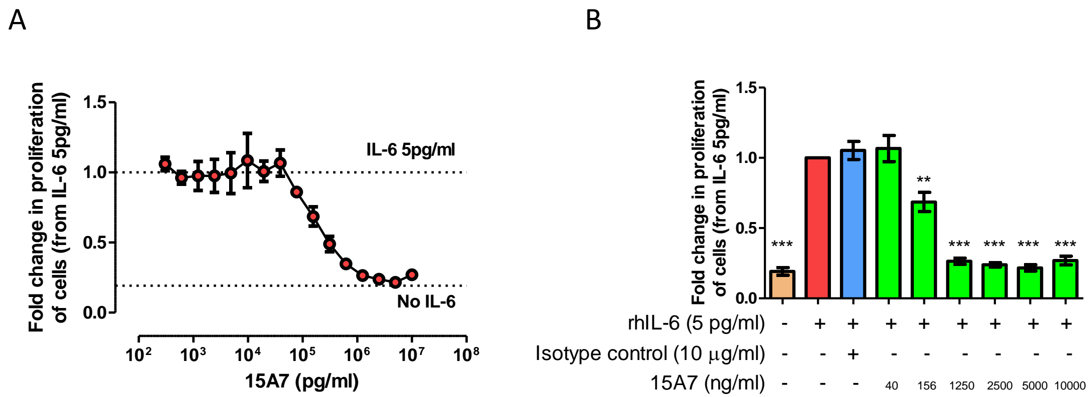


Figure 4-6: (a) Dose-response of 15A7 antibody. Concentration of antibody (pg/ml) is represented on the x-axis (log scale) while proliferation of cells, expressed as a fold change from IL-6 at a concentration of 5 pg/ml, is represented on the y-axis. The upper dotted line represents proliferation of cells in the presence of IL-6 at a concentration of 5 pg/ml (no antibody) and the lower dotted line represents cell-proliferation in the absence of IL-6. Points plotted are means (±SEM) of three independent experiments. (b) Inhibition of B9 cell proliferation in the presence of 15A7 antibody. Conditions (with respective concentrations) are represented on the x-axis while B9 cell proliferation, expressed as a fold change from IL-6 at a concentration of 5 pg/ml, is represented on the y-axis. Bars represent means (±SEM) of three independent experiments. Significance was tested by one-way ANOVA with Dunnets post-test (comparing with control column – “no IL-6”). (*** = $p < 0.0001$).

The possibility of anti-mouse sIL-6R antibody binding to and inhibiting the human sIL-6R in the clinical samples of BALF, leading to spurious results was considered. Before proceeding to measure *trans*-signalling, I conducted experiments to confirm that the anti-mouse IL-6R antibody would not cross-react with human sIL-6R. To test this, a direct ELISA was designed whereby human and mouse sIL-6R were plated onto 96-well plates and tested for binding with the 15A7 antibody. As shown in figure 4.7, 15A7 antibody did not bind to rhIL-6; however, there was significant binding to both mouse and human sIL-6R ($p < 0.0001$ compared to no antigen). Indeed, the 15A7 antibody seemed to bind to recombinant human sIL-6R more avidly than to recombinant mouse sIL-6R ($p < 0.05$) when the two were compared. These results

suggested to me that there could be significant cross-reactivity between the 15A7 antibody and human sIL-6R (in the BALF samples) which could result in inhibition of IL-6 *trans*-signalling in the B9 assay.

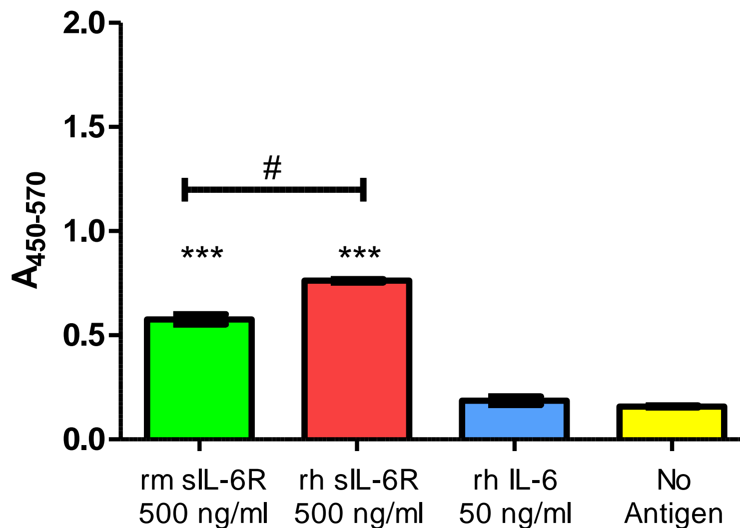


Figure 4-7: Cross-reaction of 15A7 antibody with human sIL-6R. Antigens (recombinant mouse sIL-6R in green, recombinant human sIL-6R in red, recombinant human IL-6 in blue and negative control in yellow) are depicted on the x-axis while absorbance values at 450 – 570 nm are represented on the y-axis. Bars are means (± SEM) of two independent experiments. Significance was tested by one-way ANOVA with Dunnets post-test (comparing with control column – "no antigen". (*** = $p < 0.0001$; # = $p < 0.05$).

I next searched for another appropriate anti-mouse IL-6R monoclonal antibody in order to avoid inter-species cross-reaction, but none were available commercially. A personal enquiry from Dr Simon Jones at Cardiff University identified another clone of anti-mouse IL-6R monoclonal antibody called 2B10 (Greenhill et al., 2011, Lissilaa et al., 2010) which did not cross-react with the human molecule. I obtained sufficient quantities of this antibody as a generous gift from NovImmune (Geneva, Switzerland), the company who had developed this antibody (not commercially available).

The 2B10 antibody resulted in dose-dependent inhibition of B9 cell proliferation. Similar to the 15A7 clone, inhibition started at a concentration of 40

ng/ml and above (fig 4.8). Complete inhibition (down to background levels – "no IL-6") was achieved at around 1.25 µg/ml, which was lower than that required for the 15A7 clone. To ensure complete inhibition of B9 cell proliferation, the 2B10 antibody was used at a concentration of 2.5 µg/ml in all future experiments.

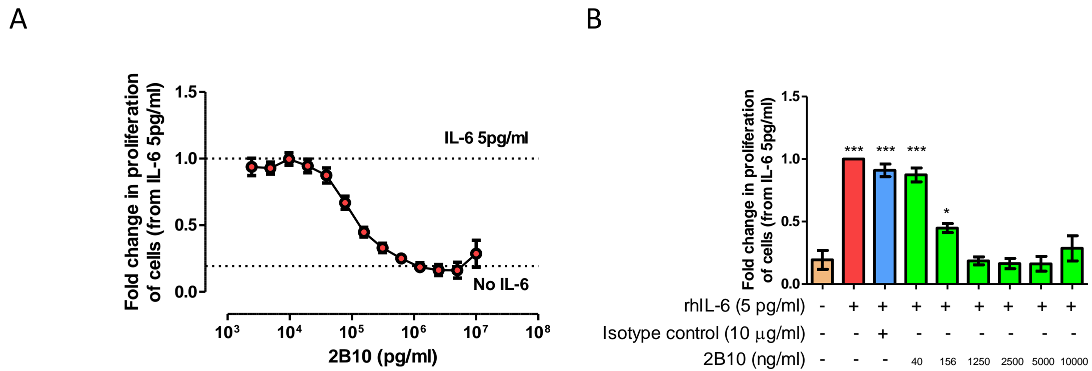


Figure 4-8: (a) Dose-response of 2B10 antibody. Concentration of antibody (pg/ml) is represented on the x-axis (log scale) while proliferation of cells, expressed as a fold change from IL-6 at a concentration of 5 pg/ml, is represented on the y-axis. The upper dotted line represents proliferation of cells in the presence of IL-6 at a concentration of 5 pg/ml (no antibody) and the lower dotted line represents cell-proliferation in the absence of IL-6. Points plotted are means (±SEM) of three independent experiments. (b) Inhibition of B9 cell proliferation in the presence of 2B10 antibody. Conditions (with respective concentrations) are represented on the x-axis while B9 cell proliferation, expressed as a fold change from IL-6 at a concentration of 5 pg/ml, is represented on the y-axis. Bars represent means (±SEM) of three independent experiments. Significance was tested by one-way ANOVA with Dunnets post-test (comparing with control column – "no IL-6"). (*** = $p < 0.0001$; * = $p < 0.05$).

I next considered the possibility of cross-reaction of the 2B10 antibody with the human sIL-6R (in the BALF samples). This was initially tested in the same direct ELISA as for the 15A7 antibody previously. The 2B10 antibody demonstrated significant binding with the recombinant mouse sIL-6R (fig 4.9). The absorbance values for a concentration of rm sIL-6R of 500 ng/ml was beyond the upper limit of detection, and only at concentrations of below 31.25 ng/ml was this within the range of detection; this was significantly higher than the negative control ($p < 0.0001$ compared to "no

antigen). When tested against rh sIL-6R coated at 500 ng/ml, 2B10 did not exhibit statistically significant binding, although the signal was higher compared to rh IL-6 or no antigen. This may reflect the 10-fold increased concentration of rh sIL-6R compared to rh IL-6. There was also a significant difference in binding of the 2B10 antibody with recombinant mouse (rm) sIL-6R and rh sIL-6R ($p < 0.05$).

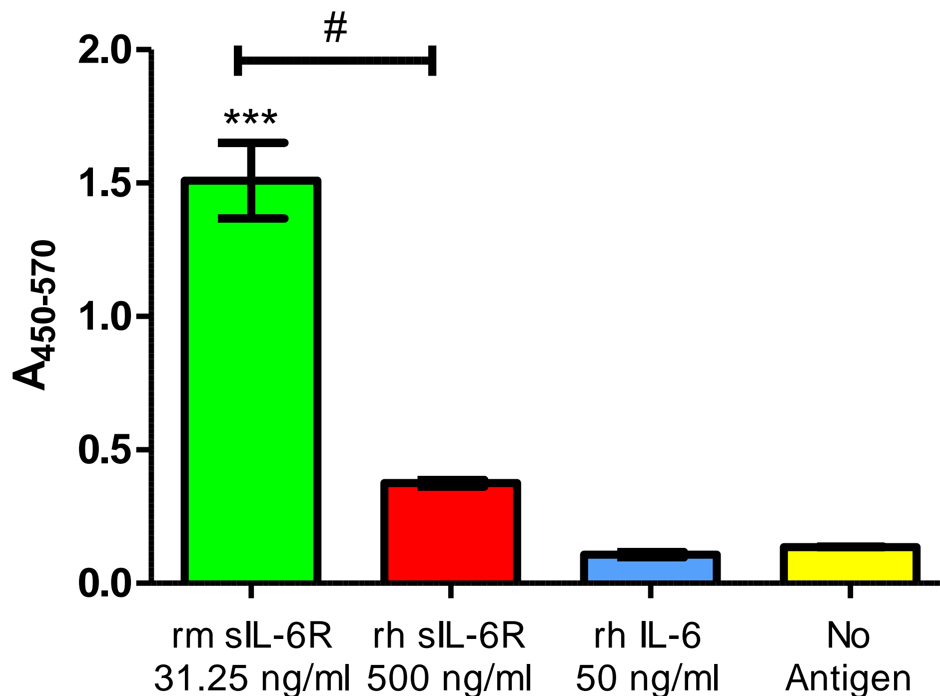


Figure 4-9: Testing cross-reaction of 2B10 antibody with human sIL-6R. Antigens (recombinant mouse sIL-6R in green, recombinant human sIL-6R in red, recombinant human IL-6 in blue and negative control in yellow) are depicted on the x-axis while absorbance values at 450 – 570 nm are represented on the y-axis. Bars are means (± SEM) of two independent experiments. Significance was tested by one-way ANOVA with Dunnets post-test (comparing with control column – "no antigen". (*** = $p < 0.0001$; # = $p < 0.05$).

I next tested this further in an activity assay using synovial fibroblast cells from rheumatoid arthritis patients. These cells do not express IL-6R on the cell-membrane but can respond to IL-6 *trans*-signalling (*via* cell-surface gp130) to selectively express MCP-1 (Nowell et al., 2003). Cells were a generous donation from Dr Mari Nowell at

Cardiff University. As expected, the cells did not respond to rh IL-6 but a combination of rh IL-6 with rh sIL-6R (complexed by pre-mixing at room temperature for 30 minutes) resulted in a dose-dependent increase in expression of MCP-1. Hyper IL-6, a specific agonist of *trans*-signalling, also resulted in a significant increase of expression of MCP-1. More importantly, this effect was preserved in the presence of the 2B10 antibody, suggesting that there was no significant binding or inhibition of the human sIL-6R by the antibody. No significant differences were observed in the expression of MCP-1 under different conditions of stimulation either in the presence or absence of the 2B10 antibody.

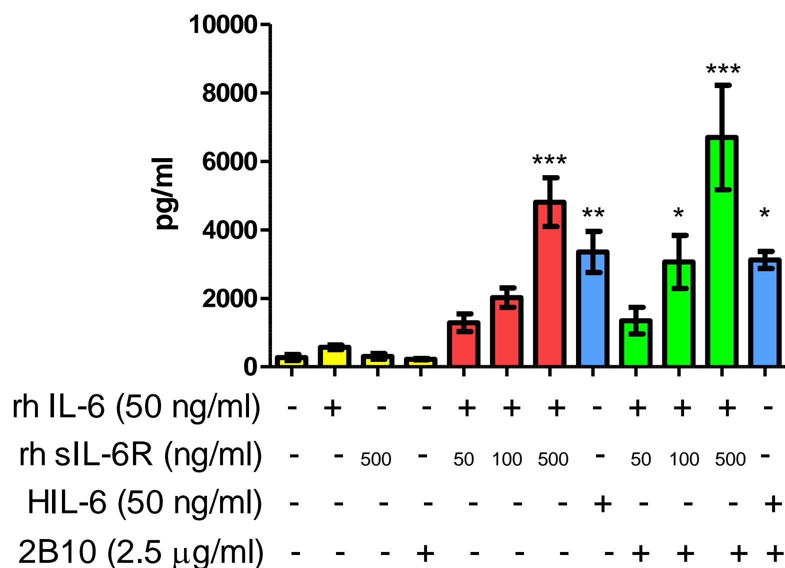


Figure 4-10: Testing cross-reaction of 2B10 antibody with human sIL-6R in RASF cells. Stimulations (presence = +, absence = -) are represented on the x-axis (with doses when appropriate) below each bar and expression of MCP-1 in the supernatant (pg/ml) is represented on the y-axis. Negative controls are depicted by yellow bars, complex of sIL-6R/IL-6 in the absence of 2B10 antibody is depicted by red bars and by green bars in the presence of 2B10 antibody. Stimulation with HIL-6 is depicted by the blue bars. Bars represent means (± SEM) of three independent experiments. Significance was tested by one-way ANOVA with Dunnets post-test (comparing with control column – "media". (*** = $p < 0.001$;** = $p < 0.01$; * = $p < 0.05$).

To show that the 2B10 antibody does not affect *trans*-signalling in the B9 cell assay, the proliferation of cells in response to HIL-6 was tested, both in the presence

and absence of 2B10 antibody. As shown in figure 4.11, no significant difference in proliferation of cells to HIL-6 was observed when cell surface mIL-6R was blocked with the 2B10 antibody. Thus, proliferation of cells in the presence of 2B10 antibody was considered to be due to IL-6 *trans*-signalling.

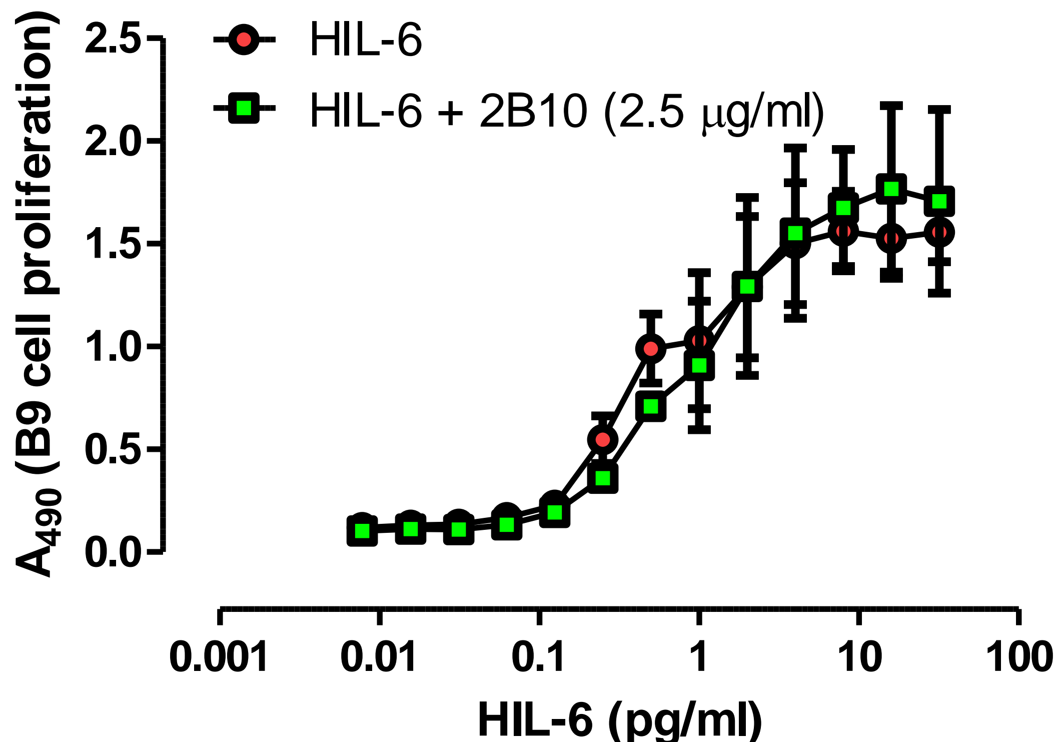


Figure 4-11: IL-6 *trans*-signalling in the presence of 2B10 antibody. Concentration of HIL-6 (pg/ml) is represented on the x-axis (log-scale) while proliferation of cells (absorbance at 490 nm) is represented on the y-axis. Red circles and green squares depict proliferation in the absence or presence of 2B10 antibody respectively. Points plotted are means (± SEM) of three independent experiments.

To confirm that the response to HIL-6 is indeed due to *trans*-signalling responses, I observed the proliferation of cells with HIL-6 in the presence of sgp130, a specific inhibitor of IL-6 *trans*-signalling. For these experiments, a chimeric protein of sgp130 linked to the Fc portion of IgG (Jostock et al., 2001b) was used as a specific inhibitor. These experiments were conducted in the presence of 2B10 antibody at a concentration of 2.5 μg/ml. As shown in figure 4.12, sgp130Fc inhibited B9 cell proliferation (in response to HIL-6 at a concentration of 4 pg/ml) in a dose-dependent

manner. Inhibition started at a concentration above 40 pg/ml of sgp130Fc (sgp130Fc: HIL-6 = 10:1) and complete inhibition was observed at around 4000 pg/ml of sgp130Fc (sgp130Fc: HIL-6 = 1000:1). These results confirmed that the proliferation of B9 cells in response to HIL-6 was indeed due to IL-6 *trans*-signalling as it could be completely inhibited by the specific inhibitor sgp130Fc.

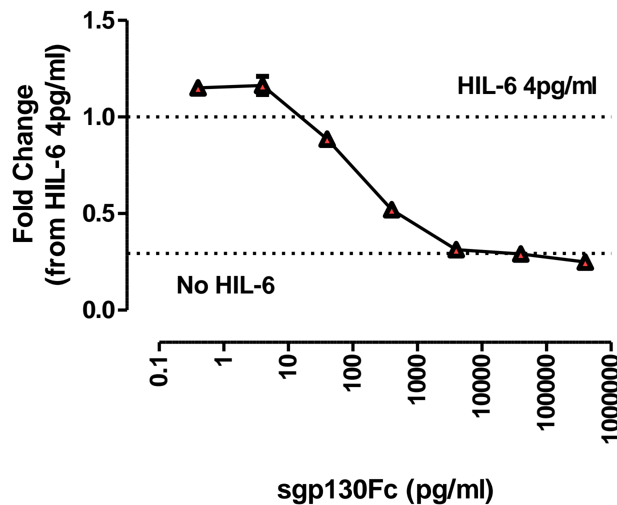


Figure 4-12: Dose-response of sgp130Fc. Concentration of sgp130Fc (pg/ml) is represented on the x-axis (log scale) while proliferation of cells, expressed as a fold change from HIL-6 at a concentration of 4 pg/ml, is represented on the y-axis. The upper dotted line represents proliferation of cells in the presence of HIL-6 at a concentration of 4 pg/ml (no sgp130Fc) and the lower dotted line represents cell-proliferation in the absence of HIL-6. All cells were exposed to 2.5 µg/ml of 2B10 antibody for 30 minutes at 37°C before stimulation. HIL-6 and sgp130Fc were pre-incubated together for 30 minutes at room temperature before adding to cells. Points plotted are means (±SEM) of three independent experiments.

4.7. IL-6 functional activity in preterm BALF

So far, I have shown that the B9 cells can be used as a sensitive and specific assay for IL-6 functional activity. I have also modified the assay in order to be able to measure specific *trans*-signalling activity. I next proceeded to measure IL-6 activity in preterm BALF.

During initial experiments with BALF, I observed that the functional activity of IL-6, as measured in the assay, was consistently greater that the estimated concentration of IL-6 in the BALF sample. As the active range of the assay (in terms of IL-6 concentration) was narrow, all future experiments were conducted after diluting the BALF samples to an estimated IL-6 concentration of 2 pg/ml (based on ELISA values) to keep the activity in the range of the assay. By using a constant concentration of IL-6 in all the BALF samples, similar proliferation of B9 cells would be expected; thus, any changes in proliferation could be attributed to differences in concentration of the *trans*-signalling molecules. Consequently, all activity is expressed as a fold-change in proliferation of B9 cells as compared to rh IL-6 at a concentration of 2 pg/ml. 24 random samples of BALF from each group (CLD and RDS) were chosen to measure functional activity.

Figure 4.13 shows a panel of controls which were run alongside each experiment. There was a predictable dose response in proliferation of B9 cells to increasing doses of IL-6 up to a concentration of 8 pg/ml. As shown in figure 4.1, the limit of the assay (maximal B9 cell proliferation) was reached at a concentration of 10 pg/ml; thus for these experiments, I chose to use IL-6 at a maximum concentration 8 pg/ml to stay within the range of the assay. IL-6 at a concentration of 8 pg/ml was used as a positive control for *cis*- and *trans*-signalling, to demonstrate that at this concentration, both anti-IL-6 antibody at a concentration of 10 ng/ml and 2B10 antibody (anti *cis*-signalling) at a concentration of 2.5 µg/ml inhibits proliferation of cells to background levels (“no IL-6”). No significant differences were noted in the proliferation of cells in the absence of IL-6 (first green bar in figure 4.13), in the presence of IL-6 at 8 pg/ml and anti human IL-6 monoclonal antibody (second red bar in figure 4.13), or in the presence of IL-6 at 8 pg/ml and 2B10 antibody (second yellow bar in figure 4.13). Similarly, no significant differences were observed in the proliferation of cells in the presence of IL-6 at a concentration of 8 pg/ml on its own and in the presence of the two isotype controls for the anti IL-6 and 2B10 antibodies.

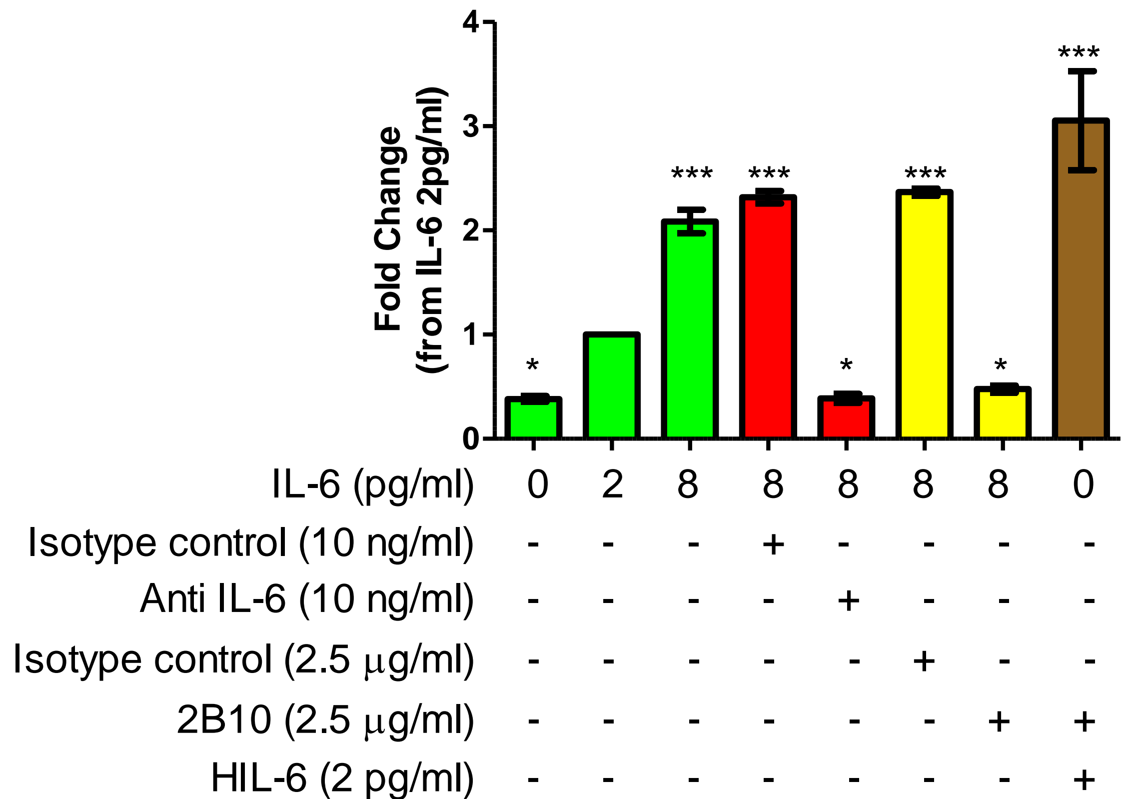


Figure 4-13: Panel of controls. Different conditions are represented on the x-axis while proliferation of B9 cells (expressed as a fold change) is represented on the y-axis. The green bars represent proliferation of B9 cells with or without IL-6 at different concentrations, red bars represent proliferation in the presence of anti human IL-6 antibody and its isotype control, yellow bars represent proliferation in the presence of 2B10 antibody and its isotype control, and the brown bar represents proliferation in the presence of HIL-6 at a concentration of 2 pg/ml and 2B10 antibody. Bars represent means (± SEM) of results of 4-5 independent experiments. Significance was tested by one-way ANOVA with Dunnets post-test (comparing with control column – IL-6 at 2 pg/ml). (*** = $p < 0.0001$, * = $p < 0.05$)

As shown in figure 4.14, total IL-6 activity was found to be about 2-fold greater than estimated from the ELISA concentrations. This increase in activity was observed equally in both groups and there was no significant difference in activity between the RDS or CLD groups. All of this functional activity was IL-6 dependent as it was inhibited

to background levels (“no IL-6”) in the presence of an anti-human IL-6 monoclonal antibody, again equally in both groups. When BALF was added to cells which had been exposed to 2B10 antibody (to block IL-6 *cis*-signalling), no increase in proliferation of cells was noted above background levels. Thus no specific IL-6 *trans*-signalling activity was noted in any of the BALF samples from the No CLD and CLD groups of ventilated preterm infants.

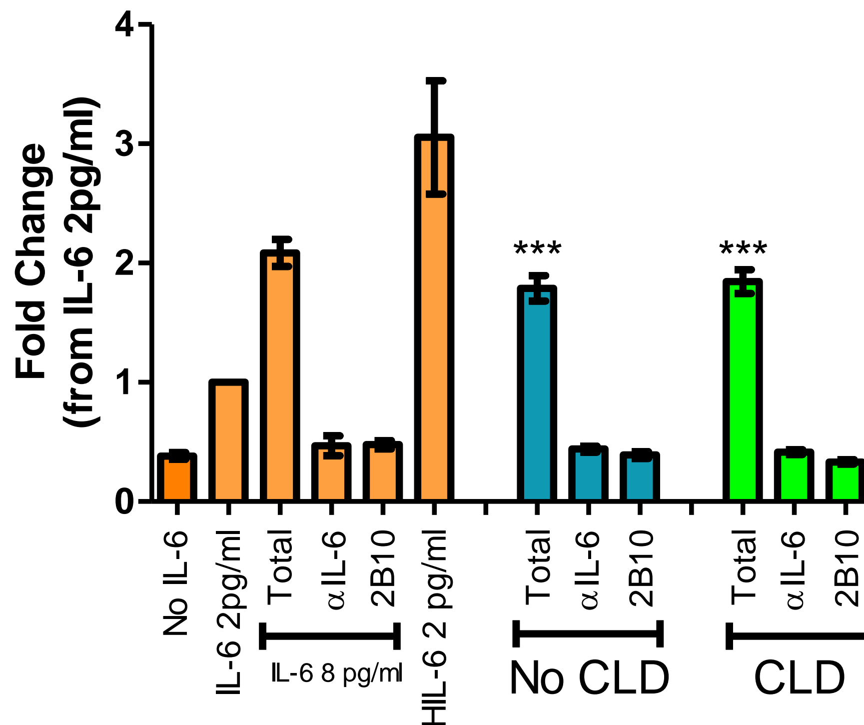


Figure 4-14: IL-6 functional activity in preterm BALF. Orange bars represent a panel of controls (as in fig 4.14), blue bars represent activity in No CLD infants and green bars represent activity in CLD infants. Stimulations (and co-stimulations) are noted on the x-axis while proliferation of B9 cells (expressed as a fold change) is represented on the y-axis. Control bars represent means (± SEM) of at least 4 independent experiments and BALF bars (No CLD and CLD) represent means (± SEM) of two experiments with 24 samples in each group, each sample analysed in triplicate in an experiment. Significance was tested by one-way ANOVA with Dunnets post-test (comparing with control column – IL-6 at 2 pg/ml). (*** = p < 0.0001)

4.8. IL-6 *trans*-signalling in B9 cells using recombinant molecules

Although the B9 cells seemed to be competent to IL-6 *trans*-signalling, no specific *trans*-signalling activity was noted in the presence of preterm BALF samples. One of the reasons for this observation could be the insensitivity of the cells to the natural complex (HIL-6 is a synthetic and stable complex and is more potent than the natural complex *in vitro*). To test whether the B9 cells do indeed respond to the natural complex, rh IL-6 at 5 pg/ml and rh sIL-6R at increasing concentrations, were premixed at room temperature for the natural complex to form before they were added to the B9 cells, both in the absence and presence of 2B10 antibody. As shown in figure 4.15, no increase in proliferation of cells were noted on addition of sIL-6R, even up to a 1000-fold excess compared to IL-6. These results suggested that, although the B9 cells are IL-6 *trans*-signalling competent, they did not seem to respond to the natural complex of sIL-6R/IL-6 at the concentrations used (within the range of the assay), although the biological activity of these molecules was validated in experiments described in RASF cells (figure 4.10), admittedly at significantly higher concentrations.

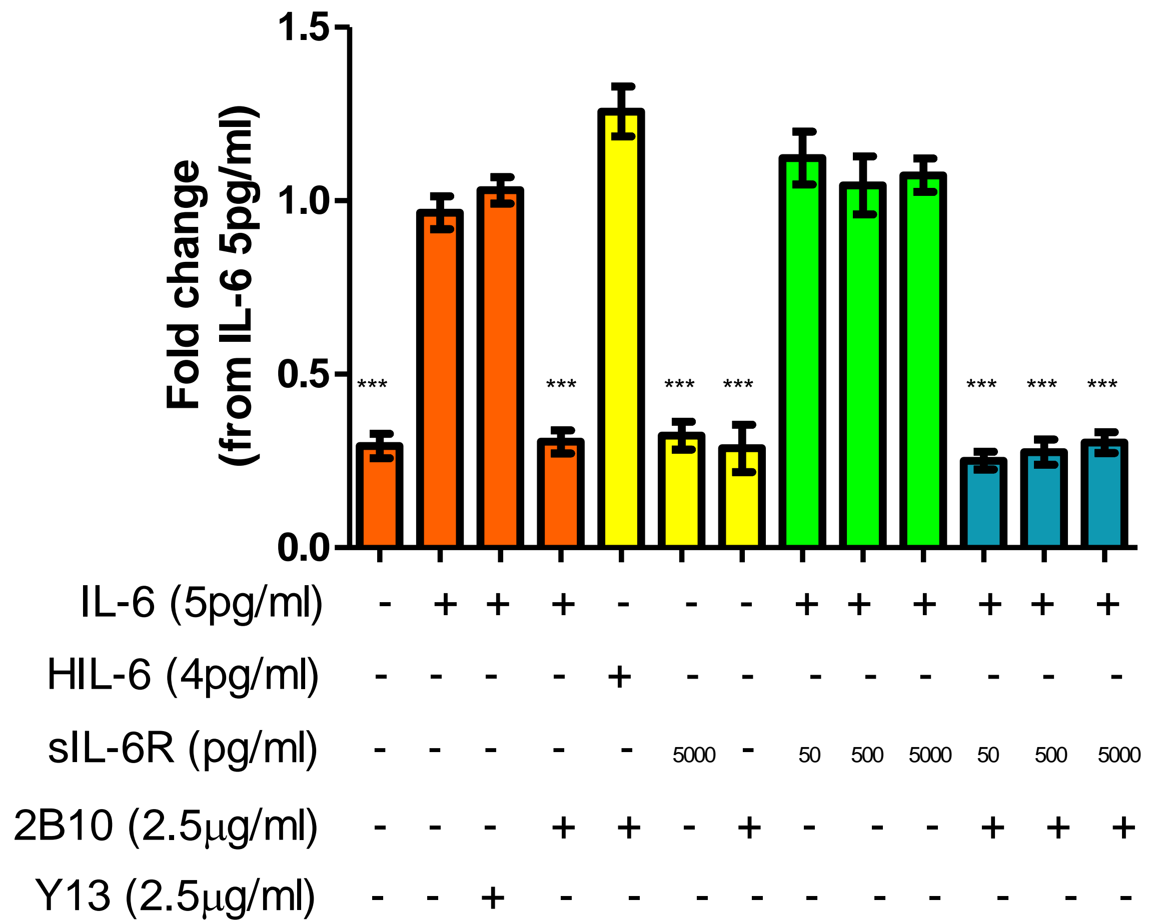


Figure 4-15: IL-6 *trans*-signalling with recombinant molecules. Effect of IL-6 (5 pg/ml) and different concentrations of sIL-6R in the absence (green bars) and presence (blue bars) of 2B10 antibody. Panel of controls with IL-6 (orange bars), HIL-6 and sIL-6R (yellow bars) are shown on the left. Bars are at means (± SEM) of at least 3 independent experiments. Significance was tested by one-way ANOVA with Dunnets post-test comparing proliferation against IL-6 standard at 5 pg/ml. (*** = $p < 0.001$). Y13 is the isotype control for the 2B10 antibody.

4.9. Discussion

4.9.1. Summary of Chapter

In this part of my work, I have confirmed the sensitivity and specificity of the B9 cell-line as an IL-6 bioassay. By modifying the assay to block IL-6 *cis*-signalling, I have also established that it is a sensitive assay for IL-6 *trans*-signalling. Stimulation of the cell with preterm ventilated BALF samples showed an increase in total IL-6 activity which was entirely IL-6 dependent. However, no specific *trans*-signalling activity was observed. Although the cells are *trans*-signalling competent, they were unresponsive to a complex of recombinant human IL-6 and sIL-6R (at the concentrations used). Further modifications of the assay would be necessary to measure IL-6 *trans*-signalling by the natural complex in clinical samples.

4.9.2. The B9 cells as an IL-6 bioassay

B9 cells, a sub-clone of the B13.29 cell-line (Lansdorp et al., 1986), were chosen for their sensitivity to proliferation in the presence of IL-6 (Aarden et al., 1987b). Since their isolation, these cells have been used as an IL-6 bioassay due to their sensitivity to human IL-6 (Nordan et al., 2001b). These cells were found to be unresponsive to a wide variety of human cytokines (expected to be present at sites of inflammation), either alone or in combination with human IL-6 (Helle et al., 1988a) including IL-1α, IL-1β, IL-2, IL-3, IL-4, GM-CSF and TNF-α. The authors found that with the exception of murine IL-4, these cells responded only to IL-6. However, several reports since then have shown interference with this assay, some of which is conflicting. A synergistic effect of IL-2 and IL-6, but not IL-2 on its own, on the proliferation of these cells has been reported (Chirmule et al., 1991). The authors noted expression of IL-2R on the cell-surface in the presence of IL-6, which is normally not present. This was proposed as the most likely mechanism of the synergistic effect noted. However, the source species of the cytokines, or the actual concentrations used in the assay were not stated. This result was disputed by other authors (Morrissey et al., 1992) who used murine antigens in defined concentrations. Using rigorous methods, the authors did

not find increased expression of IL-2 receptors on the B9 cells and speculated that non-specific staining of receptors by Chirmule *et al* may have contributed to this finding. Another report showed the interference of metals on this assay (Orupabo et al., 1992). Lead and cadmium were found to inhibit proliferation of the B9 cells which was reversible by increased concentration of IL-6. However, inhibition of proliferation by copper, cobalt and mercury were found to be irreversible. LPS from *E. coli* increased proliferation of the B9 cells in a dose-dependent manner, both on its own (> 40 ng/ml) and in the presence of IL-6 (Pedersen et al., 1995). The additive effect was noted at low concentration of IL-6 and disappeared at the higher concentrations, which presumably achieved maximal proliferation by itself. Similar results were reported by another group of researchers who showed proliferation of the B9 cells to "µg/ml" quantities of LPS obtained from *Salmonella abortus*. However, in an earlier report, LPS was not found to interfere with the B9 cell assay up to concentrations of 10 µg/ml (Soliman and Twigg, 1992). Purity of LPS may be a factor which could explain this discrepancy. I have not measured concentration of these factors in preterm BALF, which could possibly influence the assay, although notably all responses here were IL-6 dependent.

Among members of the IL-6 family, IL-11 (Burger and Gramatzki, 1993) and Oncostatin-M (Barton et al., 1994) stimulated proliferation of the B9 cells. However, the cells were reportedly 1000-fold less sensitive to both of these cytokines compared to IL-6 (Schwabe et al., 1996). Proliferation of B9 cells in the presence of other members of the IL-6 family has not been reported in the literature.

Human TNF-α and TGF-β have been reported to have an inhibitory effect on B9 cell proliferation, which could not be overcome by increasing concentrations of IL-6 (Schwabe et al., 1996). I did not have matched TNF-α or TGF-β concentrations in my BALF samples; however, TNF-α has been reported in low "pg/ml" concentrations in the lungs of ventilated preterm infants (Kazzi et al., 2008, Viscardi et al., 1997). Reported concentrations of TGF-β in the preterm lung have been in the "ng/ml" range (Kotecha et al., 1996a). My BALF samples were diluted >100 fold to keep IL-6 concentrations within the range of the bioassay. Although an inhibitory effect of either of these cytokines cannot be ruled out, all BALF samples resulted in proliferation of B9 cells

suggesting the lack of any significant effect of the inhibitory cytokines in my assay. Other cytokines and infectious agents tested in my assay did not seen to affect the cells at the concentrations used. A sub-clone of the B9 cell-line, B9-11, has also been cultured which is very sensitive to IL-11 (Lu et al., 1994). Thus, other sub-clones may exist in the population of B9 cells which may be sensitive to a specific cytokine resulting in a proliferative response; this may be a possible explanation for the increase in functional activity noted in all my samples. The synergistic effect of two or more cytokines in a clinical sample also cannot be ruled out, although combinations of cytokines have not yet been shown to have such an effect on these cells (Helle et al., 1988a, Schwabe et al., 1996).

4.9.3. Modifications for trans-signalling

In cells where both the cognate IL-6R and gp130 are expressed, as in the B9 cells, both IL-6 *cis*- and *trans*-signalling are supported. In order to assess these affects, I used IL-6 on its own for the *cis*-signalling effect. To test ability for *trans*-signalling, I used a covalent complex of IL-6 with IL-6R called hyper IL-6 (Fischer et al., 1997). The receptor binding site of IL-6 in this complex is already occupied by the covalently linked sIL-6R, thus precluding any effect on the cell-surface IL-6R. My experiments demonstrated that the B9 cells are IL-6 *trans*-signalling competent. Cells proliferated in the presence of low concentrations of hyper IL-6 with half-maximal proliferation occurring at concentrations of 0.5 – 1.0 pg/ml (figure 4.4). That this was due to IL-6 *trans*-signalling was demonstrated by inhibition of proliferation of the B9 cells when sgp130 Fc , a specific inhibitor of IL-6 *trans*-signalling, was premixed with H IL-6 (figure 4.12).

The exquisite sensitivity of the B9 cells to both IL-6 *cis*- and *trans*-signalling made this an ideal candidate assay to measure functional activity in clinical samples, where concentration of cytokines are low. If a complex clinical sample like preterm BALF, which contains free IL-6 as well as the complex of sIL-6R/IL-6, is used to stimulate these cells, then the resultant proliferation would be due to “total” IL-6 functional activity. In order to understand the relative effects of *cis*- and *trans*-signalling, I needed to block the effects of one of the pathways. My chosen strategy

was to inhibit the effects of IL-6 *cis*-signalling by blocking the cognate mouse IL-6R on the cells, but not affecting the function of human sIL-6R in the clinical samples. Although I chose monoclonal anti-mouse IL-6R antibodies, my first choice (clone 15A7) showed cross inhibition of the human sIL-6R as well (figure 4.7), making it an inappropriate molecule for my experimental system. The anti-mouse IL-6R monoclonal antibody 2B10 was generated by immunising Wistar rats with mouse IL-6R expressing CHO cells (Lissilaa et al., 2010, Greenhill et al., 2011). Using this molecule as a concentration of 1.25 µg/ml resulted in complete inhibition of B9 cell proliferation in response to IL-6 (blocks *cis*-signalling). The lack of cross-inhibition was demonstrated by two different experiments (figures 4.9 and 4.10). The 2B10 antibody did not interfere with the effects of IL-6 *trans*-signalling by H IL-6 (figure 4.11).

4.9.4. IL-6 bio-activity in BALF

Once I established the *cis*- and *trans*-signalling competence of the B9 cells, I proceeded to measure IL-6 functional activity in human preterm BALF. Observational work with the BALF samples showed me that, while the peak concentration of both IL-6 and sIL-6R were comparable in both groups of infants, sgp130 was significantly higher in the CLD infants. My main aim was to measure total IL-6 functional activity in both groups of infants and then detect any effect in modification of functional activity due to the presence of the soluble receptors, mainly sgp130. In order to be able to assess this, I equalised the estimated concentration of IL-6 in all samples from both groups to 2 pg/ml, based on the ELISA concentration. The 2 pg/ml concentration was chosen to produce a sub-maximal proliferation of the cells, so that any enhancement or inhibition of proliferation would be detectable within the range of the assay. Thus, all functional activity in BALF was expressed as a fold-change in proliferation compared to an IL-6 standard of 2 pg/ml. Also, I hypothesised that since the concentration of IL-6 was equivalent in both group of infants, any difference in proliferation between the groups could be attributed to a differences in concentration of the soluble receptors, mainly sgp130.

A panel of controls used with each experiment confirmed previous results noted during the set-up stage of the assay. It also confirmed the modifications used to

model IL-6 *trans*-signalling in this cell-line. On stimulation with preterm BALF, a two-fold increase in proliferation of cells was noted in both groups of infants; there were no statistically significant difference between the two groups. Complete inhibition of proliferation in the presence of anti-human IL-6 monoclonal antibody demonstrated that this proliferation was indeed IL-6 dependent. However, no significant proliferation of B9 cell was noted in the presence of the 2B10 antibody, suggesting a lack of any IL-6 *trans*-signalling activity in the samples. When modelled with recombinant cytokines, a similar lack of proliferation of cells was noted to IL-6 *trans*-signalling.

A two-fold increase in functional activity, compared to the expected, was noted in both groups of infants. Possible reasons could be proliferation of the cells in response to other members of the IL-6 cytokine family, such as IL-11 (Burger and Gramatzki, 1993) and oncostatin-M (Barton et al., 1994), although that seems unlikely as the B9 cells are a thousand-fold less sensitive to these cytokines compared to IL-6 (Schwabe et al., 1996). A sub-clone of the B9 cell-line, B9-11, has also been cultured which is very sensitive to IL-11 (Lu et al., 1994). More sensitive sub-clones of the B9 cells, if present, may be a possible explanation for the increase in functional activity noted in my samples. The synergistic effect of two or more cytokines in a clinical sample also cannot be ruled out, though combinations of cytokines have not yet been shown to have such an effect on these cells (Helle et al., 1988a, Schwabe et al., 1996). Overall, my data suggests that I have measured specific IL-6 bioactivity in BALF samples from preterm infants in a sensitive bioassay.

Lack of IL-6 *trans*-signalling activity could be explained by several potential theories. Concentrations of IL-6 and sIL-6R were comparable in both groups of infants, while concentration of sgp130 was significantly higher in the CLD group of infants. However, within each group, concentration of sgp130 was in excess of sIL-6R concentration in all samples. Thus, it is possible that sgp130 may have bound to any sIL-6R/IL-6 complex formed in the sample, leading to lack of any significant activity. Also, after dilution of BALF for use in the assay, the concentration of both IL-6 and sIL-6R were low in all samples. This may have prevented formation of any active complex, which has previously been shown to require much higher concentration of cytokines as well as an excess of sIL-6R *in vitro* (as discussed below).

The only other study looking at IL-6 functional activity in lung fluid from preterm ventilated infants was by Bagchi and colleagues. The authors reported higher IL-6 activity (6.6 fold) in CLD infants compared to RDS infants on the first day of life (Bagchi et al., 1994). There is a fundamental difference between their study and ours. While the study by Bagchi *et al* was to look at total IL-6 activity (modulation of IL-6 activity by the soluble receptors was not known at that time), my study was designed to primarily determine modulation of IL-6 activity by the soluble receptors. Thus, IL-6 concentration in my samples were all diluted to an estimated concentration of 2 pg/ml. Apart from this, other significant differences also exist between the study by Bagchi *et al* and ours. Their cohort of patients were recruited in the early 90s when surfactant replacement therapy was not as widespread as in my cohort. Indeed, only 27% of patients in the RDS and 74% in the CLD groups received exogenous surfactant replacement (*cf* 100% of infants in my cohort received exogenous surfactant replacement). Differences also existed in clinical characteristics of infants in the two groups (incidence of preterm labour, route of delivery and incidence of PDA). The cell-line used by the authors was also different from my cell-line. The difference in results could potentially be explained by a combination of these factors as mentioned above or this may simply reflect differences in the population under study, as it is well recognised that the pattern and severity of disease in this population has changed significantly over the past 10-20 years (Jobe, 1999).

4.9.5. Other IL-6 trans-signalling assays

The B9 cell assay for IL-6 functional activity is the most common one used (Nordan et al., 2001a). However, as this cell-line expresses membrane-bound IL-6R, it is unsuitable for measuring specific IL-6 *trans*-signalling activity without modification; my study has implemented appropriate modifications on these cells to achieve that. There is another cell-line which has been used by investigators as a model for specific IL-6 *trans*-signalling, called BAF-B03 cells. These are IL-3 dependent mouse pre-B cells, and only one of a few cell-types which does not express membrane-bound sgp130 (Hibi et al., 1990). The cells are made IL-6 *trans*-signalling competent by stably

transfecting them with human gp130 cDNA and called BAF130-9 (Hibi et al., 1990, Gearing et al., 1994). The ability of these transfected cells to respond to IL-6 *trans*-signalling was demonstrated in 1990 when they proliferated in the presence of a combination of IL-6 and sIL-6R (Hibi et al., 1990). However, it is notable that the concentration of IL-6 (2 – 200 ng/ml) and sIL-6R (5 µg/ml) used were supra-physiologic. A similarly strategy using transfected BAF-B03 cells were used to demonstrate proliferative response of these cells to other members of the IL-6 family (Gearing et al., 1994). However, the most extensive use of this cell-line as a model for IL-6 *trans*-signalling was by the German group led by Professor Stefan Rose-John (referred to as Ba/F3 cells by this group). In their report of the first description of hyper IL-6 (Fischer et al., 1997), they used the cells transfected with gp130 (Ba/F3-gp130) to demonstrate that HIL-6 was a functionally active complex capable of IL-6 *trans*-signalling. However, a difference in concentration of cytokines needed to have a proliferative effect was noted. While HIL-6, which is a stable complex of sIL-6R/IL-6, could achieve maximal proliferation of cells at a concentration of 1 ng/ml, a much higher concentration of IL-6 (100 ng/ml) and sIL-6R (100 – 1000 ng/ml) was required to achieve comparable proliferation of the cells. This has been a consistent finding of the group in several subsequent papers exploring different aspects of the IL-6 *trans*-signalling pathway (Horsten et al., 1997, Muller-Newen et al., 1998, Garbers et al., 2011).

The effect of IL-6 *trans*-signalling depends on the formation of the sIL-6R/IL-6 complex. In clinical specimens containing a mixture of the cytokines IL-6, sIL-6R and sgp130, complex formation depends on the relative presence of the individual molecules. In *in vitro* binding experiments, investigators have explored the formation of the sIL-6R/IL-6 complex and free IL-6 concentration in mixtures of cytokines with varying relative concentrations (Muller-Newen et al., 1998, Gaillard et al., 1999). Based on various starting concentration of the cytokines and their equilibrium constants, the authors demonstrated that availability of free IL-6 is affected by sIL-6R concentrations only; availability of sIL-6R/IL-6 complex increases with sIL-6R concentration and decreases with sgp130 concentration.

Measured starting values (ng/ml)			Calculated percentages (%)	
IL-6	**sIL-6R**	**sgp130**	**Free IL-6**	**sIL-6R/IL-6**
10	100	0	34	66
1	100	0	33	67
0.1	100	0	32	68
0.01	100	0	32	68
10	100	400	32	30
1	100	400	29	30
0.1	100	400	29	30
0.01	100	400	29	30
10	200	400	18	42
1	200	400	17	42
0.1	200	400	17	42
0.01	200	400	17	42
10	1000	400	4.3	76
1	1000	400	4.2	76
0.1	1000	400	4.2	77
0.01	1000	400	4.2	77
10	100	4000	30	4.0
1	100	4000	28	4.0
0.1	100	4000	28	3.3
0.01	100	4000	28	3.3
10	1000	4000	3.7	9
1	1000	4000	3.6	9
0.1	1000	4000	3.6	9
0.01	1000	4000	3.6	9

Table 4-1: Availability of free IL-6 and sIL-6R/IL-6 complex in a mixture of cytokines [modified from (Gaillard et al., 1999)]

In my cohort of infants, the peak mass concentration of IL-6 and sIL-6R was comparable in most samples. This meant that the molar concentration of sIL-6R was

lower or equivalent to that of IL-6 (figure 3.8a). Thus, based on the above table, we can predict that the majority of IL-6 in preterm BALF would be present as free IL-6 and the percentage of sIL-6/IL-6 would be minor. This could explain my observation in the functional assay where total IL-6 activity was observed in all samples but no IL-6 *trans*-signalling activity was detected.

The effect of sgp130 is complex, as measured by a functional assay. In my cohort, there was a molar excess of sgp130, compared to sIL-6R in all infants, regardless of the group they belonged to (figure 3.8b). Thus, any sIL-6R/IL-6 complex would be effectively bound to sgp130, precluding any functional *trans*-signalling activity. Recently, sgp130 has been shown to affect IL-6 classical signalling (Garbers et al., 2011); an excess of sIL-6R decreases free IL-6 and increases the sIL-6R/IL-6 complex which is bound by excess sgp130. However, free IL-6 is only affected by the concentration of sIL-6R, especially when it is in a significant excess. Thus, although sgp130 was significantly higher in the CLD infants, this did not have any effect on the functional assay as the proportion of the sIL-6R/IL-6 complexes in BALF was equivalent but minor in both groups, thus insufficient to have any effect at all. A sensitive and specific IL-6 *trans*-signalling assay (like B9 cells with membrane IL-6R expression knocked out) would be a more appropriate bioassay to measure IL-6 *trans*-signalling in this situation.

The reported effect of IL-6 *trans*-signalling by recombinant molecules in *in vitro* assays has been variable. A difference in potency between HIL-6 and IL-6/sIL-6R was noted by several investigators (Fischer et al., 1997, Garbers et al., 2011). Thus, HIL-6 has been noted to have a much higher potency compared to the natural complex. A possible explanation for that could be the fact that HIL-6 is a stable molecule where IL-6 is covalently bound to sIL-6R, whereas the natural complex of sIL-6R/IL-6 is dynamic. My experience with the B9-cell assay is similar, where low concentrations of HIL-6 was able to evoke proliferative responses but comparable doses of IL-6 (which can evoke *cis*-signalling responses) with large excesses of sIL-6R failed to do the same. Increasing the dose of IL-6 in the mixture resulted in “breakthrough” signalling, even in the presence of 2B10 antibody. Thus, the sensitivity of the assay to IL-6, which is an

attraction for clinical studies, also proved to be a drawback when attempting to elicit *trans*-signalling responses.

4.10. Summary

I have tested an existing sensitive and specific bio-assay for IL-6 and modified it to measure specific IL-6 *trans*-signalling activity by blocking the IL-6R with a monoclonal antibody. Using the assay, I demonstrated equivalent total IL-6 activity in BALF from CLD and no-CLD infants. However, no specific IL-6 *trans*-signalling activity could be elicited, probably due to the minor proportion of sIL-6R/IL-6 complexes in the samples. Using recombinant molecules, no IL-6 *trans*-signalling activity could be elicited, even in the presence of a large excess of sIL-6R, suggesting the lack of formation of a stable complex at the concentration of cytokines used. Further modifications of the assay, by using a strategy to knock-out IL-6R expression, may be beneficial in the future for measuring specific IL-6 *trans*-signalling activity in clinical samples.

5. Regulation of Interleukin-8_{77} Expression and Function in the Lungs of Preterm Ventilated Infants

5.1. Overview and background

Interleukin-8, a member of the CXC family of chemokines (CXCL8), is expressed in humans by a wide variety of cells (Remick, 2005). Due to variations in the number of amino acids (a.a.) at the amino-terminal end, several isoforms of IL-8 are expressed, of which the 72 a.a. isoform (IL-8_{72}), secreted mainly by immune cells, is the best characterised molecule. IL-8_{77} is the major isoform expressed by non-immune cells. Functionally, IL-8_{72} and other shorter isoforms are more potent than IL-8_{77} *in vitro* and *in vivo* (Nourshargh et al., 1992, Mortier et al., 2011a).

Recently, expression of IL-8_{77} has been described in circulation in extremely premature infants, which was the predominant isoform at early gestational ages (Maheshwari et al., 2009). Although total IL-8 is significantly increased in concentration in the lungs of infants developing CLD, the concentration of IL-8_{77} in the preterm lung or its proportion of the total IL-8 concentration is not known. As significant inflammation is detected in the lungs of preterm infants developing CLD, I hypothesised that the more potent shorter isoforms of IL-8 would predominate in the preterm lung. Understanding the expression of IL-8_{77} and its processing in the preterm lung could lead to novel approaches in modulating lung inflammation in the preterm infants.

5.2. Aims of the project

- To determine the expression of total IL-8 and IL-8_{77} in BALF from preterm ventilated infants.
- To compare the expression of total IL-8 and IL-8_{77} in infants who develop CLD with those who do not.
- To determine the functional potential of BALF to convert IL-8_{77} to shorter isoforms and identify possible proteases involved in the mechanisms of conversion.

- To determine the effect of proteases, potentially involved in the above conversion, on IL-8_{77} *in-vitro* and determine the functional relevance of this conversion on PMNs.

5.3. Modification and Validation of the IL-8_{77} ELISA

The IL-8_{77} ELISA has been described before using a specific pair of capture (N11) and detection (H6) monoclonal antibodies (Nashkevich et al., 2002b). Using the same pair of antibodies, I first validated the ELISA in my laboratory. As shown in figure 5.1, the N11 monoclonal antibody, when used for capture, was specific for IL-8_{77} and did not capture the shorter isoform IL-8_{72}.

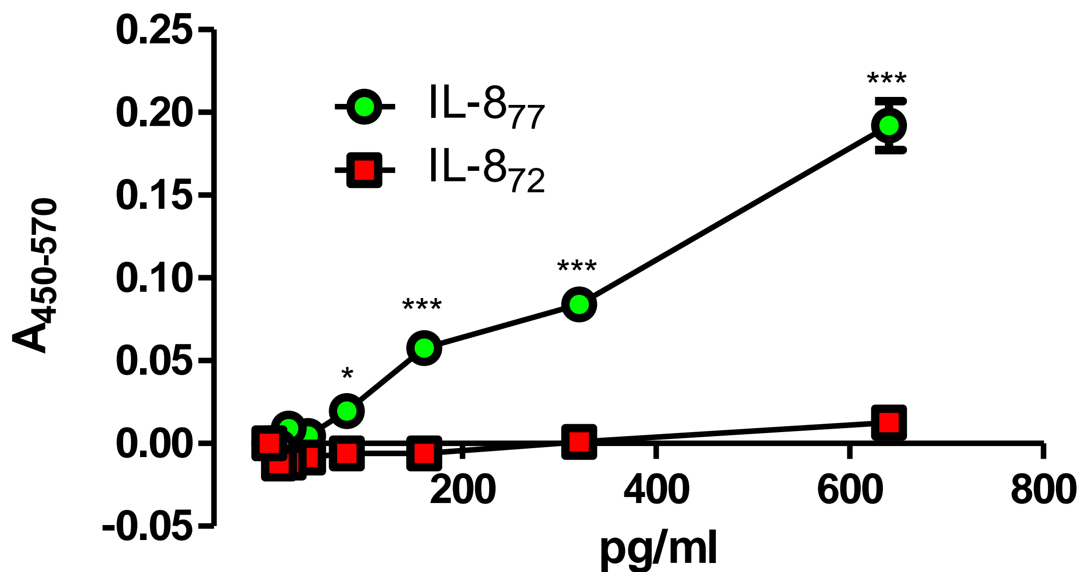


Figure 5-1: Validation of the IL-8_{77} ELISA. Plates were coated with N11 antibody and antigen was detected by H6 antibody. Concentration of the antigens IL-8_{77} (green circles) and IL-8_{72} (red squares) are represented on the x-axis (pg/ml) while absorbance at 450 nm (corrected at 570 nm) is represented on the y-axis. Points represent means (± SEM) of three independent experiments. Values were compared by two-way ANOVA with Boferroni's post-test. (*** = $p < 0.001$, * = $p < 0.05$)

To demonstrate that the commercial ELISA (BD OptEIA human IL-8 ELISA) captured both the antibodies (detects "total" IL-8), both IL-8_{77} and IL-8_{72} were individually tested in this assay. As shown in figure 5.2, the pair of capture and detection antibodies from the commercial kit detected both antigens equally at all concentrations in the range tested. The range of concentration was kept the same as for the previous experiment for easy comparison.

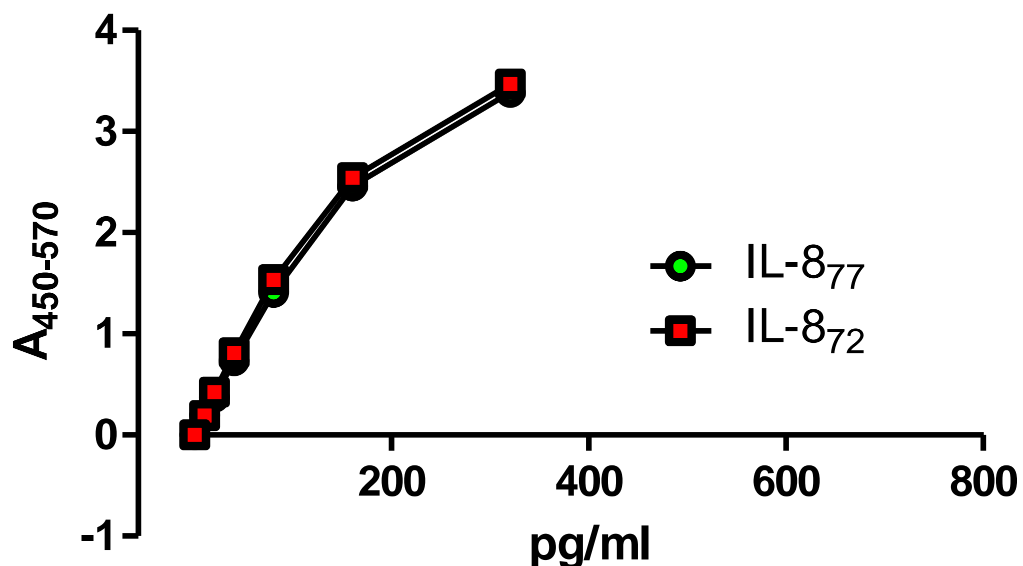


Figure 5-2: Validation of the commercial ELISA. Plates were coated with BD capture antibody and detected with BD detection antibody. Concentration of the antigens IL-8_{77} (green circles) and IL-8_{72} (red squares) are represented on the x-axis (pg/ml) while absorbance at 450 nm (corrected at 570 nm) is represented on the y-axis. Points represent means (± SEM) of three independent experiments. Values were compared by two-way ANOVA with Boferroni's post-test.

Although the two ELISAs with their own pairs of antibodies performed as expected, the original IL-8_{77} ELISA (with the N11 and H6 pair of antibodies) was found to be insensitive (low optical density values, "high" limit of sensitivity at 80 pg/ml). In an attempt to improve on this assay, an ELISA was designed with the two capture antibodies (N11 and BD capture), the BD detection antibody, and both the antigens were again tested. The N11 antibody specifically captured IL-8_{77} which was detected by

the BD detection antibody (figure 5.3a). This also improved sensitivity as better optical density values were obtained and the limit of sensitivity was lower at 3.125 pg/ml (as with the commercial kit for total IL-8). The BD ELISA capture antibody detected IL-8 isoforms with equal sensitivity in the assay (figure 5.3b). For all future IL-8_{77} ELISAs, the pair of N11 capture antibody and BD detection antibody was used.

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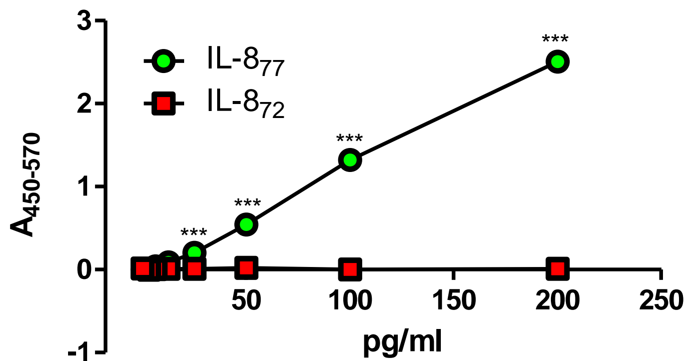

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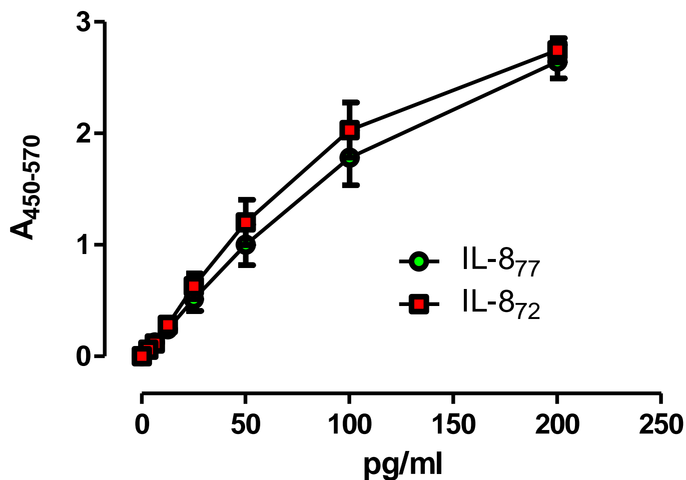

Figure 5-3 : Modification of IL-8_{77} ELISA. Antigens IL-8_{77} (green circles) and IL-8_{72} (red squares) were captured by (a) N11 and (b) BD capture antibody. Both were detected by the BD detection antibody. Concentration of the antigens are represented on the x-axis (pg/ml) while absorbance at 450 nm (corrected at 570 nm) is represented on the y-axis. Points represent means (± SEM) of three independent experiments. Values were compared by two-way ANOVA with Boferroni's post-test. (*** = $p < 0.001$)

5.4. Clinical characteristics of the patient cohort

A total of 38 samples from 22 infants (11 No CLD and 11 CLD) were analysed for the study. Clinical characteristics of the two groups are shown in table 5.1. Apart from the number of ventilation-days, which was significantly higher in the CLD infants ($p < 0.01$), the two groups were well matched for other clinical characteristics.

	No CLD	CLD	p
Total infants	11	11	
Total samples	13	25	
Gestational age (weeks)*	28^{+3} (27 – 29^{+4})	27^{+5} (25^{+4} – 29^{+2})	0.45
Birth weight (g)*	1120 (960 – 1250)	905 (798.8 – 1120)	0.14
Prolonged rupture of membranes (>24 hours)^	1 (10)	2 (22)	1.0
Antenatal steroids (≥24 hours)^	9 (82)	9 (82)	1.0
Caesarean delivery^	5 (45)	6 (55)	1.0
Exogenous surfactant replacement^	11 (100)	11 (100)	1.0
Mechanical ventilation-days*	1.0 (1.0 – 1.75)	6.5 (5.0 – 15.5)	0.004
Patent ductus arteriosus^	5 (45)	8 (73)	0.39

Table 5-1: Patient and clinical characteristics. Values are medians with inter-quartile range (*) or numbers of infants and percentage of total in group(^).

5.5. Expression of IL-8_{77} in the preterm lung

IL-8 was detected in all 38 samples analysed but IL-8_{77} was below the limit of detection in five samples (three CLD and two No CLD infants). Peak concentration of total IL-8 (table 5.2 and figure 5.4a, also figure 3.6c) was significantly higher in the CLD group compared to No CLD ($p = 0.01$), as has been consistently reported in the literature. Concentration of IL-8_{77} (corresponding to the peak concentration of total IL-8) was also significantly higher in the CLD group (table 5.2 and figure 5.4b; $p = 0.03$).

	No CLD	CLD
Total IL-8 (pg/ml)	3401 (1587 - 19080)	175600 (7064 - 202700)
IL-8_{77} (pg/ml)	144.3 (83.6 - 552.6)	2353 (140.1 - 5668)
IL-8_{77} (percentage of total IL-8)	2.9 (1.28 - 5.27)	2.29 (1.49 - 3.02)

Table 5-2: Expression of IL-8 and IL-8_{77}. Medians (IQR) of peak concentrations of total IL-8 (pg/ml) and corresponding IL-8_{77} and percentage of total IL-8 expressed as IL-8_{77} in preterm BALF from infants in the No CLD and CLD groups.

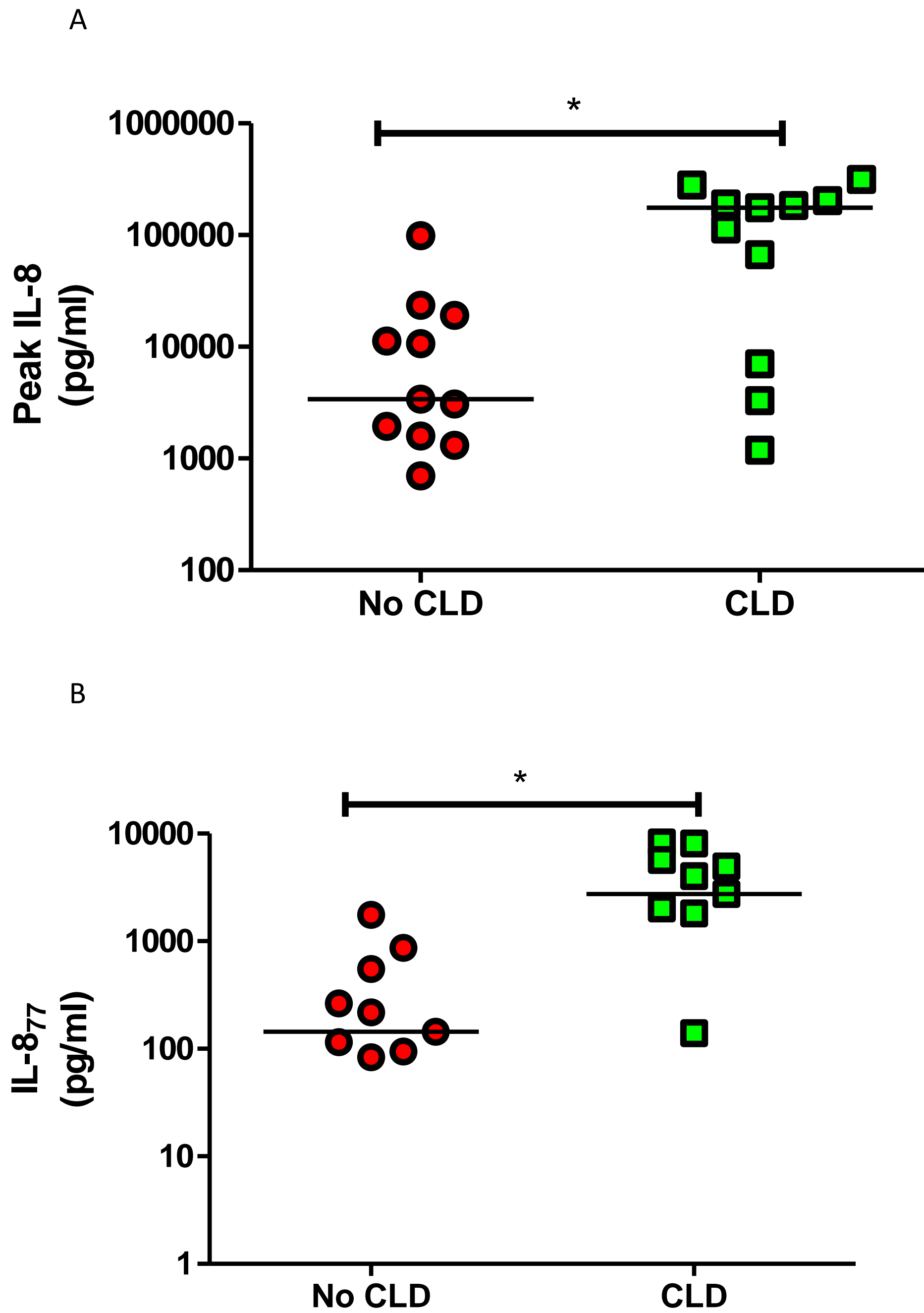

Figure 5-4: Expression of IL-8 and IL-8_{77} in preterm BALF. Concentration of (a) peak total IL-8 and (b) corresponding IL-8_{77} in preterm BALF from infants in the No CLD group (red circles) and CLD group (green squares). Groups are represented on the x-axis while concentration of protein (pg/ml) is represented on the y-axis (log scale). Each point represents peak value from a single infant and bars are at medians. (* = $p < 0.05$)

However, IL-8_{77} was a minor proportion of the total IL-8 in the samples and there was no significant difference between the two groups (figure 5.5a, table 5.2). This was reflected by a significant correlation between the concentrations of total IL-8 and IL-8_{77} ($r = 0.94$, $p < 0.0001$, figure 5.5b).

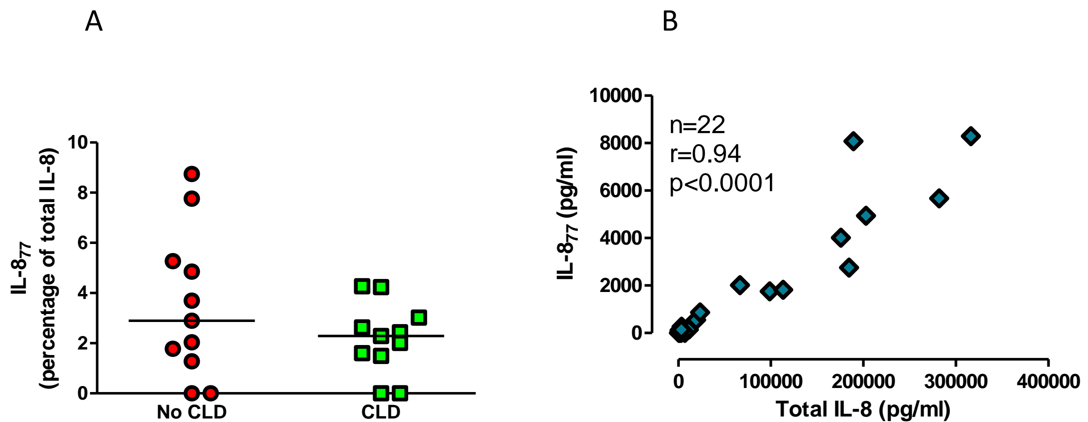

Figure 5-5: Proportion of IL-8_{77}. (a) Concentration of IL-8_{77}, expressed as a percentage of total IL-8 on the y-axis, in No CLD (red circles) and CLD (green squares) groups. Groups are represented on the x-axis while proportion of protein (percentage) is represented on the y-axis. Each point represents peak value from a single infant and bars are at medians. (b) Concentration of peak IL-8 (pg/ml) and corresponding concentration of IL-8_{77} (pg/ml) are plotted on the x- and y-axis respectively. Each point represents a single infant and correlation was tested by calculating Spearman's coefficient.

The majority of IL-8 in preterm circulation is composed of IL-8_{77}, in contrast to term or adult circulation and with maturity of the foetus, the expression of IL-8 isoforms in circulation changes (Maheshwari et al., 2009). However, in preterm BALF, no significant correlation was observed between concentration of IL-8_{77} (figure 5.6a) or the proportion of IL-8 expressed as IL-8_{77} (figure 5.6b) in the preterm lungs with the birth-gestation of infants. For this analysis, only day 1 samples were chosen to reflect in-utero concentrations as closely as possible; 13 such samples were available for analysis.

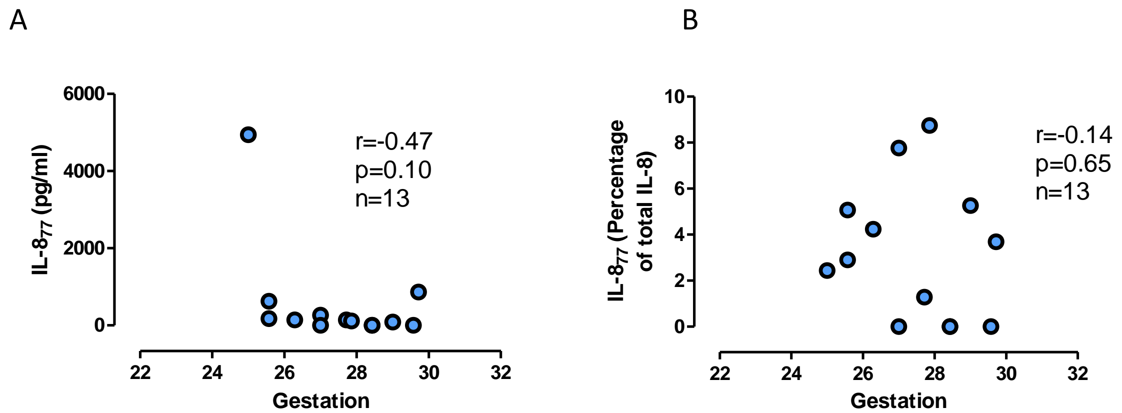

Figure 5-6: Gestation and IL-8$_{77}$ expression. Correlation of gestation at birth with (a) concentration of IL-8$_{77}$ and (b) percentage of IL-8 expressed as IL-8$_{77}$. Birth gestation of infants (weeks) is plotted on the x-axis while first day IL-8$_{77}$ concentration (pg/ml) or proportion of total IL-8 (percentage) in the preterm lung is plotted on the y-axis. Each point represents a single infant and correlation was tested by calculating Spearman's coefficient.

Thus, I have shown that IL-8$_{77}$ was expressed as a minor proportion of total IL-8 in preterm BALF. Expression of IL-8$_{77}$ was significantly correlated to total IL-8 concentration in the lung but not with the birth gestation.

5.6. Expression of IL-8$_{77}$ from specific cell-types

5.6.1. Expression in lung epithelial cells

The majority of IL-8 in preterm BALF seemed to be the more potent shorter isoforms, as I had hypothesised on analysing the inflammatory profile. Two possible explanations for this observation could be: the low proportion of IL-8$_{77}$ could be due to a lack of expression in the lungs, or conversion into the shorter isoforms. Since non immune cells, including epithelial cells (Standiford et al., 1990, Arnold et al., 1994a, Leverence et al., 2011), are known to express IL-8 under experimental conditions, I first looked at the expression of the IL-8$_{77}$ isoform in lung epithelial cells.

Due to the difficulty in obtaining neonatal lung explants, I looked at expression of IL-8_{77} in two adult lung cell-lines A549 (type II alveolar epithelial cells from adenocarcinoma) & BEAS-2B (bronchial epithelial cells), and also in primary adult small-airway epithelial cells (SAEC). Although unstimulated A549 cells expressed negligible IL-8, this increased 29-fold on stimulation, with 58% of it being IL-8_{77} (figure 5.7a and b, table 5.3). Unstimulated BEAS-2B cells expressed all of their IL-8 as IL-8_{77}; on stimulation, there was an 89-fold increase in expression of total IL-8, of which around 66% was IL-8_{77}. SAECs expressed 25% of their IL-8 as IL-8_{77} in the absence of any stimulation; this increased to 56% on stimulation which also resulted in a 10-fold increase in expression of total IL-8. Thus, all lung epithelial cells tested expressed significant proportion of IL-8 as IL-8_{77} *in vitro* under experimental conditions.

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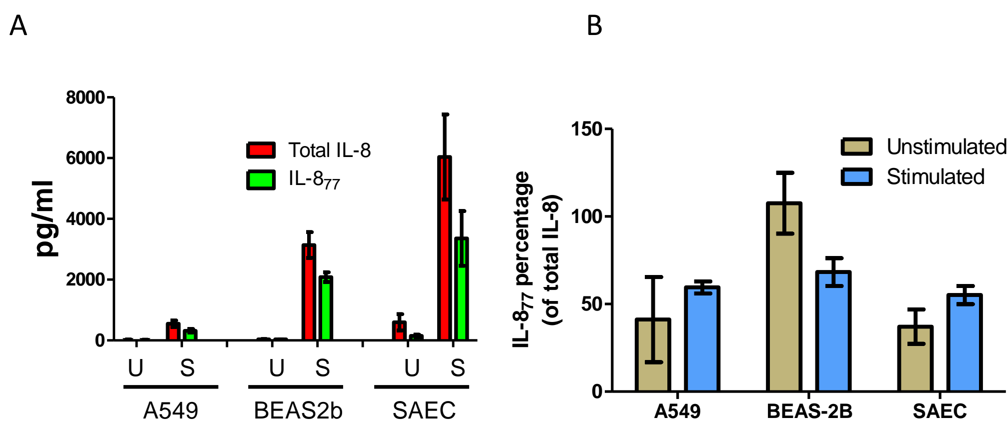

Figure 5-7: Expression of IL-8_{77} from airway epithelial cells. (a) Concentration (pg/ml) of total IL-8 (red bars) and IL-8_{77} (green bars) from airway epithelial cells both unstimulated (U) and when stimulated (S) with IL-1β. Cell-lines and incubation conditions are represented on the x-axis while concentration (pg/ml) is on the y-axis. (b) Proportion of total IL-8 (percentage) expressed as IL-8_{77} from un-stimulated (grey bars) and stimulated (with IL-1β) airway epithelial cells. Cells-lines with different conditions are represented on the x-axis while percentage of IL-8_{77} is represented on the y-axis. All bars are means (± SEM) of three independent experiments.

Airway Epithelial Cells	Un-stimulated		Stimulated	
	Total IL-8 (SEM)	IL-8_{77} (SEM)	Total IL-8 (SEM)	IL-8_{77} (SEM)
A549	19.0 (11.0)	15.6 (9.3)	548.1 (105.7)	319.3 (56.0)
BEAS-2B	35.3 (7.5)	35.7 (3.0)	3138.0 (424.0)	2084.0 (162.0)
SAEC	596.3 (267.8)	147.1 (44.2)	6037.0 (1399.0)	3358.0 (900.3)

Table 5-3: Expression of IL-8 from airway epithelial cells. Concentration (pg/ml) of total IL-8 and IL-8_{77} by airway epithelial cells, both un-stimulated and when stimulated with IL-1β. Values are means (SEM) of at least three independent experiments.

5.6.2. Expression in neonatal PMN and MNC

Expression of IL-8_{77} in adult monocytes (Lindley et al., 1988a, Yoshimura et al., 1989a) and neutrophils (Bazzoni et al., 1991, Arnold et al., 1994b, Padrines et al., 1994a) in varying proportions have been described previously. However, the proportion of IL-8_{77} expressed by neonatal immune cells is not known. Since these cells are a major source of IL-8 and they are found in large numbers in BALF from preterm ventilated infants, I next looked at expression of IL-8_{77} from neonatal PMNs and MNCs. To do so, cord blood from term infants born by elective Caesarean section for maternal non-medical causes (repeat section for previous section, breech presentation) was collected and experiments conducted on purified cell populations. Blood from preterm infants was considered inappropriate because of the possibility of activation of cells by prevailing systemic inflammation. Blood from healthy adult controls was also collected and stimulated similarly to act as controls.

Term cord-blood PMNs expressed 34% of their IL-8 as IL-8_{77} in the un-stimulated and stimulated state (figure 5.8a and table 5.4); there was a 14-fold increase in expression of total IL-8 on stimulation with LPS (figure 5.9a). Adult peripheral blood PMNs expressed 27% of their IL-8 as IL-8_{77} in the un-stimulated state, which increased to 30% when stimulated with LPS (figure 5.8b and table 5.4); this represented a 18-fold increase in expression of total IL-8 on stimulation (figure 5.9b). Unstimulated cord monocytes expressed 33% of IL-8 as IL-8_{77} which changed to 53% on stimulation (figure 5.8a and table 5.4); there was a 32-fold increase in expression of

total IL-8 on stimulation (figure 5.9c). Matched adult monocytes produced 37% of their IL-8 as IL-8_{77} in the un-stimulated state, which changed to 23% when stimulated with LPS (figure 5.8b and table 5.4); there was a 6-fold increase in expression of total IL-8 (figure 5.9d). Taken together, this data shows that more than a third of the total IL-8 expressed by term neonatal PMNs and MNCs under experimental conditions is the long isoform. The proportion of IL-8_{77} from immune cells was also higher than that recovered from BALF of preterm ventilated infants (< 5%; figure 5.5a). Thus I have demonstrated the potential of resident and infiltrating lung cells to express significant proportion of their IL-8 as IL-8_{77}.

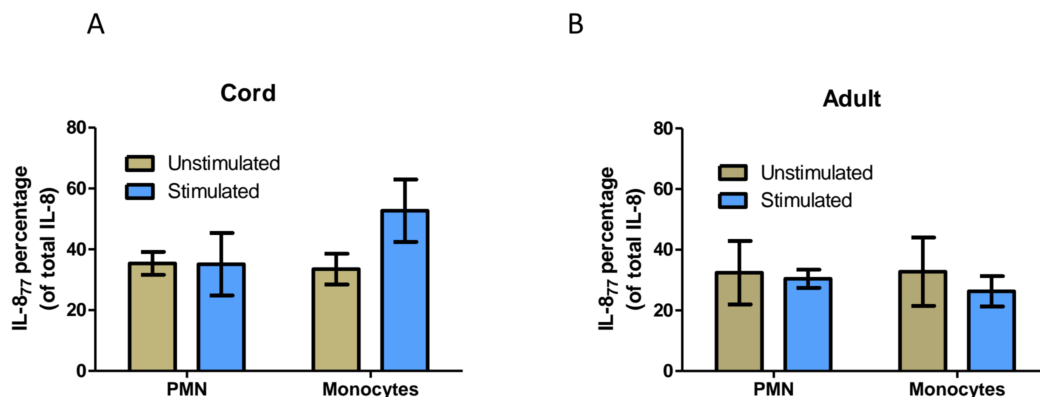

Figure 5-8: Expression of IL-8_{77} from immune cells. Proportion of total IL-8 expressed as IL-8_{77} from un-stimulated (grey bars) and stimulated (blue bars) (a) cord-blood and (b) adult peripheral-blood PMNs and Monocytes. Cells with different conditions are represented on the x-axis while percentage of IL-8_{77} is represented on the y-axis. Bars are means (± SEM) of at least three independent experiments.

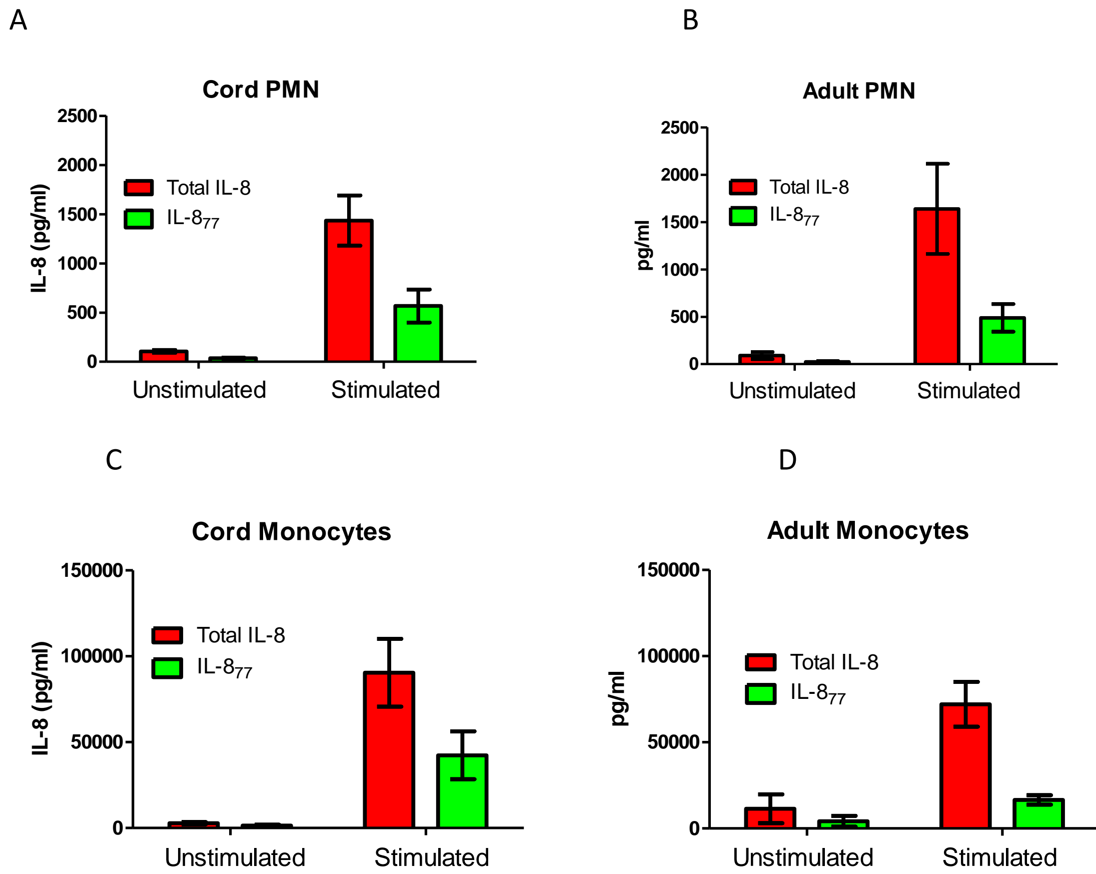

Figure 5-9: Expression of IL-8 and IL-8$_{77}$ from immune cells. Concentration of total IL-8 (pg/ml, red bars) and IL-8$_{77}$ (pg/ml, green bars) from unstimulated and LPS stimulated (a) cord-blood PMNs, (b) adult peripheral-blood PMNs, (c) cord-blood monocytes and (d) adult peripheral-blood monocytes. Cells with different conditions are represented on the x-axis while concentration of protein (pg/ml) is represented on the y-axis. Bars are means (± SEM) of at least three independent experiments.

Immune Cells	Un-stimulated		Stimulated	
	Total IL-8 (SEM)	$IL\text{-}8_{77}$ (SEM)	Total IL-8 (SEM)	$IL\text{-}8_{77}$ (SEM)
Cord PMN	105.5 (14.5)	36.0 (4.1)	1435.0 (255.0)	567.0 (167.5)
Adult PMN	89.7 (37.6)	24.5 (7.9)	1639.0 (475.8)	488.8 (145.8)
Cord MNC	2814.0 (673.4)	1433.0 (488.8)	90434.0 (19794.0)	42336.0 (13920.0)
Adult MNC	11442.0 (8358.0)	4203.0 (3149.0)	72078.0 (12994.0)	16542.0 (2766.0)

Table 5-4: Expression of IL-8 from innate immune cells. Concentration (pg/ml) of total IL-8 and $IL\text{-}8_{77}$ by PMNs and MNCs, both un-stimulated and when stimulated with LPS. Values are means (± SEM) of at least three independent experiments.

5.7. Convertase Activity of BALF

So far, I have shown that a minor proportion of the IL-8 in preterm BALF was expressed as $IL\text{-}8_{77}$, although lung epithelial cells (adult) and innate immune cells (term newborn infant cord blood) expresses significant proportions of their IL-8 as $IL\text{-}8_{77}$ under experimental conditions. This, together with reports of dominant $IL\text{-}8_{77}$ expression in preterm neonatal circulation (Maheshwari et al., 2009) suggested the possibility of conversion of $IL\text{-}8_{77}$ in the preterm ventilated lung. As several proteases have previously been shown to convert $IL\text{-}8_{77}$ to shorter isoforms (Van den Steen et al., Padrines et al., 1994b, Hebert et al., 1990b, Nakagawa et al., 1991a), I next looked for the presence of activity of such factors in preterm BALF.

Figure 5.10 shows a panel of controls where $IL\text{-}8_{77}$ (10 ng/ml = 1.1 nM) was incubated overnight (18 hours) in buffer only (tris buffered saline with 0.5% BSA), or in the presence of the protease inhibitors AAT (for neutrophil serine proteases; concentration 10 µM), PMSF (for various serine proteases; concentration 10 mM), 1-10 phenanthroline (for metallo-proteases; concentration 10 mM) and ATIII (for thrombin;

concentration 150 μg/ml). No significant loss of IL-8_{77} was observed on ELISA after incubating with buffer for 18 hours (mean ± SEM: 9498 ± 720 pg/ml) compared with concentration at 0 hours (9768 ± 356 pg/ml). Comparable concentration of IL-8_{77} was detected after incubation with protease inhibitors for 18 hours (AAT: 10342 ± 556 pg/ml; PMSF: 9764 ± 432 pg/ml; 1-10 phenanthroline: 9835 ± 536 pg/ml; ATIII: 10786 ± 407 pg/ml). Ethanol was used as a negative control for PMSF and 1-10 phenanthroline as these stock solutions were diluted in ethanol; no significant differences were observed in concentration of IL-8_{77} was detected after incubation with ethanol as compared to buffer control (ethanol: 9647 ± 611 pg/ml).

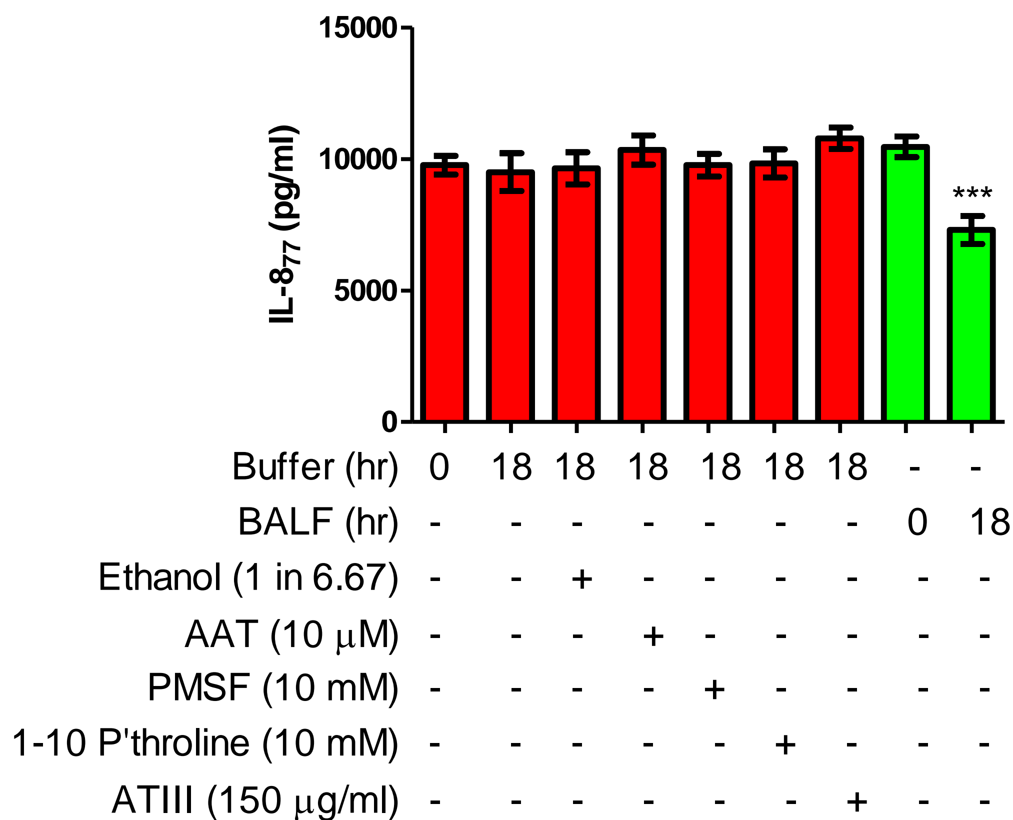

Figure 5-10: Panel of controls. Concentration of IL-8_{77} detected by ELISA at 0 hours and after incubation in different conditions for 18 hours. Conditions are detailed on the x-axis and concentration of IL-8_{77} (pg/ml) on the y-axis. Red bars represent buffer controls and green bars represent BALF samples (n = 18) at 0 hour and after 18 hours. All bars are at means ± SEM. Conditions were compared by one-way ANOVA with Dunnets post-hoc test comparing against a control column (BALF at 0 hour). (*** = $p < 0.001$)

On incubating BALF with known concentrations (10 ng/ml = 1.1nM) of exogenously added rhIL-8_{77}, recovery of this isoform after an 18 hour incubation was significantly decreased by 29% compared to starting concentration at 0 hour (71% recovery, $p < 0.001$; fig 5.11). (Green bars representing BALF at 0 and 18 hours in fig 5.10 and fig 5.11 are identical). To explore the mechanism of this conversion, IL-8_{77} was incubated with BALF and AAT, PMSF, 1-10 phenanthroline and ATIII. While both AAT (88% recovery, $p <0.01$) and PMSF (95% recovery, $p < 0.001$) resulted in significant inhibition of conversion of IL-8_{77} by preterm BALF, no similar protection was observed with 1-10 phenanthroline (68% recovery, $p > 0.05$), suggesting neutrophil serine proteases, and not metallo-proteases, are the more important group of convertases present in preterm BALF. Interestingly, inhibition of convertase activity by ATIII (88% recovery, $p < 0.01$) suggested significant activity of thrombin in the preterm lung.

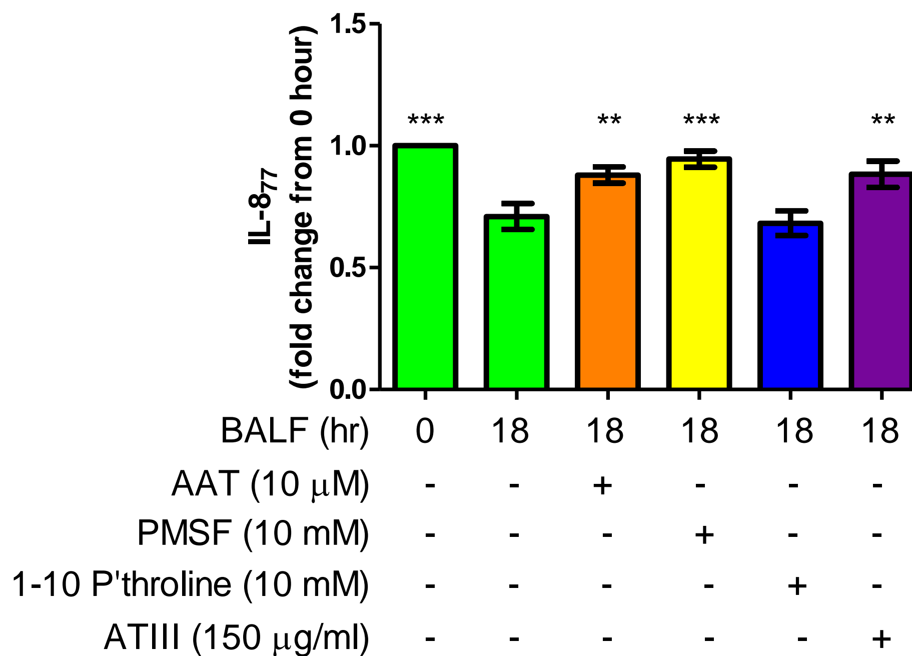

Figure 5-11: Convertase activity of preterm BALF. Concentration of IL-8_{77} detected by ELISA at 0 hours and after incubation in different conditions for 18 hours. Conditions are detailed on the x-axis and concentration of IL-8_{77} (expressed as fold change in concentration compared to BALF at 0-hour) on the y-axis. All bars are at means (± SEM). Conditions were compared by repeated measures ANOVA with Dunnets post-hoc test comparing against a control column (BALF at 18 hour). (*** = $p < 0.001$, ** = $p < 0.01$; n = 18)

There was significantly increased convertase activity in CLD infants (leading to decreased recovery of IL-8_{77}) compared to No CLD (p = 0.03, fig 5.12). Thus preterm BALF retains significant convertase activity towards IL-8_{77}, which was mainly due to the activity of neutrophil and coagulation-cascade serine proteases but not due to metallo-proteases.

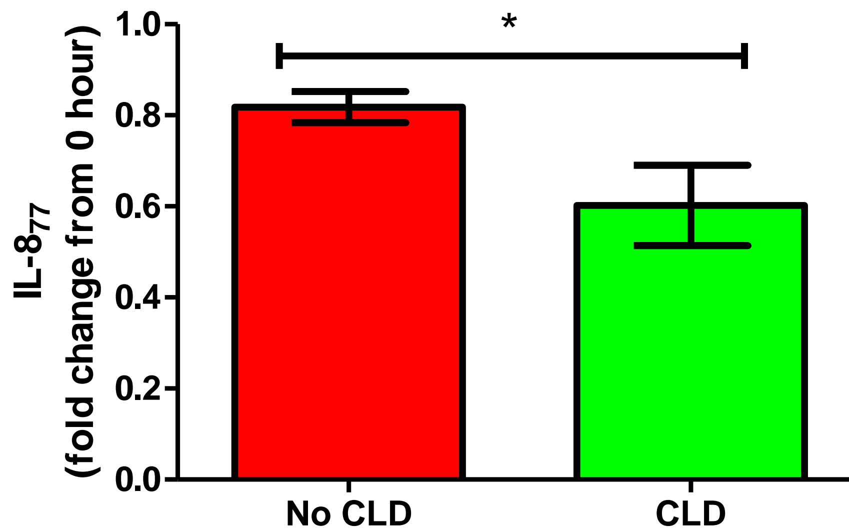

Figure 5-12: Convertase activity in No CLD and CLD infants. Concentration of IL-8_{77} detected by ELISA at 18 hours in No CLD (red bar) and CLD (green bar) infants. Infant groups are detailed on the x-axis and concentration of IL-8_{77} (expressed as fold change in concentration compared to BALF at 0-hour) on the y-axis. All bars are at means (± SEM). (* = $p < 0.05$, n = 9 in each group)

5.8. Conversion of IL-8_{77} by purified neutrophil proteases

My data suggested that active neutrophil and coagulation cascade serine proteases in BALF can significantly convert exogenously added IL-8_{77}. The role of thrombin and plasmin has been studied previously (Hebert et al., 1990b, Nakagawa et al., 1991a). I next proceeded to look at the pattern of this conversion by the three neutrophil serine proteases elastase, cathepsin G and proteinase-3 individually.

5.8.1. Conversion of IL-8_{77} by purified neutrophil proteases at different concentrations

I first looked at the effect of different concentrations of neutrophil proteases on the conversion of rhIL-8_{77}. Keeping the molar concentration of IL-8_{77} constant (300 nM), a variable concentration of proteases was added so that the enzyme: substrate ratio ranged between 10:1 (protease concentration 3 µM) to 1:10 (protease concentration 30 nM; a 100-fold change in concentration of proteases). Conversion of IL-8_{77} by the neutrophil serine proteases was found to be protease dose-dependent, with significantly decreased concentration of IL-8_{77} recovered at the higher concentration of proteases after 18 hours of incubation (fig 5.13a-c).

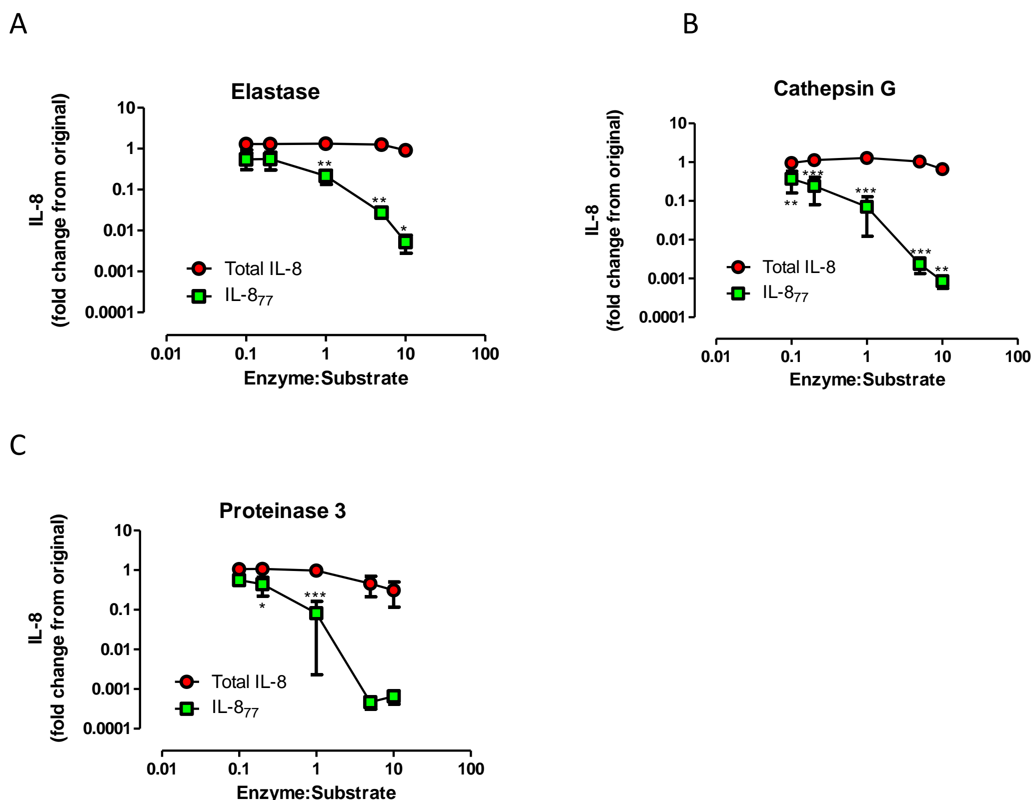

Figure 5-13: Protease dose-response. Conversion of IL-8_{77} by purified (a) elastase, (b) cathepsin-G and (c) proteinase 3 at varying concentration. Ratio of enzyme: substrate is represented on the x-axis while the fold-change (compared to concentrating at 0-hour) of total IL-8 (red circles) and IL-8_{77} (green squares) is represented on the y-axis. Points plotted are means (± SEM) of three independent experiments. Concentration of

$IL\text{-}8_{77}$ is compared to corresponding concentration of total IL-8 by two-way ANOVA with Bonferroni's post-test. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

There were no significant differences in concentration of recovered $IL\text{-}8_{77}$ between the three proteases after 18 hours of incubation, at which point they were measured (fig 5.14a). Interestingly, there was a gradual loss of total IL-8 observed at the higher concentrations of protease used (fig 5.14b). This loss was significant for proteinase 3 at an enzyme:substrate concentration of 5:1 when compared to elastase.

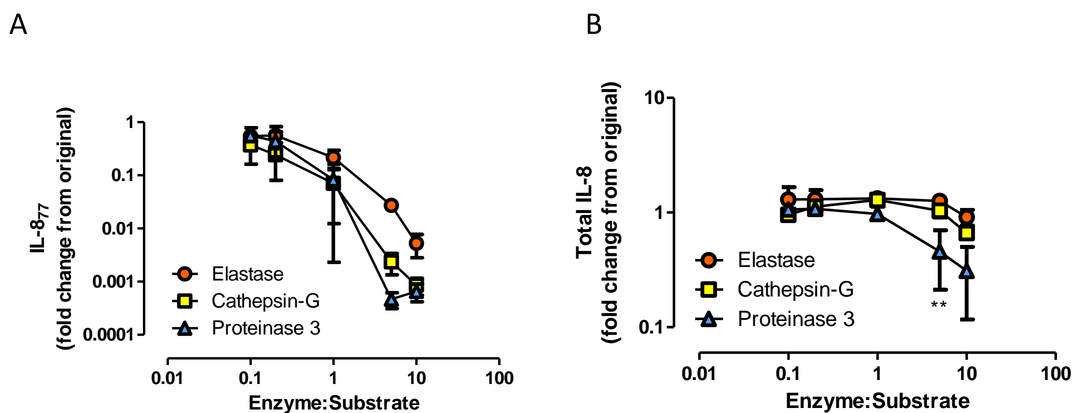

Figure 5-14: Dose-response of conversion of $IL\text{-}8_{77}$ by purified proteases. (a) Fold change in concentration of $IL\text{-}8_{77}$ and (b) total IL-8 by purified proteases at varying concentration. Ratio of enzyme: substrate is represented on the x-axis while the fold-change in concentration (compared to concentrating at 0-hour) is represented on the y-axis. Points plotted are means (± SEM) of three independent experiments. Inter-protease differences in concentration was compared by two-way ANOVA with Bonferroni's post-test. (** = $p < 0.01$)

5.8.2. Conversion of $IL\text{-}8_{77}$ by purified neutrophil proteases over time

I next explored the conversion of $rhIL\text{-}8_{77}$ by purified proteases over time. An excess of protease was used in these experiments (enzyme: substrate = 10:1) over a 24-hour time period. All three purified proteases showed significant convertase activity over time when incubated with $rhIL\text{-}8_{77}$, when compared with buffer control at same the time-point ($p < 0.001$ for all proteases at all time points beyond 0-hour; fig 5.15a).

The majority of this conversion took place by 6 hours (17% of IL-8_{77} remaining with elastase, 2% with Cathepsin G and 6% with proteinase-3). Among the three serine proteases, cathepsin G seemed to have the highest activity towards IL-8_{77}; percentage of remaining IL-8_{77} was significantly lower when compared with elastase after 2 hours ($p < 0.05$) & 4 hours ($p < 0.05$) of incubation and also on comparison with proteinase-3 after 1 hour ($p < 0.01$) and 2 hours ($p < 0.001$) of incubation. The percentage of remaining IL-8_{77} was not significantly different between elastase and proteinase-3 at any time-point. IL-8_{77} was converted to shorter isoforms of IL-8 which was detectable by immunoassay for total IL-8 (fig 5.14 b). However, after incubation with proteases for ≥ 12 hours, there was a trend towards further degradation of IL-8 to peptides which were not detected by ELISA. This was most evident with proteinase-3 where total IL-8 detected was significantly lower when compared with buffer control at 24 hours ($p < 0.01$). Such an effect has been reported previously with an excess of elastase (Leavell et al., 1997) resulting in loss of functional activity. Data is presented as fold-change in concentration compared to corresponding concentration at 0-hour.

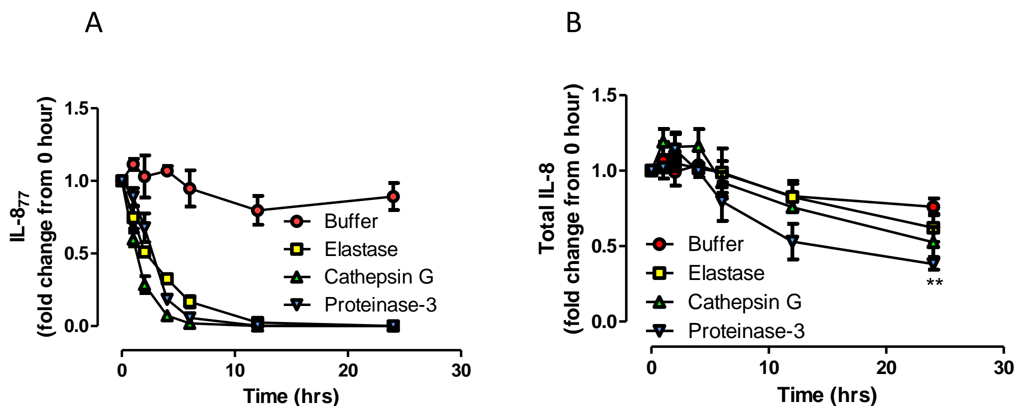

Figure 5-15: Time-course of conversion of IL-8_{77} by purified proteases. (a) Fold change in concentration of IL-8_{77} and (b) total IL-8 by purified proteases at different time-points. Time (hours) is represented on the x-axis while the fold-change in concentration (compared to concentrating at 0-hour) is represented on the y-axis. Points plotted are means (± SEM) of three independent experiments. Differences in concentration was compared by two-way ANOVA with Bonferroni's post-test. (** = $p < 0.01$)

5.9. Functional activity of IL-8 after incubation with proteases

I have shown that all the three neutrophil serine proteases efficiently convert IL-8_{77} to shorter isoforms over time. Next, I proceeded to test functional activity of the converted products.

5.9.1. Establishing the neutrophil degranulation assay

I set up a neutrophil degranulation assay, measuring expression of matrix metalloprotease-9 (MMP-9), to test for differences in functional activity between the isoforms of IL-8. Cytochalasin B (a mycotoxin which inhibits cytoplasmic division by inhibiting network formation by actin filaments) (Theodoropoulos et al., 1994) and fMLP was used as a positive control in these experiments; cytochalasin B has been shown to enhance fMLP-induced neutrophil responses, including degranulation (Honeycutt and Niedel, 1986). The estimated concentration of total IL-8 in the conversion experiments (section 5.8.2) at different time-points was 300 nM (3 X 10^{-7} M). I compared the amount of MMP-9 produced after degranulation of neutrophils by different concentration (10^{-7} M and 10^{-8} M) of the two standard isoforms of IL-8, rhIL-8_{72} and rhIL-8_{77}, in the presence of cytochalasin B. All data was normalised to the MMP-9 expression in the presence of the positive control (cytochalasin B with fMLP) and expressed as a fold-change. As shown in figure 5.16, significant increase in MMP-9 production was observed with the positive control fMLP ($p < 0.001$) and in the presence of either of the IL-8 isoforms at a concentration of 10^{-7} M ($p < 0.001$ for both), compared to expression of MMP-9 in the presence of cytochalasin B only (negative control). No significant differences were noted between the two IL-8 isoforms at this concentration. At a concentration of 10^{-8} M, significant increase in the expression of MMP-9 was noted from the neutrophils by both rhIL-8_{72} ($p < 0.001$) and rhIL-8_{77} ($p < 0.05$) compared to negative control. However, expression of MMP-9 was significantly higher when neutrophils were degranulated in the presence of rhIL-8_{72} compared to rhIL-8_{77} ($p < 0.001$). Thus, at a concentration of 10^{-8} M, the shorter isoform of IL-8 was functionally more potent than IL-8_{77} in this assay. In all future

experiments, IL-8 from all conversion experiments were diluted to a final concentration of 10^{-8} M estimated concentration for degranulation of neutrophils.

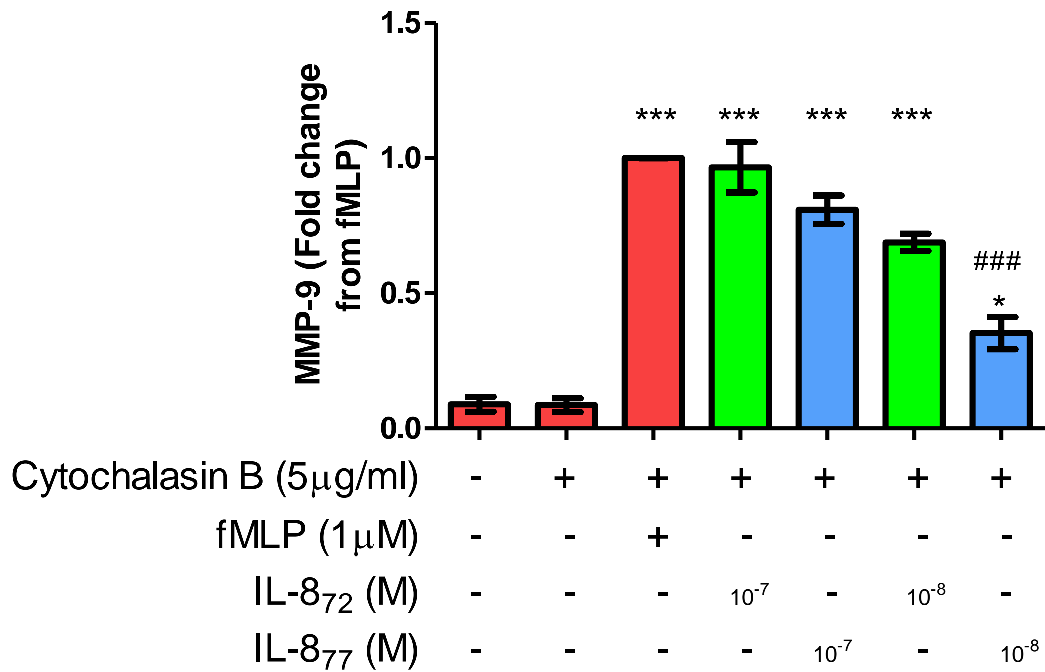

Figure 5-16: Establishing the neutrophil degranulation assay. MMP-9 expressed from neutrophils after degranulation by control conditions (red bars), rhIL-8_{72} (green bars) and rhIL-8_{77} (blue bars). Details of conditions are represented on the x-axis while expression of MMP-9 is represented on the y-axis (expressed as a fold change compared to the positive control). Bars are at means (± SEM) of three independent experiments. Difference in means was compared by one-way ANOVA with Tukey's post-test (comparing all pairs of columns). (*/*** = significant compared to negative control cytochalasin B only; ### = significant compared to rhIL-8_{72} at a concentration of 10^{-8} M. (* = $p < 0.05$, *** = $p < 0.001$, ### = $p < 0.001$)

5.9.2. Functional activity of IL-8 isoforms after processing by proteases

I then proceeded to test the functional activity of IL-8 isoforms after processing of rhIL-8_{77} by purified neutrophil serine proteases over 24 hours. For these experiments, all samples were diluted to an equivalent total IL-8 starting concentration

of 10^{-8} M (as explained above), the hypothesis being any difference (or change) in functional activity would be due to differences in functional activity between the long and shorter isoforms.

Although elastase and cathepsin G efficiently converted IL-8_{77} (fig 5.15), this was not reflected in a change of functional activity (fig 5.17 b-c); no significant differences were observed between functional activity of products from elastase or cathepsin G and buffer at any time-point. In contrast, there was a significant increase in potency in the products after incubation with proteinase-3 after 1 & 2 hours ($p < 0.01$ compared to buffer control) and 3 & 4 hours ($p < 0.05$ compared to buffer control, fig 5.17 d). Similar differences were noted in the functional potency of products on comparing proteinase-3 with elastase ($p < 0.001$ at 1 hour and $p < 0.05$ at 2 hours) or cathepsin G ($p < 0.05$ at 1 hour and 4 hours). This suggests that although all the three serine proteases can convert IL-8_{77}, conversion by proteinase-3 results in a functionally relevant isoform with increased potency, suggesting a key regulatory role for this enzyme for processing IL-8 in the preterm lung.

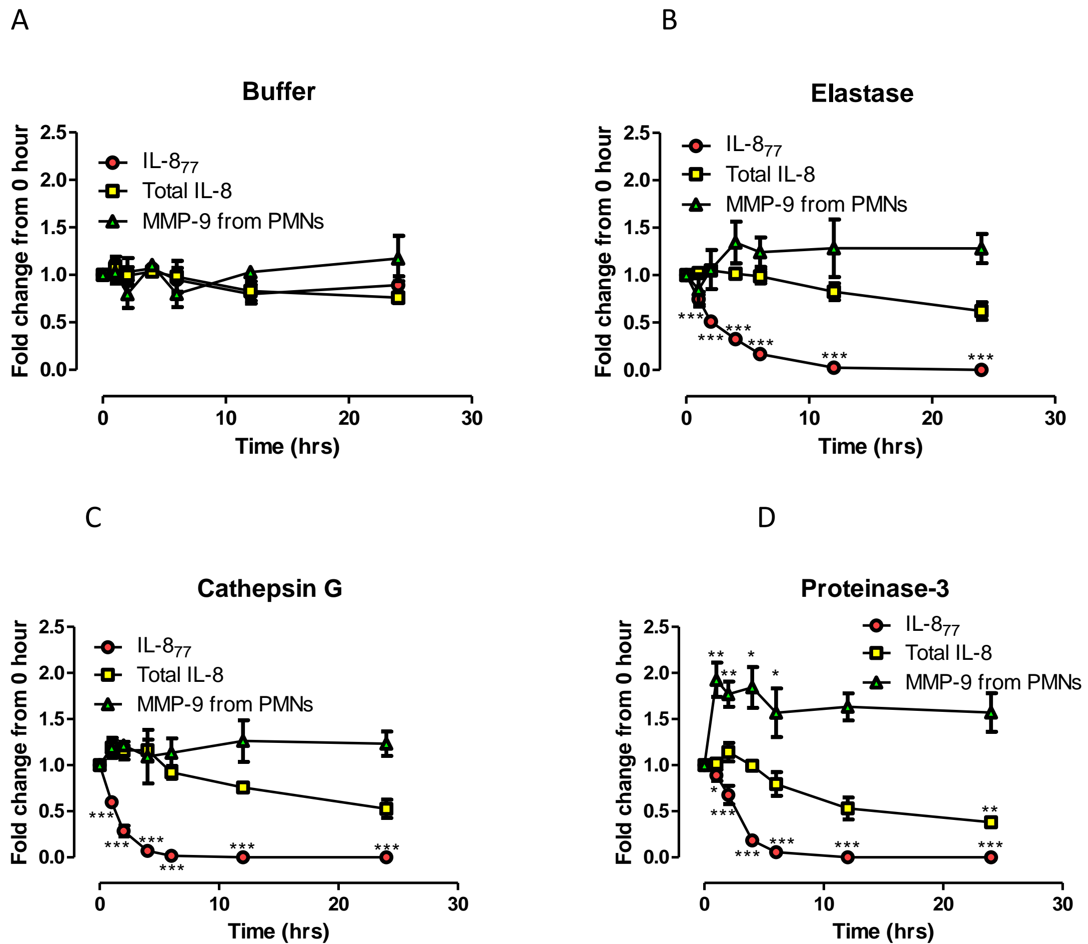

Figure 5-17: Functional activity of IL-8 isoforms. Summary of processing of rhIL-8_{77} by (a) buffer, (b) purified elastase, (c) purified cathepsin G and (d) purified proteinase-3 showing recovery of total IL-8 (yellow squares), IL-8_{77} (red circles) and MMP-9 from degranulation of neutrophils (green upright triangles) by the products of conversion. Time (hours) is represented on the x-axis while IL-8/MMP-9 (fold change from 0-hour values) is represented on the y-axis. Points plotted are means (± SEM) of three independent experiments. Statistical differences in concentration compared to buffer-control at each time-point was tested by 2-way ANOVA with Bonferroni's post-test. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

5.10. Processing of rhIL-8_{72} by proteinase-3

So far, I have shown that processing of rhIL-8_{77} by proteinase-3 results in significant reduction of recovery of total IL-8 at 24-hours. To see if this happens with the shorter isoforms of IL-8, I tested rhIL-8_{72} in the same experiments as above. As shown in figure 5.18a, on incubating rhIL-8_{72} with proteinase-3, there was a 20% loss of protein (as detected by IL-8 ELISA) at 4 hours ($p < 0.001$ compared to buffer control at same time-point) and 80% loss at 24 hours ($p < 0.001$ compared to buffer control at same time-point). On functional analysis, there was a trend towards increased activity at 4 hours but this was not statistically significant (fig 5.18b).

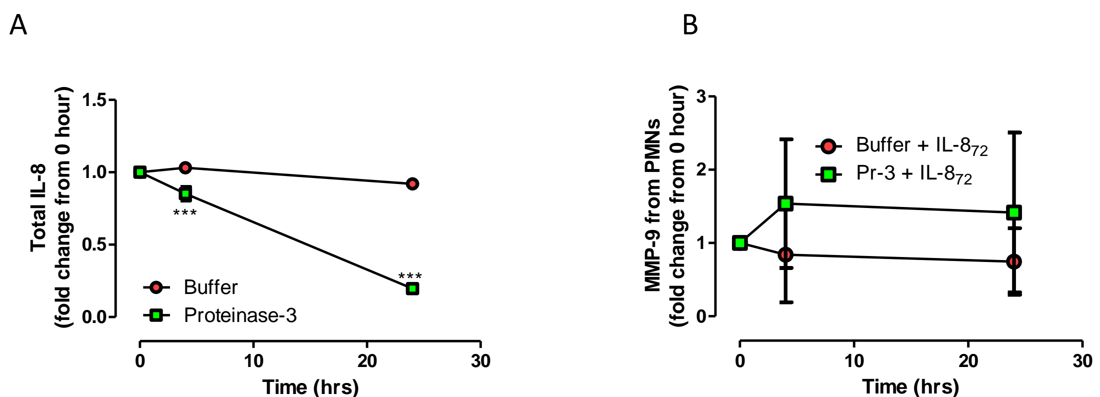

Figure 5-18: Processing of rhIL-8_{72} by purified proteinase-3. (a) Recovery of total IL-8 (expressed as a fold change from 0-hour) on incubation with buffer (red circles) and proteinase-3 (green squares) over 24 hours. (b) Expression of MMP-9 from degranulated PMNs (expressed as a fold change from 0-hour) by rhIL-8_{72} which was incubated in the presence of buffer (red circles) or proteinase-3 (green squares) over 24 hours. Time (hours) is represented on the x-axis while fold-change in protein (total IL-8 or MMP-9) is represented on the y-axis. Points plotted are means (± SEM) of three independent experiments. Differences in the expression of protein was tested by two-way ANOVA with Bonferroni's post-test. (*** = $p < 0.001$)

5.11. Expression of proteinase-3 in preterm ventilated infant BALF

My previous data shows that proteinase-3 processes rhIL-8_{77} resulting in increased functional activity. I have also shown that convertase activity is higher in BALF from CLD infants compared to RDS infants. This prompted me to look at the expression of proteinase-3 in BALF from the preterm infants in my cohort. Proteinase-3 in BALF was measured by a newly developed sandwich ELISA in-house. As shown in figure 5.19, peak expression of total proteinase-3 was significantly higher in BALF from CLD infants (median 234.4 nM, IQR 102.8 – 384.4 nM) compared to No CLD infants (median 26.5 nM, IQR 5.1 – 196.9 nM; p = 0.03).

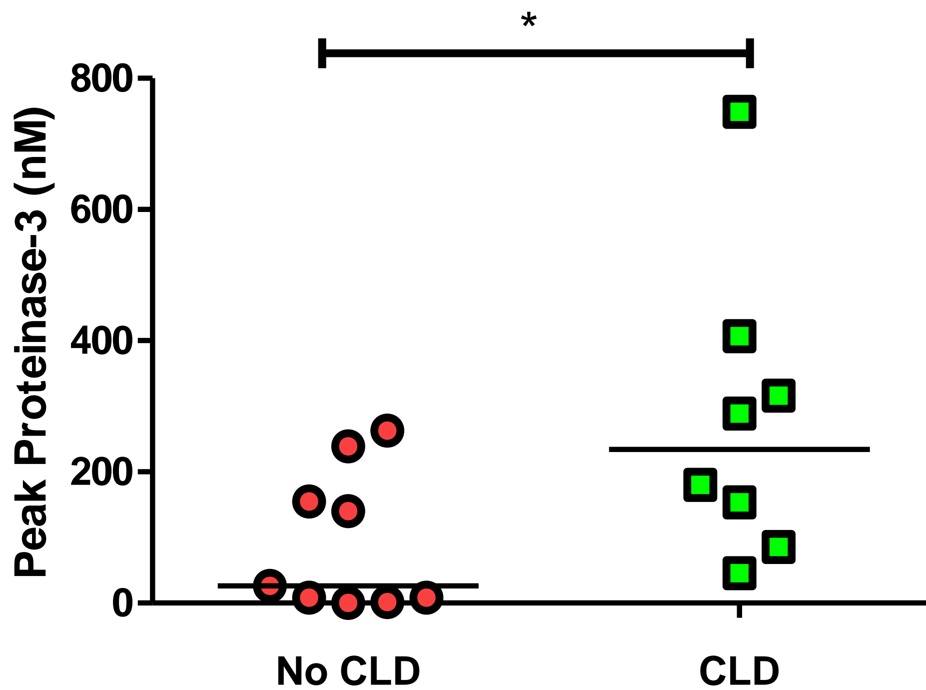

Figure 5-19: Expression of proteinase-3 in preterm BALF. Peak proteinase-3 expression (nM) in BALF from No CLD (red circles) and CLD (green squares) infants. Points plotted represent individual infants and bars are at medians. (* = $p < 0.05$)

5.12. Activity of thrombin in preterm ventilated infant BALF

I have already shown that thrombin may have a significant convertase activity in preterm BALF towards rhIL-8_{77} (figure 5.10). In order to clarify this further, we proceeded to measure specific thrombin activity in preterm BALF. Due to limited sample volume, most of the samples included in the convertase experiment (figure 5.10) could not be used for this. However, thrombin activity was measured in samples collected from the other preterm infants who were part of the same cohort but not included in this study.

As shown in figure 5.20, peak thrombin activity was significantly higher in infants with CLD (median 15.1 mIU/ml, IQR 3.8 – 52.2 mIU/ml) compared to the No CLD infants (median 0.0 mIU/ml, IQR 0.0 – 11.9 mIU/ml; $p = 0.03$).

*
150
100
50
0
Peak Thrombin Activity (mIU/ml)
No CLD
CLD

Figure 5-20: Activity of thrombin in preterm BALF. Peak thrombin activity (mIU/ml) in BALF from No CLD (red circles) and CLD (green squares) infants. Points plotted are from individual infants and bars are at medians. (* = $p < 0.05$)

5.13. Discussion

5.13.1. Summary

This is the first report of IL-8_{77} expression in the lungs of preterm infants. Although total IL-8 and IL-8_{77} concentration were significantly increased in infants who developed CLD, the majority of the IL-8 expressed seemed to be the more potent shorter isoforms. Resident (adult) and infiltrating (term newborn) cells in the lung express a significant proportion of IL-8 as the 77 a.a. isoform. I have demonstrated a significant, but inhibitable, capacity of proteases in the preterm BALF to convert IL-8_{77} to its shorter isoforms. Specifically, conversion by proteinase-3 resulted in shorter isoforms with increased functional activity.

5.13.2. Expression of IL-8 isoforms in preterm BALF

Persistent, neutrophil-driven inflammation has been strongly implicated in the pathogenesis of CLD with IL-8 being a key chemokine involved in the process. My results are in keeping with previous data that IL-8 is significantly increased in the lungs of infants developing CLD (Ambalavanan et al., 2009, Munshi et al., 1997, Kotecha et al., 1995). This reflects the pattern of lung inflammation observed in preterm infants developing CLD: persistent acute inflammation, with a poorly-resolving neutrophilia (Kotecha et al., 2003). IL-8_{77} was detected as a minor proportion of total IL-8, which agrees with my hypothesis that the shorter, and more potent isoforms, constitute the majority of the IL-8 in preterm BALF; this was observed in both groups of infants.

In a recent report (Maheshwari et al., 2009), IL-8_{77} was described as the major isoform in the preterm circulation (60%). This was found to decrease at term to 15% and then to a minor fraction in adults (8%). If the same proportions were to be reflected in the preterm lungs, it could have a profound impact on the function of IL-8 and on inflammation. The authors (Maheshwari et al., 2009) demonstrated increased transcription of IL-8 in endothelial cells compared to monocytes in an *in vitro* porcine cellular model, suggesting that endothelial cells in foetal life may be the major source of IL-8. It is already known that the major isoform of IL-8 expressed from endothelial

cells is IL-8_{77} (Gimbrone et al., 1989, Hebert et al., 1990a). Monocytes, which are the major source of IL-8_{72} (Lindley et al., 1988a, Van Damme et al., 1989b), are known to go through a "maturation" phase during foetal life (Schibler et al., 1993) but at inflammatory sites, as in the ventilated preterm lung, it is more likely that inflammatory cells (mononuclear and polymorphonuclear) are major sources of IL-8 production. However, foetal inflammation is an associated factor in preterm deliveries since chorioamnionitis has been identified as a major cause leading to preterm labour (Goldenberg et al., 2000). Thus, some degree of inflammation can be expected in preterm infants, except if they are delivered for maternal reasons. Maheshwari and colleagues (Maheshwari et al., 2009) have not specified reasons for prematurity in the cohort of infants examined in their study. Following-on from their observation, I measured the concentration and proportion of IL-8_{77} in preterm BALF on the first day of life to reflect in-utero concentrations as closely as possible. No correlation was observed in either the concentration or proportion of IL-8_{77} in BALF with the birth gestation of infants.

Taken together, the above observation suggests that in the inflamed preterm lungs, inflammatory cells (mononuclear and polymorphonuclear) may be the major producer of IL-8 (as shorter isoforms predominate), although most other cell-types in the lungs can express IL-8. Another possibility was that immune cells other than monocytes and lung epithelial-cells express significant proportions of IL-8_{77}, but it gets converted to the more potent shorter isoforms by one or several proteases. I first looked at the expression of IL-8_{77} from individual cell types.

5.13.3. Expression of IL-8_{77} from individual cell types

Pulmonary epithelial cell lines are known to express IL-8 on stimulation (Standiford et al., 1990, Leverence et al., 2011); however, expression of IL-8_{77} has not been described before. My results show for the first time that a significant proportion of IL-8 is expressed as the 77 a.a. isoform by a variety of adult airway cell-lines and primary small airway cells, both in the un-stimulated state and in the presence of inflammatory stimuli.

Another abundant cell type in the preterm lungs are neutrophils and monocytes/macrophages. Previously, IL-8_{77} expression has been reported to range from 17–38% of total IL-8 from adult monocytes (Yoshimura et al., 1989b, Lindley et al., 1988b) and mixed mononuclear cells (Van Damme et al., 1989a), and about 60% of total IL-8 from PMNs (Padrines et al., 1994b). I have described for the first time expression of IL-8_{77} from term neonatal cord-blood PMNs and monocytes. My results are similar with adult data published previously.

Taken together, this data shows that both non-immune and immune cells in the lung can potentially express significant quantities of IL-8_{77}, especially when stimulated. However, the proportion of IL-8_{77} I recovered from preterm BALF was uniformly low. This suggested that known proteases or other unknown convertases could be involved in the preterm lung to convert the IL-8_{77} to shorter isoforms. I next looked at the possibility of this in the preterm BALF samples.

5.13.4. Conversion of IL-8_{77} by preterm BALF: effect of neutrophil serine proteases

BALF from ventilated preterm infants is known to contain several inflammatory mediators (Ryan et al., 2008, Bose et al., 2008) including an imbalance in neutrophil proteases and anti-protease (Speer, 2006). Matrix metalloproteinase-9 (MMP-9) has also recently been recovered in preterm BALF (Davies et al., 2010). Since all of these proteases are known converters of IL-8_{77} to shorter and more potent isoforms (Van den Steen et al., 2000, Padrines et al., 1994a), I looked for specific convertase activity in preterm BALF. Indeed, when known quantities of exogenous rhIL-8_{77} was incubated with preterm BALF, significant conversion was noted (71% recovery of original protein). Conversion was more pronounced in the CLD infants.

To study the specific mechanisms of conversion, the same samples were pre-incubated with specific anti-proteases before rhIL-8_{77} was added. Protection from conversion by α-1 antitrypsin (AAT, 88% recovery) and anti-thrombin III (ATIII, 88% recovery) suggested neutrophil serine proteases and thrombin were involved in the conversion process. However, AAT or ATIII were not able to completely inhibit the

conversion of rhIL-8_{77} by BALF. Phenylmethanesulfonylfluoride (PMSF, recovery 95%) provided greater protection as it is known to inhibit a wider range of enzymes, including both neutrophil serine proteases and thrombin. Metallo-proteases were not involved in the convertase activity, as suggested by the lack of any protection in the presence of 1-10 phenanthroline, a non-specific inhibitor of metallo-proteases (68% recovery).

Early studies in preterm infants have suggested the involvement of neutrophil proteases in contributing to inflammation and lung damage (Merritt et al., 1983, Ogden et al., 1984a) although this has not been corroborated by more recent studies (Sveger et al., 2002). An imbalance between matrix-metallo-proteases and their inhibitors in the lungs of preterm infants has been noted (Cederqvist et al., 2001, Ekekezie et al., 2004, Sweet et al., 2004). This imbalance has the potential of damaging the lung with long-term consequences (Speer, 2006). Our group have recently reported the contribution of elastase and MMP-9 in the development of CLD (Davies et al., 2010). Similar imbalances have been suggested in cystic fibrosis, another neutrophilic lung disease (Griese et al., 2008, Gaggar et al., 2011). Apart from the direct effect of proteases, post-translational modification of chemokines (Mortier et al., 2011b) can also influence local immune function and have an impact on tissue injury. This effect is particularly important for IL-8 as cleavage by proteases results in enhanced functional activity.

The neutrophil serine proteases themselves have been shown to induce IL-8 expression from lung epithelial cells (Nakamura et al., 1992, Chen et al., 2004, Kuwahara et al., 2006) and endothelial cells (Berger et al., 1996). Some investigators have suggested the existence of a positive-feedback loop whereby proteases released from the initial influx of PMNs both induce an increase in IL-8 production from epithelial cells and also cleaves potential longer isoforms of IL-8 to shorter isoforms. Such a loop, if unchecked, has significant potential to cause lung damage.

Since protection from cleavage of IL-8_{77} in BALF was achieved by AAT and PMSF, I looked at the processing of IL-8_{77} by the three main serine proteases from neutrophils. Proteinase-3 is able to convert IL-8_{77} to IL-8_{70} (Padrines et al., 1994b) as shown previously, but the authors found that elastase and cathepsin G were not

involved in the conversion. I have shown that all three of the main serine proteases in PMNs can convert IL-8_{77} to shorter isoforms. This is effective both over time and over a dose range. Cathepsin G seems to be the most potent in this regard with elastase the least potent among the three. Interestingly, with longer incubation periods, there was a decrease in the amount of total IL-8 recovered, which was statistically significant with proteinase-3. This would suggest that there are several potential cleavage sites for the enzymes on the IL-8 molecule which are distal from the NH_2 terminal (Padrines et al., 1994b), producing smaller peptides which are undetectable by ELISA for IL-8. Elastase has previously been reported to abolish IL-8 activity by a similar process (Leavell et al., 1997), although the authors found no such effect of cathepsin G (proteinase-3 was not studied). They suggested that cleavage of IL-8 by proteases could be a mechanism by which activity of this chemokine is abolished. I have found evidence that all the three PMN serine proteases have this effect when present at an excess compared to substrate. Further studies need to be conducted to confirm the clinical implications of this effect *in vivo*.

Although I have shown that all three of the neutrophil serine proteases can convert IL-8_{77} to shorter forms efficiently, only conversion by proteinase-3 resulted in proteins which were functionally more potent than IL-8_{77}. In a detailed *in vitro* study of chemically synthesised IL-8 isoforms (Clark-Lewis et al., 1991), isoforms of IL-8 which were shorter than 72 a.a. were found to be more potent than IL-8_{72} itself. This was validated in a more recent study, where the authors defined three different categories of IL-8 isoforms based on functional potency (Mortier et al., 2011b). Thus, the most abundant and well studied isoform, IL-8_{72}, may not be the most potent. My results suggest that proteinase-3 could be an important modulator of IL-8 function in preterm lung by post-translation modification of long isoforms. Processing of both IL-8_{77} and IL-8_{72} by proteinase-3 resulted in loss of protein (as determined by immune assay) after incubation for up to 24 hours. Although I have not been able to show a clear functional correlation of this observation, it has been previously shown that a similar effect by elastase can result in abolition of activity (Leavell et al., 1997). Elastase remains the most extensively studied neutrophil serine protease in the lungs. However, my analysis

suggests that proteinase-3 may be more relevant in modulation of IL-8 biology in the preterm lungs and more research is merited to explore this.

Alpha-1 antitrypsin (Alpha-1 proteinase inhibitor, AAT) has been assessed as a therapeutic intervention in two randomised controlled trials by the same group of investigators (Shah and Ohlsson, 2001). When all the infants and all doses of AAT were pooled together, there was a reduction in incidence of CLD defined as oxygen requirement at 28 days of age (short-term outcome). However, no other statistically significant benefits were observed in any of the outcomes recorded, including prevention of CLD at 36-weeks corrected gestational age or long-term neuro-developmental outcome. As the total number of infants included in these two trials is small (195), this may have masked a true effect.

AAT has been in use in adults as a treatment for AAT deficiency since initial studies conducted in the 1980s (Wewers et al., 1987, Gadek et al., 1981). Although the authors demonstrated safety and biochemical efficacy, this study was not designed to look for clinical effectiveness. Evidence for clinical effectiveness of AAT in genetically deficient individuals have been mainly from observational studies and small randomised-controlled trials, long after the molecule was licensed for clinical use. Results from these trials and reports have been conflicting at best; more often they have failed to show clear clinical benefits (Dickens and Lomas, 2011) and as such has not been recommended for use (Gotzsche and Johansen, 2010). Several theories have been proposed to explain this apparent lack of effect. Polymers formed by abnormal AAT alleles have been shown to be pro-inflammatory and result in chronic inflammation in the lungs and slow, but persistent, damage to the liver (Dickens and Lomas, 2011), and an approach using small molecules to block this polymerisation has been proposed (Mallya et al., 2007). Moreover, as AAT is an acute-phase reactant, episodic administration could be more relevant for clinical effectiveness than a steady-state concentration. Using alternative delivery techniques like nebulisation could also prove to be both effective and economic (Siekmeier, 2010). Elafin is a potent endogenous inhibitor of elastase and proteinase-3 (Shaw and Wiedow, 2011). In a recent trial on an animal model of CLD, intra-tracheal instillation of recombinant elafin

in mechanically ventilated newborn mice was shown to reverse histological changes in lung compared to vehicle-treated controls (Hilgendorff et al., 2011).

The above evidence, along with my analysis, suggests that using antiproteases could have clinical benefits in attenuating lung inflammation and injury in preterm ventilated infants and merit assessment in well-powered clinical trials.

5.13.5. Conversion of IL-8_{77} by preterm BALF: effect of thrombin and the coagulation cascade

Links between inflammation and the coagulation cascade have recently come to light. Inflammatory cytokines (IL-6, TNF-α, IL-1β, MCP-1, CRP) increase expression of tissue factor on endothelial cells and monocytes (Lipinski et al., 2011, Mackman, 2009) leading to triggering of the coagulation cascade (Levi, 2010). Cytokines also dampen anti-coagulation by various mechanisms (Lipinski et al., 2011) and suppress fibrinolysis (Levi and van der Poll, 2008). Thrombin itself has been shown to increase expression of IL-8 from endometrial stromal cells through stimulation of MAPK (Kawano et al., 2011). Thus there is ample evidence of a pro-coagulant state during active inflammation with evidence of cross-talk between the two systems.

An imbalance in the coagulation: anticoagulation balance has been observed in lavage fluid from preterm ventilated infants (Viscardi et al., 1992). Initiation of the coagulation cascade, as demonstrated by elevated thrombin activity, was shown in BALF from a lamb model of preterm RDS (Jaarsma et al., 2001). To my knowledge, only one previous group of investigators have reported measurement of thrombin activity in BALF from preterm ventilated infants, where it was shown to be the major mitogenic stimulus in BALF for fibroblasts in culture (Dik et al., 2003). However, in this study, thrombin activity was found to be lower in infants who subsequently developed CLD compared to those who don't. My results show a significant increase in thrombin activity in BALF from preterm ventilated infants who develop CLD (figure 5.20). In a recent *in vitro* model, activation of the coagulation cascade by CLD-BALF was shown to promote fibrotic responses (Kambas et al., 2011). Importantly, the authors

demonstrated the beneficial effects of suppression of fibrosis mediators by blocking the extrinsic pathway of coagulation with ATIII in their model.

Activation of the clotting cascade has been shown to contribute to fibrotic lung diseases in adults (Scotton et al., 2009, Imokawa et al., 1997), where thrombin is part of the final common pathway. My analysis suggests that thrombin is involved in modulating lung inflammation in ventilated preterm infants by post-translational modification of IL-8, thus attracting increased numbers of neutrophils into the lungs which express serine proteases and other inflammatory mediators. Recently, inhibition of neutrophil serine proteases has been shown to improve lung growth in a murine model of BPD (Hilgendorff et al., 2011). Thus, inhibition of specific proteases could regulate IL-8 activity and curtail pulmonary inflammation in preterm infants at risk of BPD, with possible clinical benefits.

Protease-activated receptors (PAR) are a group of G-protein coupled cell-membrane proteins involved in intracellular signalling (Osso*vs*kaya and Bunnett, 2004). To date, four such receptors (1 - 4) have been characterised. A variety of cell types express these receptors including platelets, epithelial and endothelial cells, fibroblasts and neural cells. Several proteases can activate these receptors but thrombin is the best characterised among them, which can activate PAR1, PAR3 and PAR4 (van der Poll, 2008). Among the neutrophil proteases, cathepsin G has been suggested to be an activator of PAR4 (Sambrano et al., 2000). In the airways, PAR1 and PAR2 are expressed on epithelial, endothelial and smooth muscle cells (Osso*vs*kaya and Bunnett, 2004). Activation of PAR1, PAR2 and PAR4 on airway epithelial cells stimulate expression of inflammatory cytokines (Asokananthan et al., 2002). Activation of coagulation (Scotton et al., 2009, Imokawa et al., 1997), and more specifically thrombin (Howell et al., 2002) has been shown to be associated with fibrotic diseases of the lungs. In summary, these groups of receptors and their protease activators may have important roles to play in lung inflammation. A clear understanding of their role in the preterm infants and may suggest novel approaches towards lung inflammation.

The pathology of CLD has evolved over the years and can be subdivided into three broad eras. Early descriptions of CLD from the 1960s to the 1970s (Northway et al., 1967, Bonikos et al., 1976, Reid, 1979, Stocker, 1986) involving relatively late

preterm infants (by today's standards) were characterised by severe epithelial changes, extensive fibroproliferation and smooth muscle hyperplasia with hypertensive changes in the vasculature. Improvements in clinical practice in the 1980s, even before the era of exogenous surfactant replacement and antenatal steroids (Taghizadeh and Reynolds, 1976), resulted in survival of more immature infants who had lungs characterised by less epithelial and vascular changes, variable degrees of fibrosis and more simplified airspaces (Erickson et al., 1987). In the few infants with reported lung histology who received exogenous surfactant replacement (Husain et al., 1998), the major pathology was an arrest of acinar development. However, septal fibrosis was still noted among some of these infants. No reports of lung histology describing CLD from extremely premature infants who received both antenatal steroids and post-natal exogenous surfactant replacement therapy are available, presumably because most of them die before developing CLD, and very few die thereafter. However, in the face of significant and persistent inflammation documented in the lungs of these infants, healing with some degree of fibrosis is almost inevitable. Part of this effect will be contributed by activation of the clotting cascade, as noted in adult diseases (Scotton et al., 2009, Imokawa et al., 1997), and merits further research.

5.14. Limitations

I acknowledge certain limitations of this study. I was unable to confirm previously reported increased expression of IL-8_{77} in preterm circulation (Maheshwari et al., 2009) as I did not have access to matched blood samples from the infants in my study. The airway cell-lines and primary cells used in my experimental models are all from adult populations and may not reflect conditions in the preterm lung accurately. I believe that the future use of appropriate animal models may enable us to overcome some of these difficulties.

5.15. Summary

My data show that in the lungs of ventilated preterm infants, the majority of IL-8 expression seems to be the shorter (more potent) isoforms; this is also supported by the pattern of cellular infiltration into the lungs. The expression of IL-8_{77} is low, although several individual cell-types present in the lungs can potentially express significant quantities of the protein. This difference in proportion could be due to conversion of IL-8_{77} to shorter isoforms extracellularly. Imbalances in protease-antiprotease ratio could contribute to this conversion. Knowledge about intra-cellular post-transcriptional modifications during IL-8 production may lead to novel approaches toward limiting inflammation.

6. Final Summary

6.1. Introduction

Chronic lung disease (CLD) remains the most common respiratory sequelae of premature birth, especially among the extremely premature and low birth weight infants (Ali et al., 2013). Currently, CLD is a descriptive term used for a spectrum of clinical respiratory illness, from mild to severe (Jobe and Bancalari, 2001a). No single cause have been identified so far which leads to the development of CLD, apart from extreme prematurity and low birth weight, which remain the two consistent risk factors (Chakraborty et al., 2010). The cumulative effect of CLD has important medium-term health implications including respiratory (Gough et al., 2012, Kotecha et al., 2013) and neuro-developmental (Doyle and Anderson, 2009) morbidity. Although we are still in the process of understanding the long-term implications of CLD in the current population of infants born extremely preterm and who survived, our current knowledge informs us that CLD is a condition with significant short- and long-term morbidities. A better understanding of the condition, including the mechanisms and pathways involved, is essential for planning therapeutic strategies.

Lung inflammation involving the innate immune system has been strongly implicated and extensively studied in preterm infants developing CLD/BPD (Ryan et al., 2008, Chakraborty et al., 2010). A common pattern of inflammation in the lungs of preterm infants who develop CLD later is a persistent and poorly resolving neutrophilia. The chronic nature of insult to the preterm lung ensures persistence of this acute inflammation. In parallel, low numbers of mononuclear cells precludes adequate resolution of the acute inflammation. While a large body of evidence exists regarding inflammatory mediators in the preterm lungs, studies on resolution of inflammation are limited. Modulation of inflammatory process to counter acute inflammation or to initiate resolution could have profound influences on lung growth and development in preterm infants and may result in improved clinical outcomes. This forms the overall hypothesis of my research work.

Association of inflammatory mediators in the preterm lung with CLD are inconsistent, with IL-8 being a notable exception (Ryan et al., 2008, Ambalavanan et al., 2009). IL-8 is a key chemokine involved in attracting and activating neutrophils at

sites of inflammation (Remick, 2005). Significantly higher concentrations of IL-8 in lungs or BALF of preterm ventilated infants developing CLD has been reported in the literature; this is reflected in the neutrophilic lung inflammation observed in infants developing CLD. This led me to study two different pathways of possible resolution of lung inflammation in preterm infants: modulation of expression (by IL-6 *trans*-signalling responses) and function (by different isoforms) of IL-8 in BALF from a cohort of preterm ventilated infants. The study was planned to initiate with observational data, and then proceed to develop *in vitro* models to study mechanisms of each of these two pathways.

6.2. Modulation of inflammation by IL-6 *trans*-signalling

Interleukin-6 (IL-6) is a critical cytokine involved in acute inflammation. Although the majority of the pro-inflammatory functions of IL-6 are mediated by signalling through its cognate receptor, IL-6R (Jones, 2005), the pro-inflammatory effects of IL-6 *trans*-signalling have been studied in various diseases and disease models and blocking the effects of IL-6 *trans*-signalling in rheumatoid arthritis (Choy et al., 2002) and Crohn's disease (Ito et al., 2004) have resulted in therapeutic benefits.

More relevant to my research are the pro-resolution effects of IL-6 *trans*-signalling in innate immunity, as summarised in figure 6.1 and supported by experimental data from animal models of inflammation (Hurst et al., 2001, McLoughlin et al., 2004, Chalaris et al., 2007). Since the pattern of inflammation noted in these animal models reflect preterm lung inflammation closely, this led me to my first hypothesis for my research project: non-resolution of lung inflammation in preterm infants could be due to a lack of the effects of IL-6 *trans*-signalling.

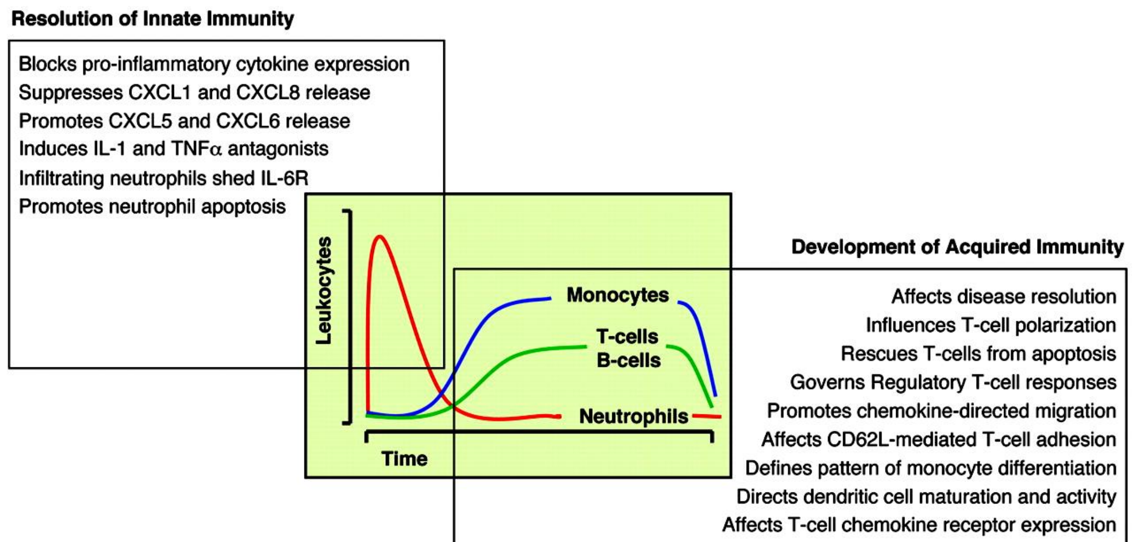

Figure 6-1: Regulation of innate and acquired immunity by IL-6 *trans*-signalling. (With permission from (Jones, 2005)) *Copyright 2005. The American Association of Immunologists, Inc.*

6.2.1. Data from the baboon model of BPD

Non-human primates share genetic and physiological characteristics with humans. They also have common susceptibilities to infection and other chronic diseases. The baboon model of BPD closely reflects the condition in human preterm infants (Coalson et al., 1999b), and has been used by several investigators to study mechanisms of the disease (Subramaniam et al., 2007, Viscardi et al., 2006a, Afshar et al., 2003, Yoder et al., 2003b, Awasthi et al., 2001) and trial therapeutic interventions (McCurnin et al., 2009, Thomson et al., 2006, McCurnin et al., 2005a, McCurnin et al., 2005c, Thomson et al., 2004, Yoder et al., 2000b). Baboons were delivered at 2/3 of term gestation (equivalent to 24-26 weeks in human infants) by Caesarean section, after receiving antenatal steroids at least 48 hours before delivery. Preterm newborn baboons received exogenous surfactant replacement and were gently ventilated on pressures and oxygen concentrations that reflects current clinical practice in preterm human infants.

To establish proof of concept, I utilised tracheal aspirate fluid (TAF) from preterm baboons, included in the control arms of therapeutic trials, to collect

observational data. I confirmed the expression of IL-6 and its soluble receptors, and the chemokines IL-8 & MCP-1, in the TAF of preterm ventilated baboons. Expression of the soluble receptors sIL-6R and sgp130 is being reported for the first time in this model.

Apart from sIL-6R, the pattern of expression of the other cytokines and chemokines showed dynamic changes usually reaching a peak (median concentration) on day 6-7 before showing signs of resolution. Similarly, the cellular infiltrate into the lungs (total and differential), as recovered in TAF, did not change significantly over the study period. Since the source of sIL-6R has been suggested to be mainly inflammatory cells, this could be a possible reason why this cytokine concentration remained unchanged.

The relative expression of the cytokines suggested a lack of any IL-6 *trans*-signalling activity in the preterm baboon lungs. This was reflected by a small, but significant, change in the pattern of recruited cells into the lungs with a predominance of mononuclear cells towards the end of the study period. Thus, data from the preterm baboons confirmed dynamic expression of the IL-6 *trans*-signalling molecules in the lungs of these animals, although suggesting lack of any significant IL-6 *trans*-signalling effects in preterm ventilated baboon TAF.

6.2.2. Data from human preterm infants

While data from the preterm baboons confirmed expression of the IL-6 *trans*-signalling molecules in the lungs, interestingly it revealed a significant increase of sgp130, the specific inhibitor of IL-6 *trans*-signalling. Observational data from the human preterm infants also showed a significant increase in expression of sgp130 and a trend towards increased expression of IL-6 and sIL-6R in bronchoalveolar lavage fluid (BALF) of preterm ventilated infants who develop CLD compared to those who do not. Anti *trans*-signalling potential (sgp130/sIL-6R) was higher in the CLD infants suggesting abrogation of IL-6 *trans*-signalling effects, my primary hypothesis.

As previously reported in the literature, expression of IL-8 was significantly higher in the CLD infants of my cohort. However, while IL-6 has been reported in increased concentration from CLD infants previously (Kotecha et al., 1996b), my

analysis showed a non-significant rise. This could be due to the limited number of infants included in each group of my cohort. However, a shift in the pattern of lung inflammation, due to changes in clinical practice, cannot be ruled out, although consistent data to support this is lacking.

The similarities and contrasting features in the data from baboons and human infants are interesting. While both are observational, the baboon data is from a cohort study while the human data is from a prospective case-control study. Thus, direct comparison between the two sets of data is not possible. However, some cautious inferences regarding the pattern of expression of molecules can be drawn from them.

1) Significant modulation of sgp130 concentration was noted in the lungs of both species. Concentration of sgp130 was in excess of both sIL-6R and IL-6 concentration in both species. This remains the key finding from my work.

2) The relative expression of sIL-6R, compared to IL-6 expression, in the baboons was noted to be different from the human infants. While sIL-6R concentration was lower than IL-6 concentration in all samples of TAF from the preterm baboons (table 3.1), they were comparable in preterm BALF from the human infants (table 3.5). Understandably, this resulted in differences in the IL-6 *trans*-signalling potential (as calculated from the ratio of the molecules) between the two species. Differences in the method of sample collection (TAF *vs* BALF), and lack of non-human primate specific ELISAs for baboons, may be possible explanations for this difference. The pattern of expression of these two molecules set up an environment where functional IL-6 *trans*-signalling would be unable to proceed in the lungs of either species.

3) While significant changes in the expression of IL-6 was noted in the baboon lungs, no significant differences were noted between the two groups of human infants.

6.2.3. Data from the IL-6 functional assay

To understand the functional implications of the previous observations, I modified a cell-base IL-6 functional assay, using mouse B9 cells, to measure specific IL-6 *trans*-signalling. My strategy to measure specific IL-6 *trans*-signalling activity was by using a monoclonal anti-mouse antibody against IL-6R (2B10), to inhibit IL-6 *cis*-

signalling. Thus, any proliferation of cells in the presence of the 2B10 antibody would be due to the effects of IL-6 *trans*-signalling. Key findings from my work are:

1. I have demonstrated the successful modification of the B9 cell assay to measure specific IL-6 *trans*-signalling using the above strategy. This can be used for clinical samples where IL-6 *trans*-signalling is thought to be involved in pathogenesis.
2. BALF samples from both groups of infants resulted in comparable proliferation of B9 cells. This suggests the presence of "free" IL-6 in the samples.
3. The significantly higher concentration of sgp130 did not seem to have an effect on the IL-6 *cis*- or *trans*-signalling activity.

My data and analysis suggests that IL-6 *trans*-signalling may not be a major pathway involved in inflammation in the preterm lungs. This inference can be drawn from the observational data in TAF from the preterm baboons and in BALF from preterm human infants, primarily due to the relative low concentrations of sIL-6R in samples from both species. An excess of sIL-6R, relative to IL-6, have been found in all diseases and disease models which seem to respond to therapeutic blockade of IL-6 *trans*-signalling (implicating IL-6 *trans*-signalling as a pro-inflammatory process) (Desgeorges et al., 1997, Kotake et al., 1996, Uson et al., 1997, Peake et al., 2006, Mitsuyama et al., 1995, Doganci et al., 2005). Binding studies conducted *in vitro* (Gaillard et al., 1999) also suggest that concentration of the sIL-6R/IL-6 complex is primarily dependent on the concentration of sIL-6R, and increases when sIL-6R is in excess relative to IL-6.

The results of the functional assay, which were designed to confirm the above observation, needs careful interpretation. I have demonstrated successfully that the B9 cells can be modified to act as a functional assay for IL-6 *trans*-signalling. This particular strategy seems to work well with hyper IL-6 (H IL-6, a synthetic stable complex of sIL-6R/IL-6), but no activity was noted with in presence of the natural complex (formed in the presence of recombinant IL-6 and sIL-6R). Although recombinant human IL-6 (used as a positive control in the assay) and sIL-6R (used for IL-6 *trans*-signalling in rheumatoid arthritis synovial fibroblasts, figure 4.10) were both

individually functionally active, no B9 cell proliferation was noted in the presence of their natural complex. Difference in functional potency between the stable (H IL-6) and natural complex, as well as inadequate concentration of the natural complex, could be possible explanations for this observation. Such a difference in potency between *cis*- and *trans*-signalling has been suggested previously in the case of signalling by the complex of IL-11 and sIL-11R, which was less potent than signalling by IL-11 through the cell-surface receptor (Curtis et al., 1997). It is also possible that the strategy of using an antibody to inhibit IL-6 *cis*-signalling (2B10) in order to measure IL-6 *trans*-signalling, may not be appropriate for clinical samples in this assay. Concentrations of IL-6 had to be kept "low" in all of these experiments to prevent "breakthrough" IL-6 signalling; possibly leading to inadequate complex formation. To overcome this specific limitation, the ideal modification should be to prevent expression of the IL-6 cognate receptor on the B9 cells (IL-6R). This can be achieved either by "knockdown" of IL-6R using translation blockers like short interfering RNA (siRNA), but more definitively by genetic "knockout" of the IL-6 receptor gene (e.g. by zinc-finger proteases). Although the BAF-B03 cells express the same phenotype ($IL\text{-}6R^{-}$ $gp130^{+}$), they only respond to supra-physiologic concentration of IL-6 and sIL-6R, making it unsuitable for use with clinical samples. Thus, I have not been able to definitively confirm or refute my hypothesis, and my work highlights need for further research in the development of a specific IL-6 *trans*-signalling assay.

6.2.4. Role of sgp130

The role of sgp130 in the preterm infant lung remains unknown. I have found significantly higher concentrations of sgp130 in preterm infants developing CLD, apparently without an obvious function. The precise mechanisms of regulation of expression of cell-surface gp130 and sgp130 in health and disease has not yet been fully elucidated. Recent evidence from a large cohort study of adults at risk for coronary artery disease found that circulatory concentration of sgp130, but not cell-surface expression, is predicted by a specific polymorphism, G148C, on the *gp130* gene (Wonnerth et al., 2014). This is intriguing, more so because polymorphisms have been suggested as a genetic risk-factor for development of CLD previously (Bhandari and

Gruen, 2006), although a genome wide association study (GWAS) did not find significant associations between CLD and single nucleotide polymorphisms (SNP) (Wang et al., 2013). On the other hand, a separate GWAS identified novel SNPs in the *IL-6ST* (IL-6 signal transducer: gp130) gene as risk loci for rheumatoid arthritis (Stahl et al., 2010), a dsease where IL-6 *trans*-signalling is involved in the pathogenesis. Taken together, sgp130 polymorphisms could explain the difference in concentration noted in BALF, and suggest novel genetic risk loci for CLD.

There are several other cytokines which belong to the IL-6 family (signal through gp130 signal transducer) including IL-11, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin like cytokine (CLC) and oncostatin-M (OSM). Like IL-6, IL-11 uses a homo-dimer of gp130 for intra-cellular signalling (along with a cognate α-receptor) while LIF, CNTF, CT-1, CLC and OSM signal through a gp130 hetero-dimer receptor (only one receptor subunit is gp130). The role of these cytokines, if any, on the development of CLD in preterm infants remain unknown. In a recent study on the growth of foetal rat lung explants, IL-6 and IL-11 (which signal through a homo-dimer of gp130) were found to enhance growth and differentiation of lungs throughout foetal life, while CNTF, CLC, CT-1 and OSM (which signal through a hetero-dimer of gp130) inhibited lung growth. It is possible that other members of the IL-6 family have a yet unknown role in the preterm lungs and in the development of CLD. Soluble gp130 (sgp130) can inhibit LIF- and OSM-induced cartilage injury (Hui et al., 2000); while IL-11 *trans*-signalling has been described (Curtis et al., 1997, Dams-Kozlowska et al., 2012), it remains unknown whether sgp130 has a similar inhibitory role as for IL-6 *trans*-signalling. Thus, the significantly high levels of sgp130 noted in preterm infants who later develop CLD could be involved in signalling by other members of the IL-6 family. This is another significant new area of research highlighted as a result of my work.

The question regarding the effects of IL-6 *trans*-signalling in the preterm lungs remains unresolved. IL-6 being such an important cytokine in innate and acquired immunity, is almost certainly involved in inflammatory process in the preterm lungs. My data seems to suggest that IL-6 *trans*-signalling currently may not be an effective pathway; however there is possibility of exploring its effect by using hyper IL-6 (H IL-6)

in an appropriate animal model. This is supported by convincing experimental evidence of the pro-resolution effects of IL-6 *trans*-signalling (Hurst et al., 2001, McLoughlin et al., 2004, Chalaris et al., 2007). It remains to be seen whether the elevated concentration of sgp130 is able to inhibit effects of H IL-6 *in vivo*.

In summary, this part of my work has confirmed some of my hypotheses, but raised several new research questions which needs to be addressed in future studies.

6.3. Modulation of inflammation by IL-8 isoforms

Interleukin-8 (IL-8) is a key chemokine in acute inflammation, involved in recruiting and activating neutrophils at sites of inflammation down a concentration gradient. It is ubiquitously expressed by almost all cell types at sites of inflammation, and increased IL-8 expression has been consistently reported in lungs of preterm infants who develop CLD, compared to those who do not. Elevated IL-8 expression could be partly responsible for the persistent neutrophilia observed in the lungs of preterm infants; however, other factors including poor resolution of inflammation likely contributes to this effect. Although IL-8 has been consistently reported in the literature in association with CLD, several other biological molecules which can attract and activate neutrophils are likely involved in lung inflammation in preterm infants. Some chemokines, like GRO-α, GRO-β and ENA-78, share receptors with IL-8, while other chemokines, like MIP-1α and RANTES, have separate receptors. Additionally, there are several other peptide molecules (e.g. complement anaphylatoxins: C5a) and lipid-derived mediators (e.g. leukotrienes and platelet activating factor) which have similar effects on neutrophils (Sadik et al., 2011). This redundancy would suggest that targeting any one of these mediators in isolation may not have a major clinical effect. However, IL-8 is the prototypical neutrophil activating chemokine and many of the other major chemokines share receptors with IL-8. Since its discovery, IL-8 has been the best studied chemokine in acute inflammation, and is known to be expressed early in the lungs of preterm infants before an influx of neutrophils (Munshi et al., 1997).

Longer isoforms of IL-8 ($IL\text{-}8_{77}$) are functionally less potent compared to shorter isoforms ($IL\text{-}8_{72}$) in terms of neutrophil chemotaxis and degranulation. Although the

shorter isoform is the better studied molecule in the context of inflammation, IL-8_{77} expression is best characterised in non-leukocytic cells. IL-8_{77} is also the most abundant isoform present in the preterm circulation (up to 60%), in contrast to term infants and adults (Maheshwari et al., 2009), raising the question of whether this is also the dominant isoform in the preterm lungs. My hypothesis for this part of the project was that persistent influx of neutrophils into the lungs of preterm infants suggests shorter, and functionally more potent, isoforms of IL-8 are the dominant molecules.

IL-8 is the link between the two parts of my thesis and research work. In my earlier work, I have already shown that total IL-8 is present at a significantly higher concentration in the lung fluid of preterm ventilated infants who develop CLD, compared to preterm infants who do not. In this part of my work, I have looked at the modulation of the effects of IL-8 by IL-6 *trans*-signalling, as IL-8 has been shown to be down-regulated by the pro-resolution effects of IL-6 *trans*-signalling. In the second part of my work, I intended to address modulation of IL-8 activity due to expression of its functionally different isoforms.

My data and analysis shows that as hypothesised, IL-8_{77} is a minor proportion of total IL-8 in broncho-alveolar lavage fluid (BALF) from preterm ventilated infants. Although all the major cell populations could experimentally express significant quantities of IL-8_{77} *in vitro*, this isoform was a minor proportion in lung fluid seemingly due to post-translational modification by neutrophil serine proteases and thrombin in preterm BALF, which retained significant enzymatic activity to cleave IL-8_{77} to shorter isoforms. Conversion by proteinase-3 (Pr-3) resulted in a functionally more potent isoform, making Pr-3 an attractive target for therapeutic intervention. Thrombin had a similar effect on IL-8_{77} as Pr-3, also making it a potential target in the preterm lung.

Strategies to inhibit activity of serine proteases in the preterm infant lung has the potential to modulate inflammation by preventing post-translational modification of IL-8_{77} and attenuating influx of neutrophils into the lungs. It is also thought that inhibition of proteases could attenuate direct lung injury caused by these proteases. Although laboratory studies have established this association between an imbalance in the protease-antiprotease concentration and the development of CLD (Davies et al., 2010) with beneficial results in experimental CLD (Hilgendorff et al., 2011), a meta-

analysis of clinical trials using AAT in preterm infants at risk of CLD was unable to demonstrate any significant benefit (Shah and Ohlsson, 2001).

My study has identified two new modulation mechanisms of IL-8 in the preterm lungs: proteinase-3 (PR-3) and thrombin. While elastase and metallo-proteases have been previously studied in preterm infants in the context of CLD, this is the first time Pr-3 has been measured in the preterm lung fluid and a potential link established with neutrophil-driven inflammation. Clear links have been found between enzymes of the coagulation cascade, including thrombin, and inflammation (Lipinski et al., 2011). Although clinical evidence from adult trials is in is still at an early stage (Lipinski et al., 2011, Wang et al., 2013b), this is clearly an under-researched area in the field of inflammation. Although anticoagulation may not be a valid option in preterm infants at risk of serious adverse effects (intra-ventricular and pulmonary haemorrhage), modified forms of antithrombin, which lacks anticoagulant properties but retains anti-inflammatory properties (Wang et al., 2013b), may offer new therapeutic opportunities.

6.4. Limitations and unresolved questions

My study originates as observations in samples collected from human infants, which leads to *in vitro* experiments. Correlation of my conclusions *in vivo* in preterm infants is difficult and appropriate animal models need to be used for this purpose. However, the fact remains that a model for current CLD, where infants with lungs in the saccular stage of development are born through chorioamnionitis after receiving a course of antenatal steroids, receive exogenous surfactant replacement, are ventilated for a variable period of time in variable concentration of oxygen, and go on to develop CLD at the time-point where alveolarisation should start, does not exist. Importantly, I have studied two specific pathways to modulate neutrophil influx in the preterm lung. Inflammation at any site is characterised by redundancy, with several different pathways converging to produce similar effects. Thus, experimental findings described here are unlikely to reflect the full clinical picture. Nevertheless, by undertaking

detailed functional studies such as this, we can begin to unpick the precise molecular events taking place in the preterm lung and leading to CLD.

To look at expression of IL-8 from cells, I have used adult cell-lines and term newborn cells. Although preterm cells are known to be transcriptionally active, we do not know whether they would express similar proportions of IL-8[77]. Lack of preterm lung cell-lines is a limitation of my study. Choosing an appropriate animal model for IL-8 work is not straightforward. Common murine models are inappropriate as they do not express IL-8. KC (mice) and CINC-1 (rats) are considered to be functional homologues of human IL-8; however, it is not known whether isoforms of these molecules exist. The closest animal model of CLD is the preterm baboon model. Again, isoforms of IL-8 have not been studied in this model.

For IL-6 *trans*-signalling, I have used a modified functional assay using the mouse B9 cells. Although lower sensitivity of IL-6 *trans*-signalling in functional assays is well known, I have had concerns whether I have used sufficient cytokine concentrations to form a stable sIL-6R/IL-6 complex. My approach has been to use an antibody for the membrane IL-6R to inhibit classical IL-6 signalling. Because of the sensitivity of these cells to IL-6, this approach only worked in the presence of a low concentration of IL-6 (< 10 pg/ml). Breakthrough signalling was observed at concentrations above this, even in the presence of the 2B10 antibody. Thus, the exquisite sensitivity of these cells to IL-6, which makes it an ideal model for use with clinical samples, may have proved to be the limiting factor in measuring IL-6 *trans*-signalling. Whether this reflects a physiologically relevant difference in the relative sensitivity of cells to *cis*- and *trans*-signalling event remains to be seen, however further work in this area would be revealing and important for therapies targeting these distinct pathways. Strategies to completely abolish classical signalling in this cell-line by appropriate modifications may prove to be useful in the future.

In the simplified experimental conditions of the laboratory, associations between a single risk-factor and a disease could become established. For the same reason, monotherapies may prove to be beneficial, when tested in the experimental models. A complex disease like CLD results from a combination of several risk factors. As is well established, nature has built-in several redundancies in the inflammatory

cascade, the factor which is strongly implicated in the development of CLD. Thus, in actual clinical situations monotherapies, especially those directed towards countering inflammation, are unlikely to demonstrate similar efficacy as under experimental conditions. Advances in clinical medicine depend on detailed research, which begins in the laboratory, progresses in animal models (which are more complex than *in vitro* conditions, but are still controlled situations) and then trialled on human patients. Many therapies, which initially show promise in the laboratory, fail on the last hurdle. However, most research advances our knowledge and understanding of diseases, and hopefully will lead to more directed interventions.

6.5. Scientific uncertainties surrounding CLD/BPD

Due to the prevalence of this condition in ex-preterm infants, CLD is commonly used in research studies as a marker of respiratory outcome. However, clinicians and researchers are still unable to fully agree on the definition of the term. In the past, CLD/BPD has been defined as a requirement for respiratory support at 28 days of life after preterm birth (Bancalari et al., 1979). This definition was changed in 1988 after a follow-up study by Shennan and colleagues who showed that the long-term respiratory outcomes of ex-preterm infants was best correlated with the need for respiratory support at 36 weeks of corrected gestational age (Shennan et al., 1988). These two clinical definitions of CLD/BPD remained in common use in the literature for a number of years; however, they were inconsistently applied by researchers in outcome studies. Predictably, comparison between studies were complicated by the lack of a consistent definition. Moreover, two major advances in neonatology – use of antenatal corticosteroids and exogenous surfactant replacement – became more common in the early to mid 1990s, leading to a shift in the natural history of the disease. Increased numbers of extremely preterm infants have survived since the routine use of these interventions (Roberts and Dalziel, 2006, Soll, 2000, Soll and Ozek, 2010), thus increasing the population of infants who are at highest risk of CLD. It was also noted that some preterm infants seem to need respiratory support, even if they have not been mechanically ventilated (Jobe, 1999). This led to a workshop to rethink the

definition of CLD and a new definition, stratified to gestation at birth and graded for severity, was proposed (Jobe and Bancalari, 2001a). A further refinement, using a room air saturation test to determine requirement for supplemental oxygen (Walsh et al., 2003), is also gaining popularity among researchers and epidemiologists. However, the definition of CLD remains a work in progress and universal acceptance is yet to be reached.

As the name implies, CLD describes a clinical condition in ex-preterm infants. Although early studies have extensively described the pathology of BPD in preterm infants (Northway et al., 1967, Bonikos et al., 1976), the advent of antenatal corticosteroids and exogenous surfactant replacement have resulted in a change in pattern of the disease (Jobe, 1999). As mortality from BPD/CLD is rare in the current population of preterm infants (Jobe, 1999), only limited information is available on lung pathology from them. One study has looked at a small number of lung specimens from human infants with CLD, who received exogenous surfactant replacement at birth (Husain et al., 1998); we have also gained useful information from a primate model of BPD (Coalson et al., 1999b, Coalson, 2003, Coalson, 2006). Due to the low rates of mortality from this condition, these studies probably describe changes from the severe end of the spectrum. Thus, for the majority of infants with CLD/BPD, the pathological changes in the lung are yet to be characterised.

Apart from extreme prematurity and low birth weight, several other risk factors have been implicated to contribute towards the development of CLD. Starting from antenatal infection, mechanical ventilation, postnatal infection, use of oxygen and patent ductus arteriosus are some of the common risk factors which have been extensively studied in the literature, although none of them have been established as having a cause-and-effect relationship with CLD. Indeed, contradictory results regarding contribution of each of these risk factors towards the development of CLD have been reported by researchers. However, lung inflammation has been proposed as the final common pathway involved in lung injury in preterm infants leading to the development of CLD (Speer, 2006, Ryan et al., 2008, Chakraborty et al., 2010).

Lung inflammation involving the innate immune system has been extensively studied in preterm infants developing CLD/BPD (Ryan et al., 2008). Pathways of

inflammation in the preterm lung, as in other inflammatory diseases, is characterised by redundancy. Thus, activation of several different pathways of the innate immune system including cytokines, chemokines and proteases has been reported in the preterm lung. As with the risk factors mentioned previously, consistent associations of inflammatory mediators in the preterm lung with CLD are lacking, with IL-8 being a notable exception. Moreover, the mechanism of how lung inflammation leads to pathological changes (a temporary growth arrest in the alveolar development) is not yet clear. While the majority of research in inflammation has been observational, there is limited evidence in animal models of BPD regarding the efficacy of interventions in countering lung inflammation to prevent CLD (Auten et al., 2001, Hilgendorff et al., 2011). This is in sharp contrast to intervention studies in human infants, none of which are yet to demonstrate any clear efficacy (Chakraborty et al., 2010).

As discussed previously, lung inflammation has been strongly implicated as a final common pathway leading to the development of CLD. Evidence for this process has been accumulated from a large number of studies looking at many different pathways of inflammation. Most of these studies have taken a reductionist approach to a complex disease, and implicated candidate molecules to be involved in lung injury. While it is true that these molecules contribute to the process, due to the redundancy in inflammatory pathways, it is unlikely that targeting single molecules or pathways in human preterm infants would result in significant clinical benefit. This is borne out by the lack of any effective therapy for the treatment of CLD (Chakraborty et al., 2010). Interestingly, introduction of a package of interventions as a quality improvement programme may result in decreasing incidence of CLD, in keeping with its multi factorial pathogenesis (Pfister and Goldsmith, 2010). Prematurity being the primary risk factor for CLD, strategies to reduce or prevent preterm delivery would be the most definitive way to reduce CLD. So far, only one therapeutic intervention, progesterone replacement, has shown some benefit in reducing preterm delivery (Iams, 2014b, Iams, 2014a).

6.6. Key points

Key findings of my research:

- Significant elevation of sgp130 concentration in BALF from preterm ventilated infants developing CLD later.
- Possible lack of IL-6 *trans*-signalling activity in the preterm lungs.
- Proteinase-3 and thrombin are key regulators of IL-8_{77} function in the preterm lungs.

Key areas of further research

- Further modifications to B9 cells for measuring specific IL-6 *trans*-signalling. This could have application in several other diseases where IL-6 *trans*-signalling has been implicated in the pathogenesis.
- Research into other members of the IL-6 family in the preterm lungs.
- *In vivo* experiments involving IL-6 *trans*-signalling (both augmenting and inhibiting) in animal models to clarify its role.
- Clinical trials of inhibition of proteinase-3 and thrombin in the preterm lungs.
- Links between inflammation and interruption of lung growth in preterm infants.

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8. Appendices

8.1. Ethical Approvals

8.1.1. Ethical Approval for Collection of BALF from Preterm Infants

Canolfan Gwasanaethau Busnes
Business Services Centre

South East Wales Research Ethics Committee - Panel D
Direct Line: 02920402420

19 April 2005

Prof Sailesh Kotecha
Professor of Child Health
Universtiy of Cardiff
Department of Child Health
Heath Park
Cardiff
CF14 4XN

Dear Prof Kotecha

Full title of study: ***Ex-vivo study to determine the efficacy of recombinant alpha 1-antitrypsin to redress proteolytic activity in lung lavage fluid obtained from preterm infants***
REC reference number: ***05/WSE04/29***

Thank you for your letter of 14 April 2005, responding to the South East Wales Research Ethics Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chairman, Dr. DEB Powell.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Canolfan Gwasanaethau Busnes
Ty Churchill
17 Ffordd Churchill
Caerdydd, CF10 2TW
Ffôn: 029 20 402402 WHTN: 1809
Ffacs: 029 20 402403
DX 121720, Caerdydd 9

Business Services Centre
Churchill House
17 Churchill Way
Cardiff, CF10 2TW
Telephone: 029 20 402402 WHTN: 1809
Fax: 029 20 402403
DX 121720, Cardiff 9

rhan o Bwrdd Iechyd Lleol Powys part of Powys Local Health Board

Document Type:	Version:	Dated:	Date Received:
Application	Parts A, B and C	11/03/2005	15/03/2005
Investigator CV	Professor Sailesh Kotecha	14/04/2005	15/03/2005
Investigator CV	Philip Lloyd Davies	14/04/2005	15/03/2005
Protocol	2	14/04/2005	15/03/2005
Peer Review	Letter from Professor Scanlon	10/03/2005	15/03/2005
Participant Information Sheet	2 - Parents/Guardians	14/04/2005	15/03/2005
Participant Consent Form	2	14/04/2005	15/03/2005
Response to Request for Further Information	Letter from Professor S Kotecha	14/04/2005	15/04/2005
Other	Letter from Arriva Pharmaceuticals Inc	03/03/2005	15/04/2005
Other	Email from Cardiff and Vale Trust - Re: Indemnity	14/04/2005	15/04/2005

Management approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Notification of other bodies

The Committee Administrator will notify the R&D Department for NHS care organisation(s) that the study has a favourable ethical opinion.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/WSE04/29	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project,

Yours sincerely

Dr D E B Powell
Chairman, Panel D
South East Wales Research Ethics Committee

8.1.2. Ethical Approval for Collection of Cord Blood from Term Infants

Canolfan Gwasanaethau Busnes
Business Services Centre

South East Wales Research Ethics Committee Panel B

Telephone: 02920 376823
Facsimile: 02920 376835
Email: Carl.phillips@bsc.wales.nhs.uk

Professor Sailesh Kotecha
Professor of Child Health
Department of Child Health
Cardiff University
Heath Park, Cardiff
CF14 4XN

3 December 2007

Dear Professor Kotecha

Full title of study:	**Developmental Regulation of Neonatal Neutrophils and Monocytes Function.**
REC reference number:	**07/WSE02/101**

Thank you for your letter of 19 November 2007, responding to the Committee's request for further information on the above research, and submitting for revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised].

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document.

You are advised to study the conditions carefully.

Canolfan Gwasanaethau Busnes
Ty Churchill
17 Ffordd Churchill
Caerdydd, CF10 2TW
Ffôn: 029 20 376820 WHTN: 1809
Ffacs: 029 20 376826

Business Services Centre
Churchill House
17 Churchill Way
Cardiff, CF10 2TW
Telephone: 029 20 376820 WHTN: 1809
Fax: 029 20 376826

rhan o Addysgu Bwrdd Iechyd Lleol Powys / part of Powys Teaching Local Health Board

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	5.5	03 October 2007
Investigator CV	O B Spiller	01 October 2007
Investigator CV	E P McGreal	01 October 2007
Investigator CV	N C Maxwell	01 October 2007
Investigator CV	L Bridge	08 November 2007
Investigator CV		08 November 2007
Investigator CV	S Kotecha	24 September 2007
Protocol	2.0	24 September 2007
Letter from Sponsor	Cardiff University	14 August 2007
Peer Review	Joint Trust/University Risk Review Committee	31 August 2007
Compensation Arrangements	UMAL	01 August 2007
Participant Information Sheet: Healthy Adult Control	3	08 November 2007
Participant Information Sheet: PreTerm Babies	3 - Parents/Guardians	08 November 2007
Participant Information Sheet: Term Babies	3 - Parents/Guardians	08 November 2007
Participant Information Sheet: Term Babies for BAL Study	3 - Parent/Guardians	08 November 2007
Participant Consent Form: Term Babies for BAL Study	3	08 November 2007
Participant Consent Form: Term Babies	3	08 November 2007
Participant Consent Form: Pre Term Babies	3	08 November 2007
Participant Consent Form: Healthy Adult Controls	3	08 November 2007
Response to Request for Further Information		19 November 2007
Future Use - Babies	1	28 November 2007
Future Use - Healthy Adults	1	28 November 2007

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

Here you will find links to the following

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service on the application procedure. If you wish to make your views known please use the feedback form available on the website.
b) Progress Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
c) Safety Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
d) Amendments. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
e) End of Study/Project. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nationalres.org.uk .

07/WSE02/101	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Carl Phillips
Executive Officer
South East Wales Research Ethics Committees

Enclosures: Standard approval conditions, SL-AC2
Site approval form

Copy to: R&D office for Cardiff University

R&D office for Cardiff & Vale NHS Trust

8.2. Parent Information Leaflets and Consent Forms

8.2.1. BALF Collection from Preterm Infants

INFORMATION SHEET FOR PARENTS/GUARDIANS Ver 2.0

Principle Investigators: Professor Sailesh Kotecha, Consultant Neonatologist

Contact Details: Neonatal Unit, 029 20 74 3374

1. **Study Title**

Ex-vivo study to determine the efficacy of recombinant alpha 1-antitrypsin to redress proteolytic activity in lung lavage fluid obtained from preterm infants

2. **Invitation**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this leaflet.

3. What is the purpose of the study?

Chronic Lung Disease of prematurity (CLD) which is also often called BPD (for bronchopulmonary dysplasia) is a common disease of premature babies. Babies with CLD require oxygen for prolonged periods and are sometimes discharged home on oxygen. Our research is trying to understand why some babies develop CLD. Our work has shown that babies who develop CLD have inflammation (redness and soreness) in their lungs. A cell type called neutrophil is responsible for the inflammation and these cells produce proteins called elastase which “dissolve” the lung tissues. Elastase is usually prevented from causing too much destruction in the lung tissue by another protein called alpha 1-antitrypsin or AAT. In babies who develop CLD, the balance between elastase and AAT is such that there is insufficient AAT thus resulting in elastase causing lung damage which may be one reason why babies develop CLD.

Arrive Pharmaceuticals in the United States of America have developed AAT (called recombinant AAT or rAAT) in their laboratories. Before moving to a clinical study to examine if rAAT can be used to treat premature babies to prevent CLD, we wish to determine if rAAT can neutralise the excess elastase activity in lung fluid obtained from babies who need help with their breathing with mechanical ventilators.

4. Why has my baby been chosen?

We wish to obtain lung lavage fluid samples from two groups of babies:

(a) babies who are born at or less than 32 weeks gestation and who need help with their breathing with breathing machines because of under-developed lungs, or
(b) babies who need help with their breathing with breathing machines because of non-breathing problems e.g. surgery.

As your baby <u>may</u> fall into one of these two groups, we would like to invite you to take part in our study.

5. Does my baby have to take part?

It is up to you to decide whether or not you want your baby to take part. If you do decide that your baby can take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide for your baby to take part, you are still free to withdraw your baby at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care that your baby receives.

6. What is the drug being tested?

The drug is called recombinant alpha 1-antitrypsin or rAAT is developed by Arriva Pharmaceuticals. Our ultimate aim is to determine if this drug can decrease the rate of CLD in premature babies. Before we proceed to a clinical trial, we want to determine in the laboratory if rAAT can "neutralise" the elastase in lung fluid obtained from babies at risk of developing CLD.

7. What will happen if my baby takes part?

We would like to obtain lung fluid from your baby whilst the baby remains on a breathing machine. The fluid will be obtained daily for the first week and twice weekly thereafter or until your baby is removed from the breathing machine, whichever occurs first. The breathing tube is often sucked out by the nurses to prevent it from blocking. We would replace this suctioning wherever possible so it does not need to be performed twice. In order to compare the results with other baby's results, we have standardised this method of suctioning: we place the baby on his/her back and turn the head to the left side to encourage the suction tube to go down the right lung. We will then gently place a suction tube through the breathing tube into the lungs and through the tube insert saline (salt water). The amount of saline is based on the baby's weight using 1 ml for each kilogram of the baby's weight (one teaspoon is 5 ml). After instilling the saline we will suck up as much fluid as possible and repeat the procedure once more. The returned fluid will consist of the saline and will also have the baby's lung fluid which we can use for our research.

We will monitor the baby's heart rate and oxygen saturation during the procedure and sometime may need a little more oxygen (usually 5 – 10%) for a short period of time.

8. What will happen to the samples collected?

We will use the fluid obtained from your babies to determine the amount of elastase activity and add rAAT to the fluid in the laboratory to determine if we can neutralise the elastase activity. The optimum dose of rAAT needed to neutralise the elastase activity will also be determined. If any fluid remains then we shall use this to measure the total amount of elastase and AAT as well as agents which promote or inhibit inflammation (named as IL-1, IL-6, TNF, IL-4, IL-10). We will analyse any cells obtained in the fluid sample for the presence of infection as infection has been shown to increase lung inflammation.

9. What are the risks of my baby taking part?

Babies who receive mechanical ventilation are monitored closely for their heart rate and oxygen levels. The risks are similar to those of routine suctioning that the baby may have. Sometimes the babies may need extra oxygen, typically 5 – 10%, for 5 – 10 minutes and sometimes especially when the suction tube is placed the heart rate may drop for a few seconds (usually less than 30 seconds). We would monitor the baby throughout the procedure and stop it if the baby becomes unwell in any way.

10. What if something goes wrong?

If your baby is harmed by taking part in this research project, there are no special compensation arrangements. If your baby is harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you or your baby have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

11. Will my baby's taking part be kept confidential?

All information which is collected about your baby during the course of the research will be kept strictly confidential. Any information about your baby which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it. The company sponsoring this study will only have access to data from which your baby's name, address and date of birth are removed. We will assign a number to each baby and use this to label the samples obtained for the study.

12. What will happen to the results of the study?

We will publish the results in reputable medical journals and will write a report on the study for the sponsors of the study. We will also present the data at scientific and medical meetings. Your baby's name and details will NOT be revealed at any stage. Please let us know if you would like a copy of the report.

13. Who is paying for the study?

The study is sponsored by Arriva Pharmaceuticals from California in the United States of America.

14. Who had reviewed the study?

This study has been reviewed by the South East Wales Research Ethics Committee and also by an expert group convened by Arriva Pharmaceuticals.

15. Who can I contact for further information?

You may contact Dr Phil Davies or Professor Sailesh Kotecha by asking one of the staff on the neonatal unit or by telephoning 029 20 74 3374 or by email (DavieP@Doctors.org.uk or KotechaS@Cardiff.ac.uk) or by mail to:

Professor Sailesh Kotecha, Neonatal Unit, Heath Hospital, Heath Park, Cardiff CF14 4XN.

Thank you for taking time to read this information leaflet at such a difficult time. Please do not hesitate to ask Dr Phil Davies or Professor Sailesh Kotecha if you would like to discuss anything further.

Dr Philip Davies	**Professor Sailesh Kotecha**
Clinical Research Fellow	**Consultant in Neonatal Medicine**

(***Form to be on headed paper)***

Patient Identification Number for this trial:

CONSENT FORM

Ex-vivo study to determine the efficacy of recombinant alpha 1 - antitrypsin to redress proteolytic activity in lung lavage fluid obtained from preterm infants

Name of Researcher: **Professor Sailesh Kotecha, Consultant Neonatologist,**
Neonatal Unit, Heath Hospital, Cardiff CF14 4XN

Please initial box

1.	I confirm that I have read and understand the information sheet dated .15th January 2005 (version 1.0) for the above study and have had the opportunity to ask questions.	☐
2.	I understand that my baby's participation is voluntary and that I am free to withdraw my baby at any time, without giving any reason, without my baby's medical care or legal rights being affected.	☐
3.	I understand that sections of any of my baby's medical notes may be looked at by The research individuals I give permission for these individuals to have access to my baby's records.	☐
4.	I agree for my baby to take part in the above study.	☐

_________________________	________________	____________________
Name of Parent/Guardian	Date	Signature
_________________________	________________	____________________
Name of Person taking consent (if different from researcher)	Date	Signature
_________________________	________________	____________________
Researcher	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes.

8.2.2. Cord Blood Collection from Term Infants

INFORMATION SHEET FOR PARENTS/GUARDIANS Version 4 (17th February 2010) –

TERM BABIES

Principle Investigators: Professor Sailesh Kotecha, Consultant Neonatologist

Contact Details: Neonatal Unit, 029 20 74 3374

Study Title

Developmental regulation of neonatal neutrophils and monocytes function.

Invitation

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1 of this leaflet tells you the purpose of this study and what will happen if you decide to take part. Part 2 gives you more detailed information about the study.

Thank you for reading this leaflet.

Part 1

1.1. **What is the purpose of the study?**

We are trying to understand why some premature babies develop the disease called chronic lung disease of prematurity or CLD. Lung inflammation (redness and soreness) is common in babies who develop CLD. However, some of our work has shown that an under-developed immune system may be one reason why premature babies develop CLD. We wish to examine blood cells (which cause lung inflammation in babies who develop CLD) from babies born on time or prematurely to determine if their cell function is under-developed when compared to similar cells from healthy adults.

1.2. **Why have I and/or my baby been chosen?**

We wish to obtain samples from three groups:

a) Babies born on time who are being delivered by an elective caesarean section
b) Babies who are born prematurely (i.e. less than 32 weeks gestation)

c) Healthy adult donors

Because your baby is due to be delivered on time by caesarean section, we would like to invite you to join our study. After the baby and placenta (after-birth) are delivered, we would like to obtain blood from the placenta to use for our studies.

1.3. **Does my baby have to take part?**

It is up to you to decide whether or not you want your baby to take part. If you do decide that your baby can take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide for your baby to take part, you are still free to withdraw your baby at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care that your baby receives.

1.4. **What will happen if my baby takes part?**

We would like to obtain blood (usually between 5-20 mls) from the placenta after the baby is born and the after-birth (placenta) is delivered.

1.5. **Will this affect my or my baby's treatment?**

The medical care of you or your baby will not be affected by this study. The information from this study will not be used to diagnose or treat you or your baby.

1.6. **What are the risks of my baby taking part?**

The cord blood sample will be taken from the placenta after the umbilical cord has been cut and is not harmful or painful for you or your baby.

1.7. **What are the benefits of my baby taking part?**

We cannot promise that the study will help your baby but the information we get from this study may help us to improve the treatment of premature babies in the future.

1.8. **What if there is a problem?**

Any complaint about the way you or your baby have been dealt with during the study or any possible harm your baby might suffer will be addressed. The detailed information on this is given in Part 2.

1.9. ***Will my baby's taking part be kept confidential?***

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making a decision.

Part 2

2.1. **What will happen if I don't want my baby to carry on with the study?**

If you decide to withdraw from the study, we would like to use the data collected up to your withdrawal. Any stored blood or fluid samples that can be identified as your baby's will be destroyed if you wish.

2.2. **What if there is a problem?**

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action against Cardiff University but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should still be available to you.

2.3. **What will happen to the results of the study?**

We will publish the results in reputable medical journals and present the data at scientific and medical meetings. Your baby's name and details will NOT be revealed at any stage. Please let us know if you would like a copy of the report.

2.4. **What will happen to the samples collected?**

From the blood samples we will separate the blood cells and the fluid (plasma). We will determine if there are differences in the function of the blood cells, including their survival, production of products which promote inflammation or tissue injury and their ability to kill germs.

Sometimes we may have some of the blood sample left over after our initial tests. We would like to ask you if we may use any remaining sample for future studies. We would have to re-apply to the ethics committee to use the sample for any new studies. If you are happy for us to use the sample in the future, you will be asked to sign a separate consent form. If you do not agree to future use of the sample then we shall destroy any remaining sample at the end of this study.

2.5. ***Will my baby's taking part be kept confidential?***

All information which is collected about your baby during the course of the research will be kept strictly confidential. All data collected will be processed on a secure, password-protected computer at Cardiff University. Any information about your baby which leaves the hospital will have your and the baby's

name and address removed so that you cannot be recognised from it. We will assign a number to each baby and use this to label the samples obtained for the study.

2.6. ***Who is paying for the study?***

The studies are being funded by the Wellcome Trust and Arriva Pharmaceuticals.

2.7. **Who has reviewed the study?**

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your and your baby's safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by the South East Wales Research Ethics Committee.

2.8. **Who can I contact for further information?**

You may contact Professor Sailesh Kotecha by asking one of the staff on the delivery suite or by telephoning 029 20 74 4187 or by email (***KotechaS@Cardiff.ac.uk***) or by mail to:

Professor Sailesh Kotecha, Neonatal Unit, Heath Hospital, Heath Park, Cardiff CF14 4XN.

Thank you for taking time to read this information leaflet. Please do not hesitate to ask Professor Sailesh Kotecha or Dr Mallinath Chakraborty if you would like to discuss anything further.

Dr Mallinath Chakraborty

Clinical Research Fellow

Professor Sailesh Kotecha

Consultant in Neonatal Medicine

CONSENT FORM – TERM BABIES

Developmental regulation of neonatal neutrophils and monocytes function.

Name of Researcher: Professor Sailesh Kotecha, Consultant Neonatologist,
Neonatal Unit, Heath Hospital, Cardiff CF14 4XN

Please initial box

1. I confirm that I have read and understand the information sheet dated 17th February 2010 (version 4) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my baby's participation is voluntary and that I am free to withdraw my baby at any time, without giving any reason, without my baby's medical care or legal rights being affected. ☐

3. I consent to my baby's cord blood being used ☐

4. I understand that sections of any of my baby's medical notes may be looked at by the research individuals I give permission for these individuals to have access to my baby's records. ☐

________________________	________________	____________________
Name of Parent/Guardian	Date	Signature
________________________	________________	____________________
Name of Person taking consent (if different from researcher)	Date	Signature
________________________	________________	____________________
Researcher	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes.