# The role of IL-6 and its soluble receptor in Chronic lung disease of prematurity

# A thesis submitted in candidature for the degree of Doctor of Philosophy

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# **Dedication**

In loving memory of mum

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Finally, this is dedicated to my late mother who was a constant support throughout my studies and sadly passed away before I completed my PhD.

# Summary

Chronic lung disease (CLD) of premature infants is an inflammatory disease characterised by the accumulation of neutrophils that fail to undergo apoptosis. Hyperoxic mechanical ventilation, needed to support the function of underdeveloped lungs, and infection are key inflammatory protagonists in CLD. Airway epithelial cells may contribute to the sustained inflammation by secreting inflammatory mediators.

Interleukin-6 (IL-6) and its soluble IL-6 receptor (sIL-6R) are known to promote resolution of the neutrophil dominated phase of inflammation *in vivo*. The IL-6/sIL-6R complex binds gp130 to initiate 'trans-signalling', which has been shown to reduce the influx of neutrophils by downregulating IL-8, promoting neutrophil apoptosis and inducing mononuclear cells infiltration by upregulating MCP-1 expression.

It is not known if IL-6 trans-signalling is capable of promoting resolution of inflammation in the airways or in neonatal leukocytes. If this role does apply, dysfunction in premature infants with CLD could contribute to some characteristic features of the disease.

Here, IL-6R and gp130 expression was compared between adult and neonatal neutrophils. IL-6R expression levels were similar whereas gp130 expression was greatly upregulated on cord blood neutrophils. This may indicate a potential difference in the way neutrophils from term infants respond to IL-6 trans-signalling however, further investigations revealed that IL-6 trans-signalling does not regulate neutrophil apoptosis *in vitro*.

IL-6 trans-signalling was investigated in transformed airway epithelial cells (A549 and BEAS2B cells) and primary small airway epithelial cells (SAEC) *in vitro*. The cells expressed gp130 but not IL-6R. MCP-1 in response to IL-6 trans-signalling was variable and generally weak. Furthermore, IL-8 release was not downregulated by IL-6 trans-signalling. Instead, an increase in MCP-1 and IL-8 release was observed in response to a combined stimulation of IL-6 trans-signalling and IL-1β. This suggests that the pro-resolution paradigm of IL-6 trans-signalling may not be the case in airway epithelial cells.

### **Abbreviations**

ACK Ammonium chloride potassium

ANOVA Analysis of variance

AVBB Annexin V binding buffer

BAL Bronchoalveolar lavage

CCU Colour changing units

CFU Colony forming units

CLD Chronic lung disease

DMSO Dimethyl sulphoxide

EDTA Ethylaminediaminetetraacetic acid

ELISA Enzyme linked immunosorbent assay

ERK Extracellular signal regulated kinase

fMLP Formyl methionyl leucyl phenylalanine

FSC Forward scatter

HI FCS Heat inactivated foetal calf serum

HPMC Human peritoneal mesothelial cells

ICAM Intracellular adhesion molecule

IFNγ Interferon gamma

IgG Immunoglobulin

IL-6 Interleukin 6

IL-6R Interleukin-6 receptor

JAK Janus Kinase

LBP LPS binding protein

LPS Lipopolysaccharide

MCP Monocyte chemotactic protein

MAPK Mitogen activated protein kinase

MMP Metalloproteinase

NE Neutrophil elastase

NF-κB Nuclear factor κB

PAMP Pathogen associated molecular pattern

PBMC Peripheral blood mononuclear cells

PBS Phosphate buffered saline

PI3k Phosphoinositide 3-kinase

PE Phycoerthrin

PFA Paraformaldehyde

PMN Polymononuclear

PRR Pattern recognition receptors

PS Phosphatidylserine

RDS Respiratory distress syndrome

RPMI Roswell Park Memorial Institute

RT-PCR Reverse transcriptase-polymerase chain reaction

SAEC Small airway epithelial cells

sCD14 Soluble CD14

sIL-6R Soluble Interleukin 6 receptor

SSC Side scatter

TAF Tracheobronchial aspirate fluid

TLRs Toll-like receptors

TNFα Tumor necrosis factor alpha

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Chapter 1

Introduction

### 1. Introduction

### 1.1 The lower respiratory system

The respiratory system consists of the upper and lower respiratory tract. The nasal and oral cavities, the pharynx, larynx and trachea make up the upper respiratory tract. The lower respiratory tract consists of the lower part of the trachea, two main bronchi and the lungs. Within the lung, the main bronchi divide into smaller bronchiole branches and the acinus (Davies and Moores 2003; McGowan, Jeffries et al. 2003).

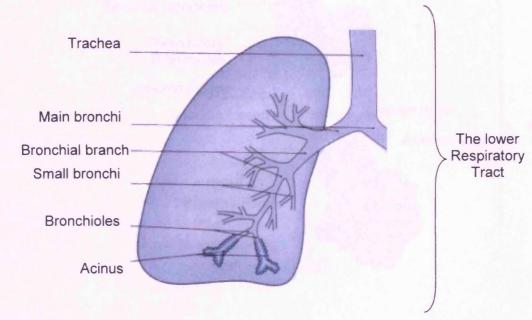
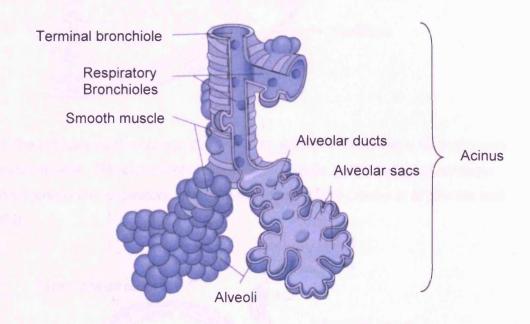


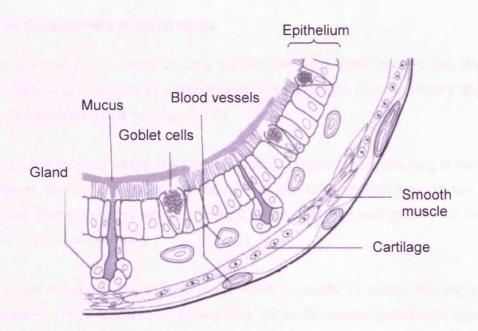
Figure 1.1: A schematic diagram of the lower respiratory tract. Adapted from McGowan et al (McGowan, Jeffries et al. 2003).

The acinus consists of the respiratory bronchioles and the alveolar ducts, which leads to the alveolar sacs. The alveolar sacs contain the alveoli (figure 1.2). Each normal lung has 150-400 million alveoli. As the respiratory tract descends, the lining and structure differs between each section. The bronchi are lined with respiratory epithelial cells (figure 1.3), which develop into ciliated cuboidal epithelium in the bronchioles. The respiratory bronchioles are surrounded by smooth muscle and contain some alveoli within the walls. The alveoli are lined with a specific type of epithelia. The surface lining of the alveoli is covered with 90-95% type I pneumocytes and 5-10% type II pneumocytes (figure 1.4). Type I cells are structurally thin and contain flattened nuclei. Type II cells are rounded in shape, contain rounded nuclei and produce surfactant. The acinus is responsible for the gaseous exchange, most of which occurs in the alveoli. A basement membrane separates type I pneumocytes and the capillary endothelium, which allows the gaseous diffusion to occur (figure 1.4). The close proximity of blood vessels allows white blood cells to enter the

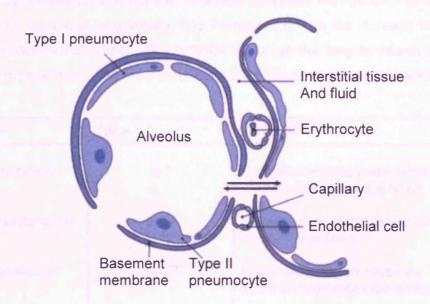
alveoli. Monocytes, macrophages and neutrophils leave the pulmonary circulation to enter the alveolar surface lining (Davies and Moores 2003; McGowan, Jeffries et al. 2003). These white blood cells help maintain a healthy respiratory tract by removing foreign material and pathogens. They form the major innate immune component of this system, which will be discussed later on in this chapter.



**Figure 1.2:** A schematic diagram of the acinus. The bronchi branch descends into the respiratory bronchioles and the alveolar ducts. Within the ducts are the alveolar sacs, containing numerous alveoli. Adapted from McGowan et al (McGowan, Jeffries et al. 2003).



**Figure 1.3:** The structure of the bronchi illustrates the epithelial lining and a discontinuous layer of smooth muscle. The bronchi contain mucosal glands, goblet cells and cartilage, which are not found in the respiratory bronchioles. Adapted from Davies et al (Davies and Moores 2003).



**Figure 1.4:** Schematic diagram of the alveolus. Type I and type II pneumocytes line the alveolar wall and are surrounded by the basement membrane. Adjacent alveoli communicate through perforations known as pores of Kohn. Endothelial cells fuse to the type I pneumocytes to aid gaseous diffusion. Adapted from McGowan et al (McGowan, Jeffries et al. 2003)

### 1.2 The development of foetal lungs

There are four main stages of lung growth that have been divided into the embryonic, pseudoglandular, canalicular, saccular and alveolar stages (brief summary given in table 1 and more detailed diagram in figure 1.5).

During the embryonic stage, the lung bud and the epithelium of the lung is formed from the endoderm. By 26-28 days of gestational age, the left and right bronchi are formed. The structural elements of the airways develop within six weeks and continue to develop during weeks 10 to 14 of gestation.

By the end of the pseudoglandular stage which is usually 17 weeks, the pre-acinar airways are present. They increase in size during 16 to 26 weeks gestational age. This is the canalicular stage in which the peripheral airways further develop in size and structure. The saccules of the alveolar ducts begin to form. Type I and II alveolar epithelial cells can be identified as they begin to line the saccular air spaces by 20 to 22 weeks of gestation.

At the alveolar stage from 28 to 32 weeks gestational age, the primitive alveoli are formed and mature by 34 weeks. The number of alveoli increases with gestational age. By term (38-42 weeks), the foetal lung volume has increased, due to the increase in the surface area and the number of alveoli. The alveolar region of the lung in infants born at term continues to grow and develop rapidly (Kotecha 2000; Greenough 2003; Kajekar 2007).

Stages	Time	Development
Embryonic	0-7	Formation of lung buds. Blood vessels connect to the heart.
Pseudoglandular	6-17	Development of pre-acinar airways and blood vessels.
Canalicular	16-27	Respiratory region develops. Type I and II pneumocytes identified.
Alveolar	27 to term	Saccules and alveoli develop.
Postnatal	Up to 18 months	Alveoli and blood vessels multiply and increase in size.

**Table 1**: The stages of lung development (Kotecha 2000; Greenough 2003).

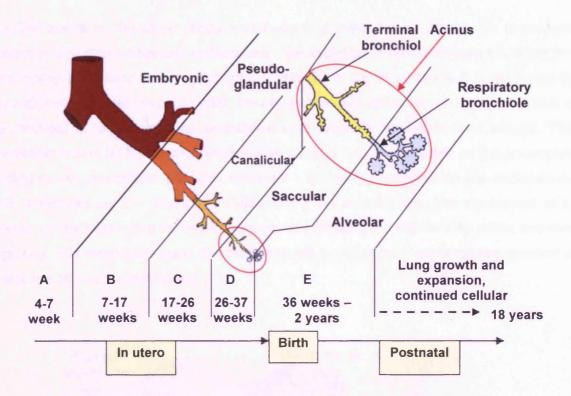
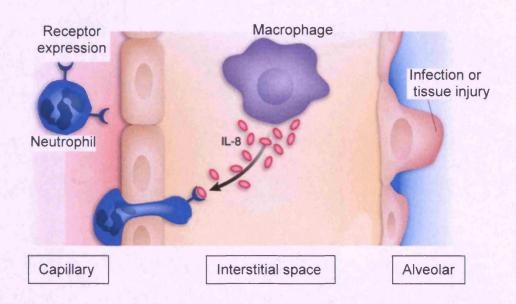


Figure 1.5: The main stages of lung development. A: Embryonic stage; lung bud differentiation, development of trachea, bronchi, pulmonary vein and artery. B: Pseudoglandular stage; development of terminal bronchioles, airways, pre-acinar vessels and immature neutral networks. C: Canalicular stage; primitive alveoli start to develop, surfactant synthesis, type I and II cells develop. D; Saccular stage, neural network mature, alveoli saccules develop. E: Expansion of gaseous exchange area, nerves and capillaries. Adapted from Kajekar (Kajekar 2007).

## 1.3 The innate immune system

Throughout the body and including the respiratory system, the innate immune response provides an immediate defence against pathogens. Leukocytes provide the first line of defence against invading bacteria, fungi and protozoa. They immediately trigger an inflammatory response and initiate the process of inflammation. Invading pathogens express receptors that are recognised by macrophages. The binding of macrophages and pathogens trigger phagocytosis, in which the macrophages engulf and digest the pathogen. Inflammatory mediators such as cytokines and chemokines are released from macrophages to attract other leukocytes to the infected or injured tissue. Tissue macrophages release interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNFα), interleukin-8 (IL-8), interleukin-12 (IL-12) and interleukin-18 (IL-18).

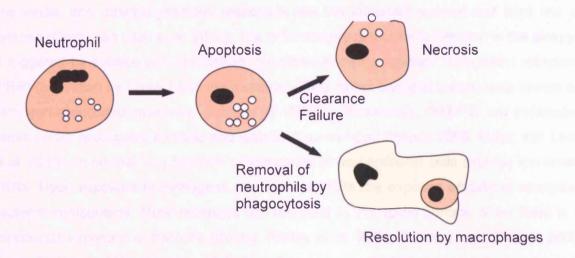
The TNFα acts on the blood vessel endothelium to make it more permeable to promote leukocyte migration across the endothelium. The secretion of chemoattractant IL-8 triggers a multi-step process to attract neutrophils to the site of infection. The IL-8 is presented by the endothelial cells to the neutrophils. This slows the neutrophils and promotes adhesion of the neutrophils to the vascular endothelial cells adjacent to the infected tissues. This interaction occurs by B₂-integrins (also known as CD11b/CD18) present on the neutrophils binding to the intracellular adhesion molecule-1 (ICAM-1) expressed on the endothelium. This is followed by the neutrophils rolling along the endothelium. The expression of L-selectin on the neutrophils becomes reduced, thus allowing the cells to stop rolling and start migrating. The neutrophils leave the blood vessels by following a concentration gradient of IL-8 to the source of the infection.



**Figure 1.6**: Simplified diagram to illustrate migrating neutrophils follow the increasing IL-8 gradient to the infected tissue. This is an example of an inflammatory response in the alveoli. Macrophages release chemokine IL-8 to attract neutrophils from the capillary. Adapted from Weathington et al (Weathington, van Houwelingen et al. 2006).

The recruitment of neutrophils to the infected tissues can occur within minutes to hours. Through recognition and binding of microorganisms, the neutrophils engulf and remove invading pathogens by phagocytosis. Neutrophils contain anti-microbial molecules, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide anion (O<sub>2</sub>). These are released to eliminate the microorganism (Brach, deVos et al. 1992; Cox 1996; Ward and Lentsch 1999; Janeway 2001; Faurschou and Borregaard 2003). Neutrophils also have azurophil granules within their cytoplasm that contain serine proteases. These proteases include neutrophil elastase (NE), cathepsin G and proteinase G (PR3) (Faurschou and Borregaard 2003; Chmura, Bai et al. 2008) and are released intracellularly during phagocytosis to aid the degradation of pathogens (Taggart, Greene et al. 2005).

Following the clearance of microorganisms, neutrophils should undergo a programmed cell death known as apoptosis. Apoptosis is an active process that is characterised by cell shrinkage, nuclear condensation, plasma membrane blebbing and activation of caspase-3 (Barazzone, Horowitz et al. 1998; Samali, Zhivotovsky et al. 1999). Cells dying by this process lose the ability to phagocytose and are not able to empty their granule contents. The mechanism in switching off this neutrophil function during apoptosis is not fully understood (Whyte, Meagher et al. 1993). Apoptosis is an essential process that is regulated by cytokines and other factors to ensure neutrophils are efficiently removed. Without this apoptotic process, dying neutrophils can release their toxic contents, potentially damaging the surrounding tissues. This is known as necrosis (Savill and Fadok 2000; Eyles, Roberts et al. 2006); (Liles and Klebanoff 1995). Although neutrophils have a short lifespan, various studies have shown that during inflammation, neutrophil life-span can be prolonged. Neutrophil survival can be promoted by pro-inflammatory signals, such as a bacterial cell wall component, lipopolysaccharide (LPS) as well as pro-inflammatory cytokines, such as granulocyte-macrophage colony stimulating factor (GM-CSF). granulocyte colony-stimulating factor (G-CSF) and IL-8 (Saba, Soong et al. 2002; Akgul and Edwards 2003) (Brach, deVos et al. 1992; Lee, Whyte et al. 1993). However, there are other cytokines such as interleukin-10 (IL-10) that have anti-inflammatory properties and can induce neutrophil apoptosis to promote the resolution of inflammation (Cox 1996). Apoptotic neutrophils are recognised by macrophages and are removed by phagocytosis to prevent tissue damage. If this clearance mechanism fails and cells are not removed, apoptotic neutrophils will undergo necrosis in this situation (figure 1.7). The neutrophil cell membrane becomes disrupted and its intracellular contents of proteolytic enzymes are released, leading to extensive tissue damage (Haslett 1999). The failure of dying neutrophils to be removed or a delay in apoptosis can cause neutrophil accumulation and subsequent tissue injury frequently associated with acute and chronic inflammatory diseases (Groneck, Schmale et al. 2001; Saba, Soong et al. 2002).

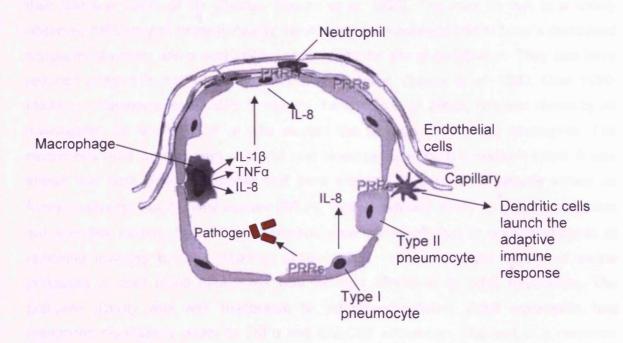


**Figure 1.7:** The resolution of an inflammatory response is aided by the clearance of neutrophils. Neutrophils undergo a programmed cell death known as apoptosis. The nucleus becomes rounded and the cell membrane shrinks. Apoptotic neutrophils are phagocytosed by macrophages. When the resolution of an inflammatory response becomes disrupted, the neutrophils may undergo necrosis. The neutrophils swell and the membrane breaks, releasing the intracellular toxic contents into the surrounding tissues.

Following the initial influx of neutrophils, a transitional change in leukocyte recruitment occurs. In the normal resolution of inflammation, a reduction in IL-8 secretion reduces the recruitment of neutrophils. The neutrophils are replaced with an influx of mononuclear cells into the infected tissue. An increased secretion of a chemokine, monocyte chemoattractant protein (MCP-1) triggers the migration of monocytes and macrophages from the bloodstream. The transitional switch in leukocyte recruitment can fail, resulting in an accumulation of neutrophils at the site of inflammation and a failed recruitment of mononuclear cells. This can lead to chronic inflammation of the airways.

# 1.4 The innate immune response within the airways

During an infection, the epithelial surface of the respiratory tract is the first line of defense. The innate and adaptive immune responses are the important second and third line of defense (Opitz, van Laak et al. 2010). The inflammatory response to infection in the airways is triggered by surface and intracellular receptors known as pattern recognition receptors (PRR) (as shown by figure 1.8 in the alveolus). They detect bacterial components known as pathogen-associated molecular patterns (PAMPS). Essentially, PAMPS are molecules derived from pathogenic bacteria and viruses (Fournier and Philpott 2005; Opitz, van Laak et al. 2010). In normal lung function, unstimulated airway epithelial cells express low levels PRRs. Upon exposure to pathogens, the airways PRRs are exposed apically to recognise bacterial components. More receptors are recruited to the apical surface when there is a continuous exposure to bacteria (Soong, Reddy et al. 2004; Fournier and Philpott 2005; Prince 2006). Toll-like receptors (TLRs) are one example of PRRs and play a central role in the response to a wide variety of endogenous and exogenous inflammatory mediators (Chaudhuri and Sabroe 2008). TLR 1 to 10 have been detected in airway epithelia and each respond to distinct PAMPS. Not all of the ten TLRs are expressed at one time on the epithelium. The activation of PRRs triggers the production of inflammatory mediators, such as IL-1β, TNFα, IL-8 and IFNγ from the epithelia. These mediators can also be released from activated resident alveolar macrophages (figure 1.8) and the epithelial cells are further stimulated. For example, IL-1β and TNFα released from macrophages can stimulate type I and II pneumocytes to produce IL-8 (Suzuki, Chow et al. 2008; Opitz, van Laak et al. 2010). Secretion of this chemokine can also be stimulated by bacteria cell wall components, such as LPS and flagellae (Gomez, Lee et al. 2004). The presence of IL-8 stimulates the recruitment of neutrophils to the site of infection.



**Figure 1.8:** The innate immune response in the alveolus. Pattern recognition receptors (PRRs) are expressed in epithelial cells, alveolar macrophages, endothelial cells, dendritic cells and other cell types. This stimulates inflammatory mediators such as IL-1 $\beta$ , TNF $\alpha$  and IL-8 from alveolar macrophages. IL-1 $\beta$  and TNF $\alpha$  may activate epithelial cells to release IL-8, which in turn stimulates the recruitment of neutrophils. The dendritic cells initiate the adaptive immune response to activate T cells. Adapted from Opitz et al. (Opitz, van Laak et al. 2010).

# 1.5. The neonatal innate immune system

Newborn infants are more likely to have frequent and serious bacterial infections during their first few weeks of life (Bektas, Goetze et al. 1990). This may be due to a widely observed deficiency of innate immunity. Neutrophils from neonatal infants have a decreased complement activity and a poor ability to migrate to the site of an infection. They also have reduced phagocytic and bactericidal functions (Bektas, Goetze et al. 1990; Quie 1990; Moriguchi, Yamamoto et al. 2006; Moriguchi, Yamamoto et al. 2006). This was shown by an investigation by Moriguchi et al who studied the function of neonatal neutrophils. The neutrophils were isolated from umbilical cord blood obtained from a newborn infant. It was shown that cord blood neutrophils that were exposed to a bacterial peptide known as formyl-methionyl-leucyl-phenylalanine (fMLP), had a reduced ability to produce a potent anti-microbial oxidant, H<sub>2</sub>O<sub>2</sub>. The neutrophils were not as efficient as adult neutrophils at removing invading bacteria following phagocytosis. Furthermore, the activity of serine proteases in cord blood neutrophils was reduced compared to adult neutrophils. The protease activity was less responsive to cytokine-stimulation. Adult neutrophils had responded significantly better to TNFα and GM-CSF stimulation. The lack of a response could be due to minimal numbers of receptors available on the cell surface to bind the cytokines. The study by Moriguchi et al had shown a reduction in cytokine receptor expression on cord blood neutrophils, particularly the expression of GM-CSF and G-CSF. From their investigation, it was concluded that neutrophil function is diminished in early newborn infants compared to adults. Their ability to adhere to endothelial cells, migrate into tissues and clear infecting microorganisms was greatly reduced compared to adults (Moriguchi, Yamamoto et al. 2006; Moriguchi, Yamamoto et al. 2006).

It was reported that neonatal neutrophils and cord blood neutrophils have a delayed ability to apoptose. This had subsequently led to neutrophil accumulation, contributing to the pathogenesis of chronic inflammation in the lung, gastrointestinal tract and other organs (Hanna, Vasquez et al. 2005). It was associated with a delay in Fas-mediated apoptosis due to reduction in FasR expression and decreased caspase-3 activity in neonatal neutrophils (Luo, Schowengerdt et al. 2003; Koenig, Stegner et al. 2005). Furthermore, pro-apoptotic molecules such as Bak, Bax and Bad were also decreased in neonatal neutrophils compared to adult neutrophils, which would also delay apoptosis (Hanna, Vasquez et al. 2005).

# 1.6 Injury and inflammation of the immature lung

Infants that are born before 37 weeks gestation are deemed as 'premature'. Premature infants have an immature lung structure, poorly developed alveoli and are deficient in surfactant (Jobe and Ikegami 1998). Infants that are born less than 30 weeks gestation are more likely to develop respiratory distress syndrome (RDS) within 4-6 hours of birth than infants who are born after 30 weeks gestational age (Greenough 2003). The incidence of RDS is inversely proportional to gestational age and occurs more frequently in infants that weigh less than 1200g. In addition, it is more common in males than females with a 2:1 ratio (Merenstein 1998). RDS is an acute lung injury that is associated with the presence of lung lesions known as hyaline membranes, which prevent oxygen from passing to the bloodstream. It is indicative of epithelial and endothelial cell damage, and can be attributed to congenital infection (Greenough 2003). Scarring and fibrosis of the airways, including the alveoli is typical and has been strongly associated with the development of chronic lung disease (CLD). The current definition of CLD is applied to infants who require oxygen supplementation beyond 28 days of age or infants more than 36 weeks gestational age (Kotecha 1999). CLD most commonly occurs in premature infants and term infants that have required mechanical ventilation and oxygen supplementation for acute RDS (Bancalari, Abdenour et al. 1979). CLD occurs in 35-40% of infants who weigh 750g to 1500g and may or may not have received surfactant therapy (Merenstein 1998).

Surfactant deficiency is a contributing factor to the development of RDS as lung volume and compliance are reduced, both of which lead to alveolar collapse. Very little air can be retained in the alveoli with no surfactant present. The presence of phosphatidylglycerol, one of the most common phospholipids in surfactant, is associated with a low risk of RDS. However, phosphatidylglycerol does not develop until around 36 weeks gestational age and then its production increases up until term (Merenstein 1998). Surfactant is vital for reducing surface tension, increasing lung compliance, alveolar stability and general protection of the epithelium (Merenstein 1998). It is made up of approximately 92% lipid and 8% protein. About 70% of the lipid are composed of phosphatidylcholine species and the main compound is dipalmitoylphosphatidylcholine (DPPC). The protein is made up of four constituents; SP-A, SP-B. SP-C, SP-D (Lynch 2004). The most common treatment for RDS is the administration of surfactant during mechanical ventilation. Although surfactant therapy improves clinical outcome, the mechanical ventilation can cause lung injury, which can lead to the development of CLD (Stevens, Harrington et al. 2007). However, studies have shown that infants at a high risk of developing RDS were to receive early surfactant replacement therapy instead of receiving surfactant when RDS has already established, their prognosis

was significantly improved, and the incidence of developing CLD was reduced (Gortner, Wauer et al. 1998; Stevens, Harrington et al. 2007). Although surfactant therapy is an established treatment for RDS, its efficacy is very much dependent on the strategy of the treatment. There are international guidelines for the preparation, dosing and timing of the surfactant replacement therapy (van Kaam, De Jaegere et al.; Stevens and Sinkin 2007).

Infants with breathing difficulties are placed on mechanical ventilation to aid inflation of the airways. They receive copious amounts of oxygen, often at a high concentration (termed hyperoxia) and a sustained pressure. The high pressure exerted by mechanical ventilation often causes physical damage to the tissues, known as barotrauma, which can lead to the development of lesions in the bronchiolar epithelial cells (Ehrenkranz 1992; Greenough 2003). Lung epithelial cells can be morphologically changed by cell flattening and stretching as a result of hyperoxia exposure, ultimately causing tissue damage and cell death (Crapo, Barry et al. 1980).

Ventilated preterm infants with RDS or early CLD were shown to have elevated apoptosis of alveolar epithelial cells with evidence of pulmonary fibrosis, associated with the development of CLD (De Paepe, Mao et al. 2005). Inappropriately activated inflammatory cells, such as neutrophils, are recruited to the site of inflammation in the airways. This often causes more damage to the cells as the neutrophils fail to be effectively removed (Fortenberry, Bhardwaj et al. 1996; Kotecha, Silverman et al. 1998). Premature neonates that developed RDS were found to have numerous inflammatory cells present in tracheal samples. This was compared to infants who were either ventilated for non-pulmonary reasons or those that had gone on to recover from RDS. These infants had fewer inflammatory cells present in the airways and a lower neutrophil cell count compared to those that went on to develop CLD (Merritt, Cochrane et al. 1983; Groneck, Gotze-Speer et al. 1994; Kotecha, Chan et al. 1995).

Furthermore, neutrophils that were isolated from the cord blood of premature infants and exposed to hyperoxic conditions *in vitro* were more susceptible to activation, superoxide production and the inappropriate release of intracellular contents compared to adult PMN (Suzuki, Yasukawa et al. 1993; Hoehn, Felderhoff et al. 2001).

Oxygen toxicity is also a major problem, as the lungs of infants with CLD are often subjected to prolonged hyperoxic conditions (Guthmann, Wissel et al. 2005) which can aggravate inflammation already present in the lungs (Fortenberry, Bhardwaj et al. 1996; Kotecha, Silverman et al. 1998). Both factors are major contributors of lung injury; strongly

associated with the pathogenesis of CLD. Other mechanisms of mechanical ventilation capable of inducing lung injury include large gas volumes (Attar and Donn 2002), alveolar collapse and re-expansion (atelectotrauma) and increased inflammation (biotrauma) (Ehrenkranz 1992; Attar and Donn 2002). The lung damage can result in blood, fluid and protein to leak into the airways, alveoli and the lung interstitium. This can impair normal lung function, prevent surfactant function and promote lung inflammation (Clark, Gerstmann et al. 2001).

Coalson *et al.* used premature baboons to investigate CLD to mimic the features of CLD observed in human premature infants with the disease. The baboons were born with lung development still in the canalicular stage and received treatment with postnatal surfactant. They received a limited and controlled use of oxygen supplementation with ventilatory strategies to prevent volutrauma and they also received good nutrition. Similarly to CLD in human premature infants weighing less than 1000g, the baboons had pulmonary lesions, fibrosis of the saccular wall, significantly reduced alveolarisation and capillary vasculature, but no airway disease. They reported an increase in inflammation in the airways of the baboons on ventilatory support. Significantly elevated levels of TNFα, IL-6 and IL-8 were observed in tracheobronchial aspirate fluids (TAFs) (Coalson, Winter et al. 1999).

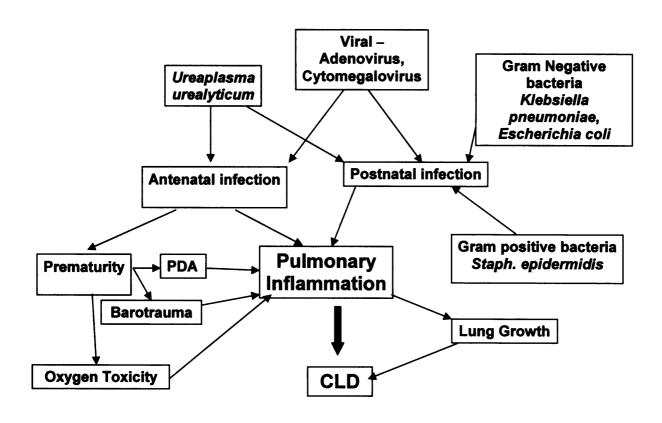
# 1.7 Inflammation in the pathogenesis of chronic lung disease

TAFs taken from premature neonates that have developed CLD often contain an abundance of neutrophils and are characterised by an increased level of pro-inflammatory cytokines. This is indicative of an inflammatory response in the airways (Groneck, Schmale et al. 2001). Significantly high levels of IL-1, TNFα, IL-6 and IL-8 were detected in TAFs and bronchoalveolar lavage fluid (BALs) obtained from premature and term neonates who were mechanically ventilated and had developed CLD (Merritt, Cochrane et al. 1983; Bagchi, Viscardi et al. 1994; Groneck, Gotze-Speer et al. 1994; Kotecha, Chan et al. 1995; Kotecha, Wilson et al. 1996; Murch, Costeloe et al. 1996; Jonsson, Tullus et al. 1997). These pro-inflammatory cytokines were detected within a few hours after birth and during 2-3 weeks proceeding (Kotecha, Wangoo et al. 1996; Kotecha, Wilson et al. 1996; Murch, Costeloe et al. 1996). Increased levels of TNFα, IL-1 and IL-6 have been detected in the amniotic fluid (Ghezzi, Gomez et al. 1998) and have been associated with the increased risk of neonatal infection and premature births (Yoon, Romero et al. 1996; Gomez, Romero et al. 1998). Elevated levels of these pro-inflammatory cytokines are also a characteristic feature of

chorioamnionitis (inflammation of the placenta). Chorioamnionitis has been associated with CLD development and is common in mothers who deliver prematurely (Yoon, Romero et al. 1996; Goldenberg, Hauth et al. 2000). Furthermore, it is often caused by *Ureaplasma urealyticum*, which has also been linked with CLD (Kundsin, Leviton et al. 1996), as it has been isolated from the lungs of infants with CLD (van Waarde, Brus et al. 1997). *U.urealyticum* was shown to be the predominant microorganism in perinatal airway infection and was associated with a broncho-alveolar inflammatory response with elevated IL-1, IL-6 and IL-8 at birth in premature infants with CLD. This was not observed in non-CLD preterm infants (Groneck, Schmale et al. 2001). It remains controversial to whether airway infection with this microbe can contribute to lung injury in preterm infants as a link between *U.urealyticum* and CLD has not been firmly established (van Waarde, Brus et al. 1997).

However, as pro-inflammatory cytokines have been linked to the pathogenesis of CLD, the early appearance of cytokines in BAL and TAF samples may help to identify preterm infants at risk of developing CLD (Jonsson, Tullus et al. 1997).

A brief overview of the factors that have been associated with the development of CLD is outlined in figure 1.9. These include antenatal and postnatal infection, mechanical ventilation (oxygen toxicity and barotrauma) and impaired lung growth. The patent ductus arteriosis (PDA) results in abnormal blood flow when the ductus arteriosis remains open. Endotracheal secretions from ventilated preterm neonates contain gram positive bacteria such as *Staphylococcus epidermidis*, and gram negative bacteria *Klebsiella pneumoniae* and *Escherichia coli*, which have also been associated with CLD incidence. Preterm infants with viral infections, mainly adenovirus or cytomegalovirus are also at risk of developing CLD (Davies, Maxwell et al. 2006).



**Figure 1.9.** Numerous factors contribute to pulmonary inflammation and the development of CLD in premature and term infants. Adapted from Davies et al. (2006).

### 1.8 The impact of hyperoxia on lung epithelial cells

Prolonged exposure to high oxygen concentrations with or without mechanical ventilation has been shown to cause tissue damage to the lining of the airways. Primary and transformed cell lines as well as animal models have been used to investigate the impact of hyperoxia on cell function. The described mechanisms of cell death caused by hyperoxia are complex as it can vary in different cell types.

Hyperoxia can affect signaling pathways involved in regulating lung development and has been implicated in the pathogenesis of CLD. Alejandre-Alcazar *et al.* showed that neonatal mice exposed to hyperoxia had reduced or arrested alveolar development due to the upregulation of transforming growth factor (TGF)-β signalling and decreased signalling of bone morphogenetic protein (BMP). Elevated levels of TGF-β have been detected in human neonates receiving oxygen therapy and diagnosed with CLD (Lecart, Cayabyab *et al.* 2000). In human and murine neonates, the disruption of the TGF-β pathway directly affects cell proliferation and apoptosis as well as elevating the expression of metalloproteinase inhibitor TIMP-1 in lung tissue. Increased TIMP-1 levels have been detected in CLD (Alejandre-Alcazar, Kwapiszewska *et al.* 2007)

Injury to lung epithelial cells caused by oxidants such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) can induce death by apoptosis (Kazzaz, Xu et al. 1996) but according to Franek *et al*, exposure to hyperoxia does not kill cells via this mode of cell death. They reported that transformed human alveolar epithelial cell line (A549) exposed to hyperoxia, were protected from oxidant-induced apoptosis. The mechanism involved is yet to be elucidated but is thought to be due to the activation of pro-survival pathways rather than through the inhibition of apoptotic pathways and is likely to involve the activation of NF-κB (Franek, Horowitz et al. 2001). NF-κB is known to be activated by pathogens, cytokines and oxidants (Schreck, Meier et al. 1992) and can protect cells from apoptosis by regulating Bcl-2 and caspases (Wang, Guttridge et al. 1999).

A non-apoptotic pathway in hyperoxia-exposed cells was also observed by Romashko *et al* who used transformed mouse alveolar epithelial cells (MLE-12) in their investigations. MLE-12 cells died as a result of exposure to hyperoxia but through a different mode of death to apoptosis. Oncosis was observed which is characterised by organelle swelling, the disruption of mitochondria structure and function but without activation of caspase-3 activity or phosphatidylserine translocation which is seen in apoptosis. Oncosis is partly mediated

by pathways thought to be involved in apoptosis which include the activation of a transcription factor AP-1 and trans-activators JNK and p38. Apoptosis and oncosis share upstream pathways but then proceed with different downstream events and are distinct modes of death. Epithelial cells exposed to oxidants exhibited downstream apoptotic events involving caspase-3 activation and phosphatidylserine translocation (Romashko, Horowitz et al. 2003). In contrast, Truong *et al* reported that epithelial cells exposed to hyperoxia undergo low levels of apoptosis and a significant number of cells died by necrosis in a caspase-independent manner (Truong, Monick et al. 2004).

Other signalling molecules, extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) are also activated by hyperoxia and have been reported to have a significant role in hyperoxia-induced oncosis that is different from JNK and p38 but this is yet to be elucidated. However, similar to p38, ERK is known to regulate the production of reactive oxygen species through activation of NADPH oxidase (Parinandi, Kleinberg et al. 2003). It has been shown to have both an anti- and pro- apoptotic role in epithelial cells (Zhang, Liu et al. 2005). However, ERK may have a protective role against hyperoxia-induced cell death as its activation delayed the death of A549 cells and acted to compensate the down-regulation of phosphoinositide 3-kinase (PI3-K) when exposed to hyperoxia. PI3-K is known as a pro-survival kinase and has been implicated in cell survival pathways. Furthermore, ERK and PI3-K inhibited apoptosis by inactivating caspase-9 (Allan, Morrice et al. 2003; Truong, Monick et al. 2004).

Lian *et al* demonstrated the activation of STAT3 pathway in preventing hyperoxia-induced inflammation and injury in the lung *in vivo*. It was shown that hyperoxia induced the activation of MMP-9 and MMP-12 in bronchoalveolar macrophages, endothelial cells and lung epithelial cells. This was inhibited by the over-expression of STAT3C (a constitutively active form of STAT3) which targeted a set of genes in the alveolar type II epithelial cells of transgenic mice, causing the release of gene products that inhibited neutrophil influx and MMP-9/MMP-12 production. It provided protection against capillary haemorrhage following exposure to hyperoxia and enhanced survival (Lian, Qin et al. 2005).

Many of the studies investigating IL-6 and hyperoxia have been in the animal lung *in vivo*. Some studies have used transgenic mice that over-express IL-6. One study in particular found that the over-expression of IL-6 enabled the mice to tolerate 100% O<sub>2</sub> exposure and all had survived for more than eight days. The wild-type mice died within 72 to 96 hours. The transgenic mice had diminished hyperoxia-induced lung injury and alveolar-capillary protein leakage. This IL-6-induced survival was associated with increased levels of anti-

apoptotic protein Bcl-2 and an MMP inhibitor, TIMP-1 (Ward, Waxman et al. 2000). This study was conducted in adult mice. Other studies investigating IL-6 and hyperoxia in neonatal animals have conflicting reports. An investigation by Choo-Wing *et al* showed the over-expression of IL-6 in neonatal transgenic mice increased their mortality. The excessive amount of IL-6 present in the hyperoxic lung increased mediators such as angiopoietin-2, angiopoietin-4 and vascular endothelial growth factor (VEGF). This contributed to the pulmonary oedema and consequent mortality of neonatal transgenic mice. Caspases -3, -6, -8 and -9 were also found to be significantly increased, resulting in cell death which according to the authors may have contributed to the lung injury observed in these mice (Lian, Qin et al. 2005; Choo-Wing, Nedrelow et al. 2007). As IL-6 has been found to be elevated in human neonatal lungs, this may explain the injury and subsequent development of CLD. Hyperoxia may be responsible for dysregulating the function of pro-inflammatory cytokines.

A further study by Chetty *et al.* also showed there was no protection against hyperoxia by IL-6. Interestingly, both the transgenic and wild-type neonatal mice in this study had elevated Bax protein as a result of hyperoxia but no Bcl-2. In the same study, they had compared an *in vitro* experiment using type II pneumocytes cultured from foetal rat lungs. In this experiment, the cells were treated with 25ng/ml IL-6 for 24 hours before oxidant injury. Rather than exposing the cells to high oxygen concentrations, they had exposed the cells to 1mM H<sub>2</sub>O<sub>2</sub> for 1 hour before assessing cell viability by trypan blue dye exclusion. They reported a 25% increase in survival in IL-6 treated cells compared to the control. The mechanism responsible for this IL-6-mediated survival had not been investigated (Chetty, Cao et al. 2008).

# 1.9 Pro-inflammatory cytokines may exert a protective effect against cell death

Studies by McNamee *et al.* into neutrophil apoptosis have reported that concentrations of TNFα or IL-1β up to 1000pg/ml and IL-6 up to 500pg/ml protect against spontaneous cell death as the rate of leukocyte apoptosis was significantly reduced (McNamee, Bellier et al. 2005). This was an *in vitro* experiment using diluted whole blood incubated for 48 hours. At 0, 24 and 48 hours, whole blood obtained from healthy volunteers was lysed with ammonium chloride lysing solution and subjected to various centrifugation steps to remove erythrocytes, leaving a purified PBMC population. Staining the cells with Annexin-V enabled apoptotic cells to be distinguished from live cells through identification of phosphatidylserine (PS), which becomes externalised on the membrane of apoptotic cells. They reported that as little as 0.01 pg/ml of IL-1β, 1pg/ml TNFα or 125pg/ml IL-6 was enough to significantly

delay neutrophil apoptosis. Lymphocyte apoptosis remained unaffected by the presence of individual cytokines. Interestingly, they also observed an increase in neutrophil apoptosis when each cytokine was administered at 1000 pg/ml (McNamee, Bellier et al. 2005), suggesting higher concentrations of pro-inflammatory cytokines induce apoptosis. A direct comparison with purified neutrophils was not made. A similar protective effect was reported by Asensi *et al.* who demonstrated that pro-inflammatory cytokines may have an antiapoptotic role in patients with an inflammatory bone infection called osteomyelitis, which is associated with increased circulating levels of IL-1, IL-6 and TNFα. This was subsequently linked with reduced apoptosis of neutrophils observed in patients with this inflammatory disease (Asensi, Valle et al. 2004).

IL-6 has been known to rescue T cells from entering apoptosis (Teague, Marrack et al. 1997; Kovalovich, Li et al. 2001) through the upregulation of STAT3 as well as the activation of anti-apoptotic molecules Bcl-2 and Bcl-x (Ivanov, Bhoumik et al. 2001; Narimatsu, Maeda et al. 2001). Studies by Ward et al have shown IL-6 to have a protective role in the lung when exposed to 100% oxygen. It can reduce the extent of injury to the airways caused by hyperoxia and is able to exert this protective effect alone in the lungs of transgenic mice. They reported that this protective effect was associated with an anti-apoptotic molecule Bcl-2 and a metalloproteinase inhibitor, TIMP-1. Both of which are involved in inhibiting cell death (Ward, Waxman et al. 2000). In contrast, Tsan et al reported that IL-6 was not capable of a protective role by itself and instead it mediates the actions of TNF and IL-1 which both act together to protect against hyperoxia within endothelial cells of rats (Tsan, White et al. 1992). The protective actions of TNFα and IL-1 can be exerted through a specific type of enzymes called superoxide dismutases (SOD) (Groneck, Schmale et al.). There are three types of SOD that catalyse the formation of reactive oxygen species. superoxide (O<sub>2</sub>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radical (•OH). One type, known as MnSOD, is located in the mitochondria. The protein levels and enzymatic activity of MnSOD is increased upon stimulation with TNFα and IL-1 and has been demonstrated to have an important role in the protection against O<sub>2</sub> toxicity (Fox, Hoidal et al. 1981; Freeman and Crapo 1981; Wong and Goeddel 1988; Tsan, White et al. 1992). IL-6 does not appear to mediate its protective role through the activation of MnSOD but does interact with TNFα and IL-1 to enhance their protection through MnSOD (Tsan, White et al. 1992; Ward, Waxman et al. 2000). Similar to Ward et al, investigations by Rollwagon et al have also shown IL-6 to inhibit cell death by upregulating bcl-2 in rat intestinal epithelial cells following tissue damage by oxygen free radicals (Rollwagen, Madhavan et al. 2006). Although many studies have shown IL-6 to delay apoptosis, the opposite effect has been observed in a study by Afford et al, who demonstrated that IL-6 induced the apoptosis of human neutrophils and the human monocytic cell line, U937. It was claimed that the rate of apoptosis increased in neutrophils and U937 cells when incubated with IL-6 compared to the control without IL-6. Elevated apoptosis was observed after 24 hours incubation for neutrophils and 48 hour incubation for U937 cells. However, this effect was seen in a small proportion of cells and not the entire population in culture (Afford, Pongracz et al. 1992). An *in vitro* study by Brach *et al* revealed that IL-6 had no effect on delaying neutrophil apoptosis but found that GM-CSF prolonged survival for 216 hours. Neutrophils were purified by dextran sedimentation and centrifugation on Ficoll-Hypaque. The purified neutrophils at 1 x 10 $^6$  cells/ml were incubated for up to 216 hours in RPMI media supplemented with serum, with or without treatment. Apoptosis was determined by eosin dye exclusion test and also confirmed by DNA fragmentation analysis. After 24 hours, 80%  $\pm$  9 of neutrophils treated with 1nmol/L rh-GM-CSF survived compared to 70%  $\pm$  8 of neutrophils with 1nmol/L rhIL-6 and the control without treatment, 68%  $\pm$  4. By 48 hours, rh-GM-CSF had kept 81%  $\pm$  9 alive compared to 18%  $\pm$  3 for the control and 18%  $\pm$  8 for IL-6. Interestingly, incubating the cells with a bacterial peptide fMLP had not altered neutrophil survival (Brach, deVos et al. 1992).

#### 1.10 IL-6

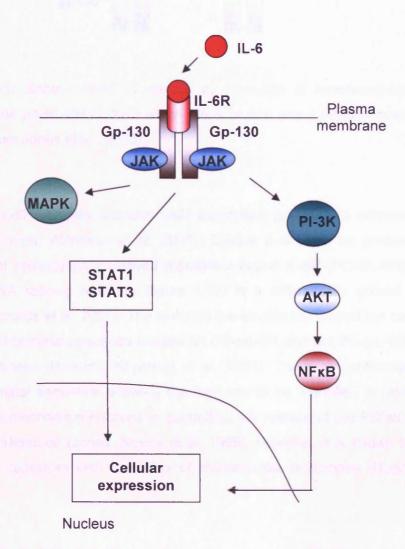
IL-6 is a pleiotropic cytokine that has both pro- and anti-inflammatory functions. It is important in regulating the local and systemic acute inflammatory response, as it affects numerous activities of the immune response. This includes regulating macrophage gene expression, complement receptor expression (C5aR), the activation of polymorphonuclear neutrophilic leukocytes (PMNs) and cell apoptosis (Xing, Gauldie et al. 1998; Ward, Waxman et al. 2000; Gomez, Sokol et al. 2005). IL-6 is a member of a family of cytokines that consist of oncostatin-M (OSM), interleukin-11 (IL-11), interleukin-27 (IL-27), Leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and cardiotrophin-like cytokine (CLC), which are all involved in regulating the acute phase response (Heinrich, Behrmann et al. 2003).

#### 1.10.1 IL-6 and its receptors

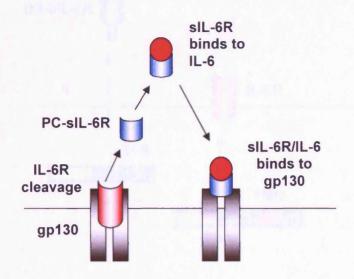
IL-6 belongs to a family of cytokines that act through receptor complexes containing at least one subunit of the signal transducing glycoprotein gp-130. The expression of the membrane bound IL-6 receptor (80kDa) is limited to specific cell types such as hepatocytes and leukocytes whereas gp130 (130kDa) is more widely expressed on the majority of cells in the body. Cells expressing IL-6R respond to IL-6 by the recruitment of two gp130 molecules, as

demonstrated in figure 1.10. Gp130 can not be activated or bound to by IL-6 or IL-6R alone. IL-6 must be bound to IL-6R before signal transduction via gp130 can occur. IL-6R itself does not appear to have independent signalling capacity (Grotzinger, Kernebeck et al. 1999; Jones, Richards et al. 2005).

Cells that express gp130 but not IL-6R are not responsive to IL-6 but become responsive when IL-6 is bound to the naturally occurring soluble form of this cognate receptor (sIL-6R). This mode of activation depends on the recruitment two molecules of gp130 (Hurst, Wilkinson et al. 2001) (Figure 1.11).

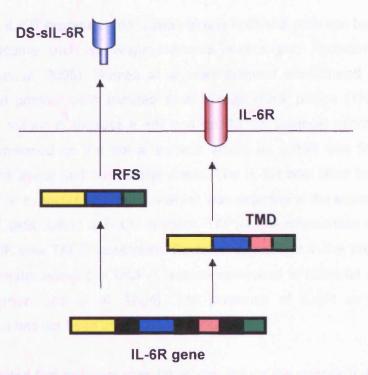


**Figure 1.10:** *IL-6 binds to its membrane bound receptor IL-6R and forms a complex with two gp-130 molecules. Three major pathways are activated that include MAPK, PI3-kinase, JAK-STAT pathway and transcription of target genes. Adapted from Hodge et al (Hodge, Hurt et al. 2005).* 



**Figure 1.11:** Soluble IL-6R production by cleavage of membrane-bound IL-6 receptor produces the pc-sIL-6R isoform which binds to IL-6 and gp-130, termed 'trans-signalling'. Adapted from Jones et al. (2005).

The IL-6/sIL-6R complex activates cells expressing gp-130 in a process known as 'transsignaling' (Hurst, Wilkinson et al. 2001). Soluble IL-6R can be produced either through shedding of proteolytically cleaved membrane-bound IL-6R (PC-sIL-6R), or by differential IL-6R mRNA splicing resulting (figure 1.12) in a differentially spliced form (DS-sIL-6R) (Jones, Richards et al. 2005). The isoforms are structurally related but can be distinguished by a COOH-terminal sequence located on DS-sIL-6R and not PC-sIL-6R as a result of the splicing process (Horiuchi, Koyanagi et al. 1994). There are antibodies specific for the COOH-terminal sequence allowing the isoforms to be identified in normal and diseased states. The mechanism involved in controlling the release of the PC-sIL-6R isoform is not properly understood (Jones, Novick et al. 1999). However, it is known that an increase in DS-sIL-6R correlates with the influx of mononuclear leukocytes (Hurst, Wilkinson et al. 2001).



**Figure 1.12:** Differential mRNA splicing produces two distinct transcripts that encode separately for membrane bound IL-6R and the DS-sIL-6R isoform. There is a reading frameshift (RFS) in the DS-sIL-6R transcript so that it lacks the coding sequence for the transmembrane domain (TMD) and incorporates a 10 amino acid sequence at the COOH terminal tail. Adapted from Jones and Rose-John, 2002.

### 1.10.2 Shedding of IL-6R

The activation of proteases, including TNFα converting enzyme (TACE), a disintigrin and metalloprotease (Underwood, Osborn et al. 2000) and aminopeptidase regulator of TNFR1 shedding (ARTS-1) can cause the release of sIL-6R from the membrane bound IL-6R (Franchimont, Lambert et al. 2005). Investigations into the inhibition of IL-6R cleavage involving hydroxamic acid-based inhibitors have indicated that ADAM 17 (also known as TACE) and ADAM10 are involved in IL-6R shedding (Matthews, Schuster et al. 2003). Transcription and expression of ADAM17 can be stimulated by bacteria (Gomez, Sokol et al. 2005) but it is also upregulated by apoptosis. Following activation, it subsequently translocates to the cell membrane where it induces IL-6R shedding (Kobayashi, Braughton et al. 2003; Walcheck, Herrera et al. 2006; Chalaris, Rabe et al. 2007). The activation of ADAM17 is dependent on the combined activation of caspases 3, 8 and 9 (Chalaris, Rabe et al. 2007).

IL-6 release and IL-6R expression on human airway epithelial cells can be induced following exposure to bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Gomez, Sokol et al. 2005). Gomez *et al* used cultured transformed cell lines 16HBE, 1HAEo-cells and primary cells isolated from human nasal polyps (THP-1) and by flow cytometry, were shown to express IL-6R and gp130. By confocal microscopy, IL-6R was shown to be expressed on the apical surface where as gp130 was found to be evenly distributed on the apical and basolateral areas. The IL-6R was shed from the membrane following a 24 hour exposure to *S.aureus* which was detected in the supernatants harvested from the 16HBE cells. Using a TACE inhibitor, TAPI-1, the mechanism responsible for the shedding of IL-6R was TACE-dependent. Bacterial stimulation in the presence of IL-6/sIL-6R induced a greater amount of MCP-1 release compared to bacterial stimulation without IL-6/sIL-6R (Gomez, Lee et al. 2004). The presence of IL-6R on epithelial cells is controversial as it has not been previously described.

It has been reported that bacterial stimulation can induce the release IL-6R to produce sIL-6R from macrophages or PMNs. Furthermore, bacterial peptide fMLP is also known to induce IL-6R shedding from activated neutrophils (Jones 2005).

The mechanism of sIL-6R production by physiological activators is not properly understood particularly during inflammation. Franchimont et al studied the release of sIL-6R from an oesteoblast-like cell line MG63 that endogenously expresses IL-6R. They found untreated cells released sIL-6R through alternative splicing of the IL-6R mRNA. However, the elevated sIL-6R observed in response to stimulation by 10ng/ml IL-1β or 10ng/ml TNFα was due to shedding rather than alternative splicing. Surprisingly, RT-PCR and inhibitor studies involving RNA polymerase II inhibitor DRB or protein synthesis inhibitor cyclohexamide revealed IL-1β and TNFα had no direct effect on sIL-6R mRNA levels. In addition, IL-1β and TNFα-induced release of sIL-6R was completely blocked by calcium chelating agent EGTA suggesting that the mechanism required a protease to cleave the receptor. Therefore, IL-1β and TNFα could induce IL-6R shedding through the activation of TACE in an oesteoblast cell line. Calcium chelating agents were shown to completely inhibit sIL-6R production following stimulation with IL-1β and TNFα indicating the involvement of calcium-requiring ADAMs (Franchimont, Lambert et al. 2005). In a separate in vitro investigation of a monocytic cell line, Hargreaves et al reported that IL-1β, TNFα or IL-6 had not elevated expression or the release of IL-6R from IL-6R-expressing THP-1 cells. In the same study, MCP-1 also had little effect on IL-6R shedding (Hargreaves, Wang et al. 1998).

### 1.11 IL-6 and sIL-6R regulate the inflammatory response

In vivo investigations using a murine model of peritoneal inflammation revealed IL-6 transsignalling has a crucial role in regulating leukocyte recruitment. The neutrophil influx is reduced by IL-6/sIL-6R downregulating IL-8 and upregulating MCP-1, to recruit mononuclear cells. IL-6 trans-signalling has not been shown to affect the initial neutrophil influx (Hurst, Wilkinson et al. 2001; Gomez, Sokol et al. 2005; Jones 2005). In an in vitro study by Hurst et al, using growth arrested and serum starved human peritoneal mesothelial cells (HPMC), stimulation with 10ng/ml IL-6 and 50ng/ml sIL-6R significantly induced MCP-1 release. This suggests that it may not be IL-6 dependent as treatment with sIL-6R alone also significantly elevated MCP-1 release. However, Witowski et al showed HPMC release basal IL-6 that may be sufficient to form an active IL-6/sIL-6R complex (Hurst, Wilkinson et al. 2001). MCP-1 was also induced by a genetically engineered IL-6/sIL-6R fusion protein known as Hyper-IL-6. This protein was designed to have human IL-6 covalently attached by a flexible peptide chain to human sIL-6R. It is a highly active molecule in gp130-expressing cells in vitro (Peters, Blinn et al. 1998) and has potential therapeutic benefits (Rakemann, Niehof et al. 1999). In contrast to the in vivo scenario, baseline IL-8 production from HPMC was unaffected by IL-6/sIL-6R, sIL-6R alone or by hyper IL-6 in vitro. However, IL-1βinduced IL-8 was downregulated by IL-6 trans-signalling in this study (Hurst, Wilkinson et al. 2001).

In an *in vitro* experiment using endothelial cells, IL-6/sIL-6R stimulated copious expression of MCP-1, where-as IL-6 or sIL-6R alone had no affect. Similar results were obtained for IL-8 and IL-6 release in response to IL-6/sIL-6R. Human endothelial cells (HUVEC) were cultured from extracted umbilical cord veins. Romano *et al* reported MCP-1 release was induced following 24 hour treatment with 10ng/ml IL-6 in the presence of sIL-6R at concentrations ranging from 10-500ng/ml. In addition, MCP-1 mRNA expression was observed after 4 hours in endothelial cells cultured with 10ng/ml IL-6 and 100ng/ml sIL-6R. Tyrosine phosphorylation of STAT3 was observed after a 10 minute treatment with IL-6/sIL-6R but not with IL-6 or sIL-6R alone (Romano, Sironi et al. 1997).

#### 1.12 Signalling pathways activated by IL-6

IL-6 signalling through gp130 leads to the activation of Janus kinases (JAK), such as JAK1, JAK2 and Tyk2 (figure 1.11). These in turn induce the phosphorylation of STAT1 and STAT3, which form homodimers or heterodimers and translocate to the nucleus. This initiates the transcription of target genes (Horiuchi, Koyanagi et al. 1994; Heinrich, Behrmann et al. 1998; Matthews, Schuster et al. 2003). STAT3 has diverse functions and is known to be involved in regulating cell proliferation, differentiation, survival, apoptosis and inflammation. In addition it has been shown to have an important role in regulating surfactant homeostasis and lung function during hyperoxic-induced injury but is not required for normal lung development or function (Bromberg and Darnell 2000). In an *in vivo* study, STAT3-deletion in mice that were exposed to high oxygen had epithelial cell damage, an increased inflammatory response and decreased surfactant proteins (Hokuto, Ikegami et al. 2004). In chronic and acute inflammation, the dysregulation of STAT3 was observed leading to the over-expression of STAT3 (Hanada, Yoshida et al. 2001; Gao, Guo et al. 2004). The mechanism behind the dysfunction has yet to be identified.

Mitogen-activated protein kinase (MAPK) is another major signalling pathway activated by IL-6. In particular, ERK1/2, p38 and JNK members of MAPK have been known to be activated. ERK1/2 is involved in regulating cell survival where as p38 and JNK are stress-activated. In certain cell types such as HepG2 hepatoma cells, PI3K activation has been observed. This activation in multiple myeloma cells by IL-6 leads to cell survival by inhibiting apoptosis (Horiuchi, Koyanagi et al. 1994; Heinrich, Behrmann et al. 1998; Jones, Horiuchi et al. 2001; Matthews, Schuster et al. 2003; Jones 2005; Jones, Richards et al. 2005). It is currently unknown if the signalling mechanisms outlined here are activated by IL-6 in human airway epithelial cells. However, there is a potential for IL-6 activation of pathways to be dysregulated in premature neonates with CLD.

#### 1.13 Soluble gp130

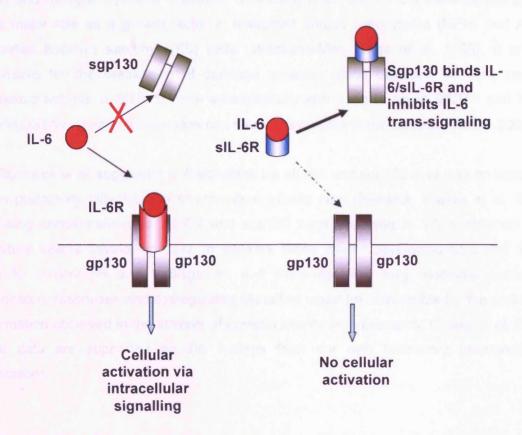
Soluble gp130 is a natural inhibitor of IL-6/sIL-6R complex. IL-6 trans-signaling is selectively counteracted by sgp130 as it binds IL-6/sIL-6R and exerts an antagonistic affect (Taga, Hibi et al. 1989). Soluble gp130 only interacts with IL-6 in the presence of sIL-6R and cannot bind to membrane bound IL-6R/IL-6 complex or IL-6 alone (figure 1.13) (Jostock, Mullberg et al. 2001). Muller-Newen reported that sIL-6R acts as an antagonist that increases the inhibitory affect of sgp130 enabling sgp130 to trap IL-6 in a soluble tertiary complex and

together can prevent IL-6 signalling through its cognate membrane-bound receptor (Muller-Newen, Kuster et al. 1998). Studies by Hurst *et al.* using a murine model have shown that sgp130 can regulate leukocyte recruitment during acute inflammation by inhibiting the cellular responses induced by the sIL-6R/IL-6 complex (Muller-Newen, Kuster et al. 1998; Hurst, Wilkinson et al. 2001; Jostock, Mullberg et al. 2001).

Circulating soluble forms of gp130 have been detected in plasma and at concentrations of 100-300ng/ml in human sera. Three different forms of sgp130; 50-kDa, 90-kDa and 100kDa have been purified from human sera (Honda, Yamamoto et al. 1992; Narazaki, Yasukawa et al. 1993; Jones and Rose-John 2002). A fourth isoform has been identified but its expression has been found in embryonic cells only (Sharkey, Dellow et al. 1995). Soluble gp130 is produced by membrane-bound receptor shedding or by differential mRNA splicing (Muller-Newen, Kuster et al. 1998; Tanaka, Kishimura et al. 2000). The 100kDa isoform is released from the cell membrane where as the 50kDa isoform is produced from alternatively spliced mRNA and contains a COOH-terminal sequence. This 50kDa isoform known as gp130-RAPS (gp130 of the Rheumatoid arthritis Antigenic peptide-bearing soluble form) and has been identified as an autoantigen in rheumatoid arthritis (Tanaka, Kishimura et al. 2000). The synovial fluid and plasma in patients with rheumatoid arthritis contain the 50-kDa isoform but its role in arthritic disease has yet to be determined. A recombinant form of human gp130-RAPS produced using the baculovirus expression system has been reported to be therapeutically effective in experimental arthritis (Richards, Nowell et al. 2006). In addition, it has been shown that the gp130-RAPS isoform can bind IL-6/sIL-6R and modulate leukocyte infiltration through inhibiting IL-6 trans-signalling and effecting local chemokine expression (Hurst, Wilkinson et al. 2001; McLoughlin, Hurst et al. 2004).

It is not clear which stimuli induce gp130 shedding or splicing. The amount of sgp130 produced by shedding is almost negligible compared to IL-6R shedding and the loss of membrane-bound gp130 would render the cell unresponsive to cytokines that require gp130 to elicit cellular activation (Mullberg, Dittrich et al. 1993). Mullberg *et al* showed that stimulating IL-6R and gp130-expressing Madine Darby canine kidney cells (MDCK) with PMA did not significantly induce shedding of gp130, where as a significant increase in IL-6R shedding was observed. They reported protein kinase C (PKC) activation was required for the IL-6R shedding but was not required for gp130 shedding (Mullberg, Schooltink et al. 1992). In a separate study, gp130 release was significantly induced from human melanoma cell lines by an IL-6 related family member, oncostatin-M. In a comparison study, normal leukocytes treated with oncostatin-M had not produced sgp130. IL-6 itself did not induce

gp130 release from melanoma cell lines or from normal leukocytes (Montero-Julian, Brailly et al. 1997).



**Figure 1.13:** The selective inhibition of IL-6 by sgp130. IL-6 trans-signaling is inhibited by sgp130 binding to IL-6/sIL-6R complex and prevents IL-6 binding to its cognate membrane bound receptor IL-6R and gp130. Soluble gp130 is not able to bind IL-6 alone or IL-6/IL-6R complex. Adapted from Scheller et al (Scheller and Rose-John 2006).

#### 1.14 IL-6 trans-signalling in inflammatory diseases

IL-6 is relatively undetected in serum from healthy individuals (Klein, Zhang et al. 1995) whereas sIL-6R is present in the plasma of healthy individuals at 25-35ng/ml. IL-6 levels have been known to rise to over 5ng/ml (Klein, Zhang et al. 1995) and elevated levels of sIL-6R have been detected in many diseases (Jones and Rose-John 2002). This is in contrast to findings by Montero-Julian *et al.* who stated there was little difference in serum concentrations of sIL-6R from healthy individuals or those with disease (Montero-Julian, Klein et al. 1995). It has been suggested by Kallen *et al* that 98% of IL-6/sIL-6R complex in the serum is bound to sgp130, making it inactive (Kallen 2002). In contrast, it has also been suggested that many of the pathophysiological activities of IL-6 are mediated through sIL-6R/gp-130 trans-signalling (Hurst, Wilkinson et al. 2001). It has been reported that the

dysregulation of IL-6 signalling contributes to the development of many diseases including rheumatoid arthritis, inflammatory bowel disease, oesteoporosis, multiple sclerosis, prostate cancer and multiple myeloma (Heinrich, Behrmann et al. 2003). IL-6 trans-signalling also has a major role as a growth factor in malignant fibrous histocytoma (MFH) and AIDS-associated Kaposi's sarcoma (KS) cells (Murakami-Mori, Taga et al. 1996). It is also responsible for the breakdown of cartilage collagen which destroys the joint causing rheumatoid arthritis. IL-6/sIL-6R acts synergistically with IL-1α to cause MMP-1 and MMP-13 upregulation. These collagenases can cause irreversible tissue damage (Kallen 2002).

Von Bismarck *et al*, suggested IL-6 signalling via sIL-6R and sgp130 may play an important role in pulmonary inflammation of premature infants (von Bismarck, Claass et al. 2008). Increasing concentrations of sIL-6R and sgp130 were observed in TAFs obtained form premature infants developing CLD. In addition, those infants developing CLD had higher ratios for IL-6/sIL-6R and IL-6/sgp130. IL-6 trans-signalling may modulate pulmonary inflammatory responses and dysregulated signalling could be responsible for the prolonged inflammation observed in the airways of preterm infants (von Bismarck, Claass et al. 2008). These data are supported by the findings from our own laboratory (manuscript in preparation).

### 1.15 Summary and hypothesis

The current understanding of IL-6 trans-signalling stems from the research of Hurst et al, in which they had shown IL-6 trans-signalling to have a crucial role in the transitional change in leukocyte recruitment in the in vivo murine model of peritonitis. IL-6 trans-signalling was shown to downregulate IL-8 and IL-1β-induced IL-8 production, which in turn reduced the influx of polymorphonuclear cells to the site of infection (Hurst, Wilkinson et al. 2001). Furthermore, IL-6 trans-signalling was shown to induce neutrophil apoptosis in vivo (McLoughlin, Hurst et al. 2004). This was followed by an increase in MCP-1 and an influx of mononuclear cells. This role was also demonstrated in human peritoneal mesothelial cells in vitro (Hurst, Wilkinson et al. 2001). The regulation of neutrophil apoptosis by IL-6 in vitro was described by Biffl et al and Afford et al (Afford, Pongracz et al. 1992; Biffl, Moore et al. 1996). However, there are currently no reports of IL-6 regulating neutrophil apoptosis through its soluble receptor in vitro. In addition, it is not known if IL-6 trans-signalling can regulate chemokine production and leukocyte recruitment in the airways or whether airway epithelial cells respond to IL-6 trans-signalling. If this role does occur, it could be impaired in premature infants with CLD, which is a plausible explanation for the accumulation of aging neutrophils failing to undergo apoptosis. BAL and TAF samples obtained from such infants have shown the abundance of neutrophils present in the airways, as well as the presence of IL-6 (Kotecha, Wilson et al. 1996; Groneck, Schmale et al. 2001), sIL-6R and a specific inhibitor of IL-6 trans-signalling, sgp130 (von Bismarck, Claass et al. 2008). More recent observations from our own laboratory have shown the significant high ratio of sgp130/sIL-6R in infants who developed CLD (Chakraborty, M et al. Manuscript in preparation).

The hypothesis is that IL-6 trans-signalling regulates the transitional switch in leukocyte recruitment by regulating chemokine release from airway epithelial cells and promoting neutrophil apoptosis. The *in vitro* investigations will help decipher a role of IL-6 transsignalling in the airways and contribute to the understanding of CLD pathogenesis.

#### 1.16 Project Aims

The overall aim is to understand the potential role of IL-6 trans-signalling in chronic lung disease of prematurity and will involve investigating the following:

#### 1.16.1 Chapter 3

IL-6 trans-signalling requires IL-6 and sIL-6R bound to gp130. Soluble IL-6R can be produced by shedding of the membrane bound IL-6R. For IL-6 trans-signalling to occur, IL-6 and sIL-6R must be bound to gp130. Neutrophils are known to express IL-6R and gp130. The expression and shedding of IL-6R and gp130 on neutrophils from adults and term infants will be compared. This will help determine if neonatal infants respond differently than adults to IL-6 trans-signalling, as has been reported for other stimuli.

The specific aims of this chapter were to:

- 1. Establish the most suitable methodology for investigating receptor expression on neutrophils
- 2. Determine the membrane receptor expression of IL-6R, gp130, CD16 and CD11b on adult neutrophils.
- 3. Determine the membrane receptor expression of IL-6R, gp130, CD16 and CD11b on cord blood neutrophils obtained from healthy term neonates.
- 4. Determine the susceptibility of receptor expression to be altered in response to neutrophil stimulation with a bacterial peptide or pro-inflammatory cytokine.
- Compare and contrast receptor expression on adult neutrophils with cord blood neutrophils.

### 1.16.2 Chapter 4

IL-6 and IL-6R is known to protect T cells, osteoblasts and neutrophils against apoptosis. There are no studies investigating the effect of IL-6 and sIL-6R on neutrophil apoptosis in adults or neonates. The aim of this chapter was to establish the role of IL-6 trans-signalling on neutrophil apoptosis. Flow cytometry will be used to assess the impact of IL-6 transsignalling on neutrophil apoptosis using Annexin-V as a marker of apoptotic neutrophils. Necrosis will also be measured by the staining of ToPro-3 dye which binds to the DNA in necrotic cells. ToPro-3 is unable to enter cells that have not necrosed and therefore allows apoptotic and necrotic cells to be distinguished.

#### 1.16.3 Chapter 5

IL-6 trans-signalling in adult and neonatal airways is poorly understood. The impact of IL-6/sIL-6R on transformed human airway epithelial cells A549s and BEAS2Bs will be investigated by measuring chemokine release. This will help provide an insight into the role of IL-6/sIL-6R as a potential regulator of leukocyte recruitment in the airways. In addition, the role of IL-6/sIL-6R during hyperoxia will also be investigated. Premature infants on mechanical ventilation receive oxygen at high concentrations, which subsequently damages the airway epithelial cells. Hyperoxia may alter the IL-6/sIL-6R regulation of chemokine production and leukocyte recruitment.

The specific aims of this chapter were to:

- 1. Establish if airway epithelial cells express gp130, an essential component of IL-6 trans-signalling.
- 2. Determine if airway epithelial cells produce MCP-1 in response to IL-6 transsignalling
- 3. Evaluate the modulation of cellular responses to IL-6 trans-signalling by exposing the cells to high oxygen concentrations (hyperoxia) and proinflammatory cytokines, such as IL-1β.
- 4. Establish if airway epithelial cells produce IL-8 and whether IL-6 trans-signalling downregulates IL-8 and IL-1β-stimulated release of IL-8 in airway epithelial cells.

#### 1.16.4 Chapter 6

The role of IL-6 trans-signalling in primary airway epithelial cells SAEC will be determined to establish the validity of the results obtained from the transformed epithelial cell lines.

The specific aims of this chapter were to:

- 1. Establish is primary airway epithelial cells express membrane IL-6R and gp130
- Determine if the primary airway epithelial cells produce MCP-1 in response to IL-6 trans-signalling
- 3. Determine if IL-6 trans-signalling can downregulate IL-8 and IL-1β-induced IL-8 in primary airway epithelial cells.
- 4. Compare and contrast the response from SAEC with a positive control line human peritoneal mesothelial cells (HPMC).

Chapter 2
Methodology

## 2. Methodology

#### 2.1 Buffers and solutions

### Phosphate buffered solution (PBS):

1 x phosphate-buffered saline tablet (Oxoid) dissolved in 100ml distilled water.

### Flow cytometry buffer solution:

1 x PBS, 1% (w/v) bovine serum albumin (BSA) (Sigma), 10% (v/v) heat-inactivated foetal calf serum (HI-FCS), 5mM ethylene-diamine-tetracetic acid (EDTA) (Sigma). The blocking buffer consisted of flow cytometry buffer without HI-FCS and 1% (v/v) heat-inactivated mouse serum (Sigma).

# Ammonium chloride-potassium (ACK) 10 x solution:

200ml distilled water, 8.29% (w/v) ammonium chloride (Sigma), 1% (w/v) potassium bicarbonate (Sigma), 0.2% (v/v) 0.5M EDTA (Sigma).

# 2.2 Antibodies

Antigen	Clone	Isotype	Conjugate	Supplier	Working concentration (dilution factor)	
Gp130	28126	lgG1	PE	R & D systems	1/10	
IL-6R/CD126	M91	lgG1	PE	Immunotech	1/5	
CD11b	CRf44	lgG1	PE	Southern Biotech	1/500	
CD16	3G8	lgG1	PE	BioLegend	1/200	
CD59	MEM-43	lgG2a	PE	BioLegend	1/50	
TLR 2	TL2.1	lgG2a	Biotin	eBioscience	1/100	
TLR 4	HTA125	lgG2a	Biotin	eBioscience	1/100	
CD14	UCHM.1	lgG2a	Biotin	Southern Biotech	1/100	
Gp130	D2	lgG1	Biotin	BD Biosciences	1/50	
Streptavidin	-	-	PE-Cy5.5	eBioscience	1/1000	
isotype control	-	lgG1	PE	R & D systems	Diluting to the	
Isotype control	-	lgG2a	PE	R & D systems	same concentration	
Isotype control	-	lgG1	Biotin	eBioscience	as the corresponding	
Isotype control	-	lgG2a	Biotin	eBioscience	antibody	
p-Stat 3	pY705	lgG2a	PE	BD Biosciences	1/20	

 Table 2: List of antibodies. All isotypes are mouse-derived. PE = Phycoerythrin

# 2.3 Inflammatory stimuli

Treatment	Company	Typical stock concentration
Tumor necrosis factor-α (TNFα)	Peprotech	2mg/ml
Formyl-methionyl-leucyl-phenylalanine (fMLP)	Sigma	10µM
Lipopolysaccharide (LPS)	Sigma	2mg/ml
Human recombinant interleukin-6 (rh-IL-6)	R & D systems	30µg/ml
Human recombinant soluble interleukin- 6 receptor (rh-sIL-6R)	R & D systems	30µg/ml
Hyper interleukin-6 (Hyper IL-6)	Kindly provided by Prof Rose-John	30µg/ml
Interleukin-1β (IL-1β)	Peprotech	100µg/ml

Table 3: List of inflammatory stimuli used.

#### 2.4 Leukocyte isolation

Blood was obtained from healthy adult donors or from umbilical cord blood into anti-coagulated sodium citrate (Sigma) at 10% of total blood volume. Anti-coagulated blood was mixed with 6% dextran (Baxter Healthcare) at 20% of total blood volume unless otherwise stated.

### 2.4.1 Neutrophils isolation by dextran and histopaque

Anti-coagulated and dextran treated blood was left to sediment at room temperature for 45-60 minutes. The top layer of plasma and white blood cells was carefully removed and gently layered over 7ml of Histopaque-1077 (Sigma) in a 25ml universal tube. After centrifuging at 200 x g for 25 minutes at room temperature (brake off), four separate layers were obtained. The layers containing platelets and peripheral blood mononuclear cells (PBMC) were carefully removed and discarded. A pellet of polymononuclear (PMN) cells and contaminating erythrocytes was retained and immediately resuspended in 1ml HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> with 10mM EDTA (HBSS-EDTA) (Lonza). The erythrocytes were lysed with 3ml sterile water for 30 seconds and then topped up with HBSS-EDTA. After centrifuging at 500 x g for 4 minutes, the supernatant was poured off and the PMN pellet was washed twice in HBSS-EDTA prior to cell counting.

### 2.4.2 Neutrophil isolation by dextran and percoll

Anti-coagulated and dextran treated blood was centrifuged at 20 x g for 20 minutes. The top layer of plasma and white blood cells was removed and transferred to a fresh 50ml tube. After pelleting the white blood cells at  $150 \times g$  for 7 minutes, the plasma was removed, leaving 3ml of plasma surrounding the cell pellet.

Percoll (Sigma) was diluted with 10% 1.5M saline. The diluted percoll was further diluted with sterile PBS to make 81%, 70% and 55% (of stock concentration) percoll solutions. To conical tubes, 4.5ml of 81% percoll was added and over-layed with 3ml of 70% percoll. The 3ml plasma/cell pellet was resuspended in 3ml of 55% percoll and 3ml of the 55% percoll/plasma/cell pellet mixture was gently over-layed on the 70% percoll layer. Density gradients were formed following centrifugation at 1500 x g for 25 minutes (brake on). The top layer containing PBMCs was gently removed in a circular motion using a pasteur

pipette. The second layer containing PMNs was gently removed in the same manner and transferred to a fresh tube. The remaining lower layer with a pellet of erythrocytes was discarded. The tube with the PMNs was topped up with 10ml of sterile 1 x PBS and centrifuged to pellet the cells at  $500 \times g$  for 4 minutes. The PBS was discarded and the pellet resuspended in fresh 10ml 1 x PBS to repeat the wash step. The pellet was resuspended in 2ml HBSS containing  $Ca^{2+}$  and  $Mg^{2+}$  prior to cell counting.

### 2.4.3 Leukocyte isolation

Anti-coagulated and dextran treated blood was centrifuged at 20 x g for 20 minutes. The plasma-leukocyte layer was carefully extracted without disturbing the lower layer of red blood cells and transferred to a fresh 50ml tube. The leukocytes were pelleted at 150 x g for 7 minutes and the plasma was removed leaving 3ml surrounding the pellet. The cell pellet was resuspended in RPMI media (Lonza) and centrifuged 400 x g for 5 minutes. The supernatant was discarded. The cell pellet was resuspended in 3-5 ml of cold 1 x ACK solution for a few minutes to lyse the erythrocytes. RPMI was added and centrifuged at 400 x g for 5 minutes. The cells were washed twice in RPMI before resuspending in 2-3 ml RPMI prior to cell counting

#### 2.4.4 Stimuli-treatment of isolated neutrophils and leukocytes

Isolated neutrophils or leukocytes were counted on a haemocytometer and a cell density of 2 x 10<sup>6</sup>/ml was used per treatment. The appropriate tubes containing 1ml of cells was primed with 2ng/ml TNFα and incubated in a 37°C waterbath for 15 minutes. For a further 15 minutes, 1μM fMLP was added to the appropriate tubes. During both incubation periods, the tubes were gently swirled 2-3 times to disperse the treatments. The concentration of stimulants were selected based on a previous study by Williams *et al.*, in which 1μM fMLP was shown to directly activate leukocytes, whilst 2ng/ml TNFα primes the cells in preparation for activation (Williams, Pettitt et al. 2007). Following centrifugation at 500 x g for 4 minutes, the supernatant was removed and transferred to 1.5ml tubes and stored at -80°C for future analysis. The cell pellet was retained and prepared for flow cytometry, as described in 2.4.6.

#### 2.4.5 Stimuli-treatment and ACK lysis of whole blood

Anti-coagulated blood was not treated with dextran. Instead, the blood was diluted by 50% with HBSS containing Ca2+ and Mg2+ (HBSSCa2+ Mg2+) (Sigma). To a tube, 2ml of diluted blood was added and treated with 2ng/ml TNFα for 15 minutes at 37°C. For a further 15 minutes at 37°C, the appropriate samples were incubated with 1µM fMLP. The concentration of stimulants were selected based on a previous study by Williams et al., in which 1μM fMLP was shown to directly activate leukocytes, whilst 2ng/ml TNFα primes the cells in preparation for activation (Williams, Pettitt et al. 2007). The tubes were gently swirled to disperse the treatments. Samples were centrifuged at 500 x g for 4 minutes and the supernatant containing plasma was removed. The pellet was resuspended in 2-3ml of cold 1 x ACK and incubated at room temperature for 15-20 minutes. A plastic pasteur pipette was used to gently resuspend the blood and was transferred to a fresh tube. The tubes were topped up with 3ml flow cytometry buffer and centrifuged at 500 x g for 4 minutes. The supernatant was removed and discarded. Samples were further lysed with 1ml 1 x ACK solution for 5-10 minutes depending on the erythrocytes susceptibility to lyse. Samples were washed once in flow cytometry buffer and centrifuged. The cell pellets were retained and prepared for analysis by flow cytometry, as described in 2.4.6.

#### 2.4.6 Preparation of cell pellets for flow cytometry

The cell pellet (obtained as described in 2.4.4 and 2.4.5) was resuspended in flow cytometry buffer and centrifuged at 500 x g for 5 minutes. After the supernatant was discarded, the cell pellet was resuspended in 1ml flow cytometry buffer. The appropriate wells of a 96-well U-bottomed plate received 100µl cells/well. After pelleting the cells at 500 x g for 5 minutes, the buffer was removed. The cell pellets were resuspended in 100µl of blocking buffer and incubated at 4°C for 25 minutes. After centrifugation at 500 x g for 5 minutes, the blocking buffer was removed and cells were washed again in flow cytometry buffer. Following another centrifugation and wash step, the cells were resuspended in the appropriate PEconjugated antibody and incubated at 4°C for 30 minutes. The centrifugation and wash step was repeated twice to ensure removal of unbound antibody. The cell pellet were resuspended in 100µl of flow cytometry buffer and transferred to the tubes containing 100µl cold flow cytometry buffer. Samples were analysed immediately on a BD FACS calibur.

### 2.5 Leukocyte isolation method used for investigating neutrophil apoptosis/necrosis

Two methods were used to assess neutrophil apoptosis in a mixed leukocyte population in the absence and presence of human serum.

### 2.5.1 Dextran isolation of leukocytes to retain the serum

Anti-coagulated and dextran treated blood was left to stand for approximately 45-60 minutes at room temperature to sediment the erythrocytes. The top layer of leukocytes and plasma was transferred to a fresh tube and pelleted at 500 x g for 4 minutes. The supernatant was removed and the pellet was treated with 1 x ACK to lyse any remaining erythrocytes. The cells were washed in RPMI, pelleted at 500g for 4 minutes and resuspended in media prior to cell counting.

#### 2.5.2 Dextran isolation of leukocytes to remove the serum

Anti-coagulated and dextran treated blood was centrifuged at  $20 \times g$  for 20 minutes. The top layer of leukocytes and plasma were removed and transferred to a fresh tube. After centrifuging for 7 minutes at  $150 \times g$ , the plasma was removed and the pellet was resuspended in  $1 \times ACK$  to lyse the erythrocytes. The cells were centrifuged at  $500 \times g$  for 4 minutes. The cells were washed in RPMI media, centrifuged twice before resuspending in media prior to cell counting.

### 2.5.3 Preparation of leukocytes for apoptosis/necrosis analysis

The isolated leukocytes were counted on a haemocytometer and cells at a density of 2 x10<sup>6</sup> per ml were added to a well of a 24-well plate. Leukocytes were either untreated or were treated with 100ng/ml LPS or 30ng/ml IL-6 and 30ng/ml sIL-6R. The cells were incubated at 37°C for 5 or 17 hours. Following the incubation step, the wells were washed thoroughly in HBSS<sup>Ca2+ Mg2+</sup> to lift off adherent leukocytes. As control samples, isolated leukocytes that had not received treatment nor had been incubated, were included. The cells were transferred to tubes and centrifuged at 500 x g for 5 minutes. After the cells were resuspended in 1ml HBSS<sup>+Ca2+ and Mg2+</sup>, 100µl was transferred to a U-bottomed 96 well plate. The cells were pelleted at 500 x g for 5 minutes and the supernatant was removed. The cells were either resuspended in 100µl of Annexin V binding buffer (AVBB) (BD Biosciences) containing 3% Annexin V-PE (MBL) to fluorescently label apoptotic cells or in 100µl AVBB containing 20mM EDTA with 3% AV-PE or 100µl AVBB only as controls.

Following a 30 minute incubation at 4°C in the dark, cells were washed in AVBB or AVBB/20mM EDTA and centrifuged at 500 x g for 4 minutes. The cells were resuspended in the appropriate buffer and either 100µl or 200µl was transferred to tubes. To the tubes containing 100µl of cells, 100µl of To-pro-3 (Molecular Probes) was added to the appropriate sample and immediately analysed by flow cytometry.

### 2.6 Analysing receptor expression and apoptosis by flow cytometry

Samples were analysed by flow cytometry on a FACS calibur (Becton Dickinson). The neutrophil population was gated (a region was drawn around the population) on the forward scatter (FSC-H) and side scatter (SSC-H) parameters of the flow cytometer. FSC determines the size of a cell and SSC depends on the density and granularity of a cell. Neutrophils can be identified on the FSC-H/SSC-H dot plot from other cell populations by their large size and granularity.

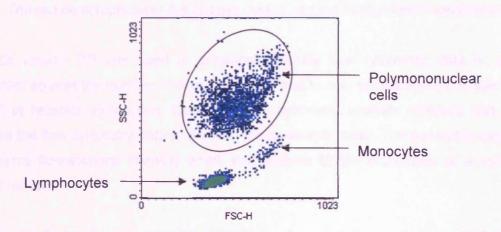
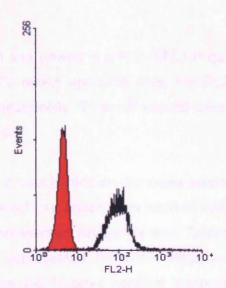


Figure 2.1: An example of an SSC-H/FSC-H plot.

#### 2.6.1 Receptor expression

The number of events within the gate were counted (10,000 events were collected) and displayed in a histogram plot (FL2-H on the x-axis) against the number of events (counts on y-axis) to reflect the fluorescence properties of the gated cell population (in this case only the neutrophils). The histogram highlights the relative fluorescence intensity of each receptor expressed on the gated neutrophils. The FL2-H channel corresponds to the wavelength required to detect cells stained with PE-conjugated antibodies.



**Figure 2.2:** An example of a histogram plot to demonstrate receptor expression (black peak). The red peak represents the isotype control and the background fluorescence.

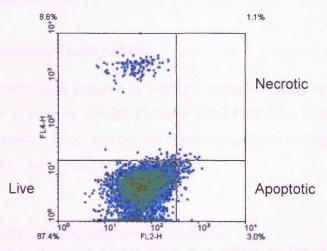
WinMDI version 2.9 was used to graphically display flow cytometric data on a FL2-H histogram against the number of events (counts) and to over-lay histograms to demonstrate a shift in receptor expression. CellQuest flow cytometry analysis software was used to analyse the flow cytometry data and obtain the geometric mean. The geometric mean value represents fluorescence intensity which corresponds to the expression of a membrane-bound receptor.

In Microsoft Excel, the geometric mean for the isotype negative control was deducted from the geometric mean of the corresponding receptor antibody. The geometric mean values were plotted on a scatter graph to compare fluorescence intensities (receptor expression) on untreated and treated neutrophils for each individual experiment to highlight low receptor expression or variation between each experiment. The geometric mean was presented as percentage difference ± SEM to compare receptor expression on treated neutrophils against untreated adult neutrophils set at 100%. Graphically representing the data as percentage difference highlights changes in receptor expression in response to a stimulus compared to unstimulated neutrophils.

### 2.6.2 Apoptosis

The neutrophils were gated and viewed in a FL2-H/FL4-H quadrant to determine apoptotic and necrotic neutrophils. To detect apoptotic cells, the FL2-H channel was required to detect Annexin-V stained neutrophils. To-pro-3 stained cells were detected in the FL4-H channel to determine necrosis.

Data from the FL2-H channel was plotted on the x-axis against the FL4-H channel data on the y-axis of a dot plot to detect live, apoptotic or necrotic cells in a quadrant. The lower left section of the quadrant represented Annexin-V and To-pro-3 negative cells (live cells) where as the upper right represented Annexin-V negative and To-pro-3 positive cells (necrotic). Annexin-V positive and To-pro-3 negative (apoptotic) were detected in the lower right section. A small proportion of Annexin-V negative/To-pro-3 positive cells were detected in the upper left section of the quadrant and probably represents cells which had non-specifically taken up To-pro-3 dye.



**Figure 2.3:** An example of a FL4-H/FL2-H quadrant plot to show percentage of apoptotic, live or necrotic cells.

The percentage of apoptotic, necrotic and live neutrophils in each section of the quadrant was obtained from CellQuest software analysis and expressed as the mean percentage of apoptotic, necrotic or live neutrophils.

### 2.7 Epithelial cell culture

### 2.7.1 Culture of transformed epithelial cells

The transformed epithelial cells used in this study are listed in table 4, along with their media requirements. The cells were cultured in 75cm³ flasks containing media supplemented with L-glutamine and 5% heat-inactivated foetal calf serum (HI-FCS) (Sigma). Cells were grown to 80% confluency in a humidified incubator at 37°C and 5% CO<sub>2</sub>. The cells were detached with 0.25% (w/v) trypsin-Ethylenediaminetetraacetic (EDTA) solution. The cells were sub-cultured at splitting ratios of 1:3 to 1:6 depending on individual experiments. The medium was changed every other day.

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

Table 4: List of transformed cells

### 2.7.2 Small airway epithelial cells (SAEC)

SAEC (Lonza CC-2547) were cultured in T-25cm<sup>2</sup> flasks in SAEC basal media containing growth supplements (Clonetics SAGM BulletKit CC-3118). The vials contained 5 x 10<sup>6</sup> cells/ml. Cells were seeded at an appropriate density specific to individual experiments. The recommended seeding density is 2,500 cells/cm<sup>2</sup>.

### 2.7.2.1 Thawing process and the initial culture of SAEC

An appropriate volume of media at 1ml/5cm<sup>2</sup> was added to the flasks and allowed to equilibrate at 37°C, 5% CO<sub>2</sub> humidified incubator for 30 minutes. The cryovial was wiped with ethanol. The cap was loosened to relieve pressure and was retightened, before rapidly thawing the cryovial in a 37°C 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 37°C, 5% CO<sub>2</sub> incubator.

#### 2.7.2.2 Sub-culturing SAEC

The cells were sub-cultured when they had reached 60-80% confluency. For a 25cm2 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 following equation was used to determine the total number of viable cells:

Total number of viable cells = <u>Total cell count x percent viability</u>

100

The total number of flasks to be inoculated was determined. The number of flasks required depended on the cell yield and the seeding density. The cells were seeded into well plates at a recommended density of 10,000 cells/cm<sup>2</sup>. The following equation was used:

Total number of flasks = <u>Total number of viable cells</u>

Growth area x seeding density

The following equation was used to calculate the volume of cell suspension to seed the flasks:

Seeding volume = <u>Total volume of diluted cell suspension</u>

Number of flasks as determined in the above equation

The cells were resuspended and the appropriate density of cells was added to the new flasks containing the warmed up media. To each flask, 1ml of growth media was added for every 5cm<sup>2</sup> surface area of the flask. The new flasks were returned to the incubator.

#### 2.7.2.3 SAEC maintenance

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/5cm². When confluency reached 25-45%, 1.5ml per 5cm² was added. When the cells were more than 45% confluent, the cells received 2ml/5cm² 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.

#### 2.7.3 Human peritoneal mesothelial cells (HPMC)

Serum starved HPMC were kindly donated by Dr. C Fielding and Miss C Colmont from the Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University. The procedure for the isolation and culture of HPMC are outlined by Topley *et al* (Topley, Brown et al. 1993). Briefly, HPMC were isolated by serial tryptic (0.1% w/v trypsin and 0.02% w/v EDTA) digestion of omental tissue from consenting patients undergoing elective abdominal surgery (Topley, Brown et al. 1993). HPMC were cultured in Earle's buffered-199 medium containing 10% (v/v) FCS, 2mM L-glutamine, 100U/ml penicillin, 100ug/ml streptomycin, 5ug/ml transferrin, 5ug/ml insulin and 0.4ug/ml hydrocortisone at 37°C in a humidified 5% CO<sub>2</sub> incubator. HPMC on the second passage were growth arrested for 48 hours in the absence of FCS. HPMC can remain viable under these conditions for up to 96 hours (Topley, Brown et al. 1993). All stimulations were performed in the absence of serum and on cells no older than the second passage. The HPMC were used in comparison to the SAEC and the responses of both cell lines were to be compared.

#### 2.8 Preparation of transformed epithelial cells

Epithelial cells were seeded at the density appropriate to individual experiments, into 25cm<sup>2</sup> 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 HIFCS or additives.

# 2.9 Cytokine treatments

Cytokines were diluted to the appropriate concentrations in serum-free media before stimulation of neutrophils or transformed and primary epithelial cells. Cells were incubated for various times at 37°C, 5% CO<sub>2</sub> and 21% O<sub>2</sub>. For the hyperoxia experiments, cells were exposed to 80% O<sub>2</sub>.

#### 2.10 Enzyme-linked immunosorbent assay (ELISA)

Supernatants were aspirated from cell lines at specific time points, pelleted at 13,000 x g for 5 minutes and transferred to fresh tubes before storing at -80°C. Samples were diluted accordingly for each ELISA in buffer specific for the ELISA kit.

### 2.10.1 Monocyte chemoattractant protein-1 (MCP-1) ELISA

Two different ELISA kits were used for detecting MCP-1.

### 2.10.1.1 MCP-1 (eBioscience ELISA)

The MCP-1 ELISA (Human CCL-2 ELISA Ready-SET-Go! eBioscience) was performed according to the manufacturer's guidelines. Briefly, a NUNC maxisorp 96 well plate was coated with 100µl/well capture antibody (purified anti-human CCL2; clone 5D3-F7) diluted 1:250 in coating buffer (coating buffer powder reconstituted in 1 litre distilled water) and incubated overnight at 4°C. The washing steps were performed as outlined on the eBioscience protocol with freshly made wash buffer (1 x PBS, 0.05% Tween-20). The assay diluent was diluted to 1X in sterile water and used in the blocking step and to dilute the antibodies, supernatants and standards accordingly. The standard (recombinant human MCP-1) at 1µg/ml was diluted to 1000pg/ml and 2-fold serial dilutions were performed. The sensitivity of the ELISA was 15pg/ml. Following an incubation and wash step, the detection

antibody (biotin-conjugate anti-human CCL2; clone 2H5) was diluted 1:250 and  $100\mu$ l/well was added and incubated. Separately,  $100\mu$ l/well of enzyme (Avidin-HRP) was added following a wash step and proceeding the addition of  $100\mu$ l/well substrate solution (1 x TMB). Sulphuric acid was used to stop the reaction and the plate was read at 570/450nm on a spectrophotometer

### 2.10.1.2 MCP-1 (BD Bioscience ELISA)

MCP-1 ELISA (BD Biosciences OptEIA<sup>TM</sup>) was performed according to the manufacturer's guidelines. The protocol was similar to that of the eBioscience MCP-1 protocol previously outlined with the following differences in reagents and amendments. The assay diluent (1 x PBS with 3% BSA) was freshly made and filtered. Two different wash buffers were used; wash buffer 1 contained 1 x PBS with 0.05% tween 20, and wash buffer 2 contained 1 x PBS. Wash steps were performed as outline on BD protocol with 2-3 washes with wash buffer 1 followed by 2 washes with wash buffer 2. The top standard of 500pg/ml was prepared from recombinant human MCP-1 and a 1:2 serial dilution performed using assay diluent. The sensitivity of the ELISA was 7.8pg/ml. At the detection antibody step, biotinylated anti-human MCP-1 monoclonal antibody (1:1000) was added at the same time as the enzyme reagent (Streptavidin-horseradish peroxidase conjugate). The substrate solution was made up in 12ml distilled water with 4 OPD tablets and 5.2μl H<sub>2</sub>O<sub>2</sub>. After stopping the reaction, the absorbance was read at 490nm.

#### 2.10.2 Human Interleukin-8 ELISA

The IL-8 ELISA (BD Biosciences) was performed according to the manufacturer's guidelines. Briefly, a NUNC maxisorp 96 well plate was coated with 100µl/well capture antibody diluted 1:250 in coating buffer and incubated overnight at 4°C. The washing steps were performed as outlined in the BD Biosciences protocol with freshly made wash buffer (1 x PBS, 0.05% Tween-20). The assay diluent (refer to ebioscience MCP-1) was diluted 1:5 using sterile water and used to block the wells for 1 hour at room temperature. Supernatants and standards were diluted accordingly with assay diluent. The lyophilized recombinant human IL-8 stock standard was diluted to 200pg/ml and 1:2 serial dilutions were prepared. Assay diluent was used as the control. Following a 2 hour incubation and wash step, the detection antibody (biotinylated anti-human IL-8) was diluted 1:250 in assay diluent, the Streptavidin-horseradish peroxidase conjugate was diluted 1:250 in the diluted detection antibody and 100µl/well was added. After a 1 hour incubation, the wells were washed and

 $100\mu$ I/well of substrate solution added (1 x TMB). Sulphuric acid was used to stop the reaction and the plate was read at 570/450nm on a spectrophotometer.

#### 2.10.3 Human Interleukin-6 sR ELISA

The IL-6 sR Duoset ELISA (R&D systems) was performed according to the manufacturer's guidelines, Briefly, NUNC maxisorp 96 well plates were coated with 100µl of 2µg/ml mouse anti-human IL-6 R (capture antibody stock is 360µg/ml) diluted in PBS and incubated overnight at 2-8°C. Wells were aspirated and washed with PBS containing 0.05% Tween-20 pH 7.2 and blocked with assay diluent (freshly prepared and filtered 1% BSA in PBS) for 2 hours at room temperature. The wash step was repeated as before prior to 100µl of standard, supernatant and control being added in duplicate. A top standard of 1000 pg/ml was prepared from recombinant human IL-6 sR (85ng/ml) and 1:2 serial dilution was performed using assay diluent. Supernatants from A549 and BEAS2B cells were diluted 1:2. Control wells contained assay diluent. Following 2 hour incubation at RT, wells were aspirated as before and 100µl of 100ng/ml of biotinylated goat anti-human IL-6 R (detection antibody stock at 18µg/ml) diluted in assay diluent was added. After 2 hours incubating at room temperature, the aspiration step was repeated and 100µl Streptavidin horseradishperoxidase conjugated enzyme was added for 20 minutes at RT in the dark. Following the wash step, 100µl of substrate solution (for a full plate, 12ml distilled water, 4 OPD tablets and 5.2ul H<sub>2</sub>O<sub>2</sub>) was added. Sulphuric acid was used to stop the reaction once the standards had developed. Absorbance readings were obtained at 490nm on a spectrophotometer.

# 2.10.4 Human sgp130 ELISA

Sgp130 ELISA (R&D systems DuoSet) was performed according to the manufacturer's guidelines. Briefly, NUNC 96 well plates were coated with 100µl of 4µg/ml capture antibody (stock 720µg/ml) diluted in coating buffer and incubated at 37°C for 1 hour. Wells were washed with PBS containing 0.05% Tween-20 and blocked with assay diluent (freshly prepared and filtered PBS with 1% BSA) for 1 hour at room temperature. The wash step was repeated as before prior to 100µl of standard, supernatant and control being added in duplicate. Top standard of 10,000 pg/ml was prepared from recombinant human sgp130 (80ng/ml) and 1:2 serial dilution was performed using assay diluent. Supernatants from A549 cells were diluted 1:3 and supernatants from BEAS-2B were added undiluted. Control wells contained assay diluent. Following 2 hour incubation at RT, wells were aspirated as

before and 100 $\mu$ l of 400ng/ml of detection antibody (stock 72 $\mu$ g/ml) diluted in assay diluent was added. After 2 hours incubating at room temperature, the aspiration step was repeated and 100 $\mu$ l Streptavidin horseradish-peroxidase conjugated enzyme was added for 20 minutes at RT in the dark. Following the wash step, 100 $\mu$ l of substrate solution (for a full plate, 12ml distilled water, 4 OPD tablets and 5.2 $\mu$ l H<sub>2</sub>O<sub>2</sub>) was added. Sulphuric acid was used to stop the reaction once the standards had developed. Plate was read at 490nm on a spectrophotometer.

#### 2.11 Viability assay using MTS

An MTS assay (CellTiter 96® AQ<sub>ueous</sub> One Solution Cell Proliferation Assay) is a colourimetric assay and was used to determine the number of viable proliferating cells, which take up the MTS tetrazolium compound and is reduced to a coloured formazon product.

A549s were seeded at 3 x 10³ per 100μl onto 96 well plates and left to adhere. After 12 hours, cells were washed twice in saline to remove serum before serum starving in Kaign's F12 media overnight. Cells were washed again and received 100μl of fresh serum free media containing treatments of 30ng/ml IL-6 and sIL-6R or 20ng/ml IL-1β or were left untreated. Treatment was set up in duplicate wells. Control wells were also included which contained 100μl media only and no cells. Cells were incubated for 12, 24 and 48 hours at 37°C at either 21% or 80% O₂. At each time point, 20μl MTS solution was added to the wells containing media only and all wells containing A549s. After wrapping the plate in foil, the cells were returned to the incubator for 2 hours at 37°C 21% O₂. Absorbance readings were obtained at 490nm on a spectrophotometer. In Microsoft Excel, a mean absorbance was taken from the duplicate set of the control 'no cells' wells, depicting background absorbance. This was deducted from the averaged cell absorbance readings. The corrected absorbance values of cells were set against the absorbance of control wells containing unstimulated cells (set at 100%) and expressed as percentage changed in proliferation.

### 2.12 Preparation of cells for a paracrine experiment and flow cytometry analysis

Neutrophils were isolated by histopaque as outlined in section 2.4.1. To a flat bottomed 96 well plate,  $100\mu$ l of neutrophils at 2 x  $10^6$ cells/ml were added to  $50\mu$ l of supernatant harvested from A549 cells. Following 18 hour incubation at 37°C 21%  $O_2$ , cells were transferred to a U-bottomed 96 well plate, washed in AVBB solution, centrifuged at  $500 \times g$  5 min and stained with 3% Annexin-V for 30 min. After washing and centrifuging, cells were resuspended, transferred to tubes with a final volume of  $200\mu$ l. The appropriate samples stained with  $100\mu$ l 1:10,000 To-pro-3.

# 2.13 Culture and preparation of Escherichia coli (E.coli)

LB broth was inoculated with a viable *E.coli* colony and cultured overnight at 37°C. Following an initial 1:100 dilution, a 1:10 serial dilution was performed and segments of agar plates were inoculated with 20µl of each serial dilution. The 1:100 dilution of *E.coli* was centrifuged at 13,000 x g for 10 minutes and washed twice in PBS. After the final wash, the pellet was resuspended in 1ml PBS. E.coli was inactivated by heat-killing at 56°C for 30 minutes. Airway epithelial cells were incubated with and without heat-killed *E.coli* for 24 hours with and without cytokine treatments.

#### 2.14 Culture and preparation of *Ureaplasma Parvum*

U.parvum was cultured from a frozen aliquot kindly provided by Dr. M Beeton, Department of Child Health, School of Medicine, Cardiff University. U.parvum was inoculated into 10ml ureaplasma selective media (USM) (Mycoplasma Experience Ltd) and cultured for 48 hours. The U.parvum culture was evenly distributed into 1.5ml microcentrifuge tubes and centrifuged at 13,000 x g for 10 minutes. The supernatants were removed and the pellets pooled together in one tube. Following two wash steps in PBS, the pellet was resuspended in 1ml PBS. A serial dilution of 1:10 was performed to inoculate the media. Following 48 hours incubating, the colour changing units (CCU) were determined to indicate the density of U.parvum, as described by Beeton et al (Beeton, Chalker et al. 2009). The 1ml of PBS containing U.parvum was centrifuged and resuspended in the appropriate culture media for airway epithelial cells (DMEM or F12K media with and without HI-FCS. SV3 was inactivated by heat-killing at 56°C for 30 minutes. Killing was confirmed by subsequent culturing of U.parvum. Airway epithelial cells were incubated with and without heat-killed U.parvum for 24 hours with and without cytokine treatments.

#### 2.15 Intracellular staining for phosphorylated STAT3

A549 and SAEC cells were grown in six well plates to 70-80% confluency and A549 cells were serum starved before the addition of cytokines. HMPC were cultured in 6 well plates as outlined in 2.9.6 and serum starved for 48 hours. Cells were stimulated for 15, 20, 30 or 60 minutes dependent on cell line, with the appropriate stimuli in serum free media. Cells were removed with trypsin/EDTA, centrifuged at 200 x g for 2 minutes and resuspended in 2% paraformaldehyde (PFA) .To a U-bottom 96 well plate, 100µl of the cell suspension was added and the cells were fixed for 15 minutes at 37°C. PFA was removed by centrifugation at 200 x g for 2 minutes before resuspending the cells in 100µl ice-cold 90% methanol and permeabilising on ice for 30 minutes. Cells were centrifuged and washed twice in flow cytometry buffer, and stained with pSTAT3 or isotype controls (BD Bioscience) diluted 1:20 in 100µl flow cytometry buffer. Cells were centrifuged and washed twice in flow cytometry buffer, and analysed by flow cytometry.

### 2.16 Analysing receptor and intracellular expression by flow cytometry

Epithelial cells were analysed by flow cytometry on a BD flow cytometer calibur. The cells were viewed on the FSC-H/SSC-H graph as outlined in section 2.6. Expression was determined as outlined in 2.6.1. Briefly, a histogram plot was obtained by WinMDI to graphically display receptor expression. CellQuest analysis software package was used to analyse the flow cytometry data and obtain the geometric mean. The geometric mean value represents the fluorescence intensity, which corresponds to the expression of a receptor.

#### 2.17 Statistical analysis

For all mean results presented on a graph, the error bars represent SEM.

In chapter 3, statistical analysis to compare treatments with untreated was performed using a non-parametric ANOVA test and the Kruskal-Wallis test with Dunn's multiple comparisons post test. Setting the untreated data to 100% created a standard deviation of zero, which could not be used in a parametric test. This statistical test was also used to compare selected conditions where the sample size was small and the standard deviation was significantly different between conditions. A parametric statistical test was used when the data passed the normality test and followed Gaussian distributions. In addition, the Bartlett's test was used to show there was no significant difference in the standard deviations between the conditions. For this, statistical analysis was performed using one way ANOVA with Bonferroni post test to compare selected conditions.

In chapter 4, statistical analysis of experiments conducted in the presence of serum was performed using Kruskal-Wallis non parametric ANOVA test with Dunn's multiple comparisons post-test. Due to the small sample size and the difference in sample number, a parametric one way ANOVA test could not be performed. For experiments performed in the absence of serum, statistical analysis was performed using a one way ANOVA using Tukey-Kramer multiple comparisons test as the sample size was larger.

In chapter 5 and 6, statistical analysis was performed by the Friedman or Kruskal-Wallis non-parametric repeated measures ANOVA test with Dunn's multiple comparison post-test, depending on the individual experiment. The statistical analysis of experiments of larger sample size were performed by using the repeated measures ANOVA and Tukey-Kramer comparison test or Bonferroni post test, depending on the individual experiment.

For all statistical tests performed here, a p value of less than 0.05 (p = <0.05) is considered significant.

# **Chapter 3**

A comparison of IL-6R and gp130 expression and shedding on adult neutrophils and umbilical cord blood neutrophils *in vitro* 

#### 3.1 Introduction

The role of IL-6 trans-signalling in premature infants with chronic lung disease (CLD) remains unknown. Furthermore, its role in healthy term infants and adults has not been fully investigated. During episodes of inflammation, IL-6 trans-signalling has been shown to have a crucial role in regulating leukocyte recruitment during peritoneal inflammation in a murine model. Peritoneal inflammation is a complication of end-stage renal failure in patients on dialysis. An in vivo murine model of peritoneal inflammation and in vitro experiments using human peritoneal mesothelial cells (HPMC) have both shown IL-6 trans-signalling to stimulate MCP-1 upregulation and IL-8 downregulation. This triggered a transitional change in leukocyte recruitment from the early predominant neutrophil influx to the later sustained mononuclear cell influx (Hurst, Wilkinson et al. 2001). It is not known if this process occurs during an inflammatory response in human airway cells. As previously stated, IL-6 transsignalling requires IL-6 to be bound to its soluble IL-6 receptor (sIL-6R) and membrane bound gp130 in order for trans-signalling to occur. One mechanism by which sIL-6R can be freely available for IL-6 trans-signalling is by shedding of the IL-6R from cell membranes. Hurst et al demonstrated sIL-6R cleavage from leukocytes during peritoneal infection and upon binding to IL-6 was found to act as a regulator of MCP-1 and IL-8 expression. IL-6R is expressed by specific cell types, such as neutrophils and sIL-6R accumulation in peritonitis correlates with neutrophil influx. CLD is characterised by an abundance of neutrophils and high levels of pro-inflammatory cytokines, such as IL-6. Increased sIL-6R levels have also been detected (von Bismarck, Claass et al. 2008). The sIL-6R shedding from the neutrophils could be contributing to the increased sIL-6R in the airways of premature infants with CLD. It is not known if the IL-6 and sIL-6R are functional and capable of transsignalling. The possibility of defective expression of IL-6 and sIL-6R, therefore preventing IL-6 trans-signalling, could be responsible for the retention of neutrophils in the airways.

This first results chapter will investigate IL-6R expression on neutrophils in response to stimuli, to determine if they are the source of sIL-6R production. Expression of gp130 on neutrophils will also be assessed. IL-6R and gp130 expression will be compared between adults and babies to determine if trans-signalling may differ between term infants with an immature immune system compared to healthy adults with a fully developed immune system. Neutrophils will be obtained from the umbilical cord blood collected from term infants born 38-40 weeks gestation and from venous blood of healthy adult donors.

An appropriate methodology for investigating receptor expression on neutrophils would need to be established. Four different methods of isolating neutrophils and leukocytes were used to compare and analyse expression of IL-6R and gp130. Two of those methods were selected to compare receptor expression against neutrophils from term umbilical cord blood. Receptor expression in response to a bacterial peptide known as formyl-met-leu-phe (fMLP) and a pro-inflammatory cytokine, tumor necrosis factor-α (TNFα) was assessed. Neutrophils are known to be activated by fMLP and TNFα (Williams, Pettitt et al. 2007). It is not known how IL-6R and gp130 expression on neonatal neutrophils will be altered in response to these stimuli.

In addition, two receptors CD16 and CD11b are constitutively expressed on neutrophils were also investigated as controls. CD16, also known as FcyRIII, binds IgG immune complexes during an inflammatory episode and is expressed at high levels on neutrophils, making it an ideal positive control for flow cytometry. Shedding of CD16 from neutrophils has been observed in response to fMLP stimulation (Huizinga, Kerst et al. 1989; Huizinga, van Kemenade et al. 1989). Neonatal neutrophils may express substantially less CD16 than adult neutrophils (Maeda, van Schie et al. 1996). CD11b is expressed on unstimulated neutrophils and becomes upregulated 3- to 10- fold by fMLP stimulation. CD11b forms a membrane heterodimeric complex with CD18 and mediates neutrophil adherence to vascular endothelium during an inflammatory response (Vedder and Harlan 1988). CD11b was shown to be significantly elevated in neonates with a bacterial infection (Weirich, Rabin et al. 1998). CD11b is therefore an ideal marker of neutrophil responsiveness to stimulation.

The overall aim was to establish if IL-6R, gp130, CD16 and CD11b expression on neutrophils differed between adult and term neonates. Receptor expression was also to be monitored on adult and term neonatal neutrophils in response to stimuli. This would help identify potential differences in IL-6 trans-signalling between adults and neonates.

#### **3.2 Aims**

The specific aims of this chapter were to:

- 1. Establish the most suitable methodology for investigating receptor expression on neutrophils
- 2. Determine the membrane receptor expression of IL-6R, gp130, CD16 and CD11b on adult neutrophils.
- 3. Determine the membrane receptor expression of IL-6R, gp130, CD16 and CD11b on cord blood neutrophils obtained from healthy term neonates.
- 4. Determine the susceptibility of receptor expression to be altered in response to neutrophil stimulation with a bacterial peptide or pro-inflammatory cytokine.
- 5. Compare and contrast receptor expression on adult neutrophils with cord blood neutrophils.

The Department of Child health have the approval of the South-East Wales Research Ethics Committee to obtain adult and cord blood for use in the experiments conducted in this study.

#### 3.3 Results

#### 3.3.1 Receptor expression on neutrophils isolated by dextran and histopaque

#### 3.3.1.1 Methodology

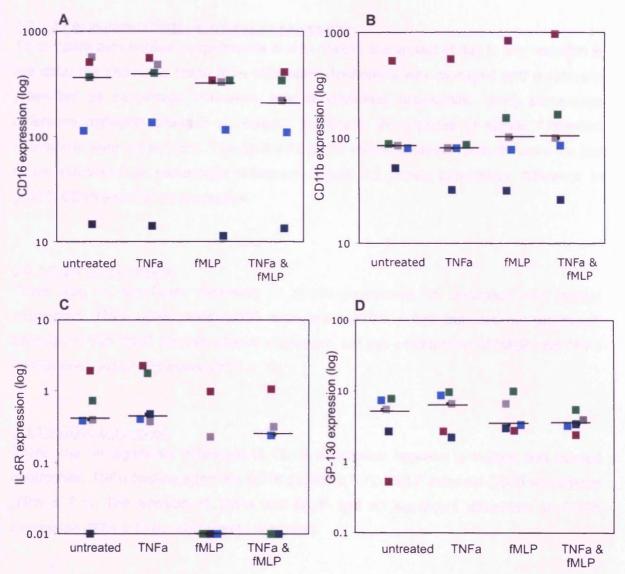
Blood was obtained from healthy adult donors and treated with dextran to remove the erythrocytes. The remaining layer of leukocytes was treated with histopaque to isolate the neutrophils. The histopaque method for neutrophil isolation has been widely used in the Child Health research group (Davies, Maxwell et al. 2008; McGreal, Davies et al. 2010) and is described in detail in Methodology section 2.4.1. Briefly, isolated neutrophils were treated with 2ng/ml TNF- $\alpha$  or 1µM fMLP or received both treatments prior to cell staining with PE conjugated antibodies specific for IL-6R, gp130, CD16, CD11b. Neutrophils were also stained with the corresponding isotype controls, IgG1 or IgG2a to measure background fluorescence. Membrane bound receptor expression was analysed by flow cytometry using CellQuest analysis software package. A geometric mean value was obtained which corresponded to the fluorescence intensity of receptor expression. The background fluorescence measured by the isotype controls were deducted from the geometric means for IL-6R, gp130, CD16 and CD11b. Flow cytometry data analysis is described in Methodology section 2.6.

Variation in the data between experiments was apparent. To analyse the data closely, geometric mean values obtained in response to stimuli from individual experiments were plotted on scatter graphs on a log scale. Figure 3.1 shows four scatter graphs representing IL-6R, gp130, CD16 and CD11b.

#### 3.3.1.2 Receptor expression

There was no significant difference between untreated and treated neutrophils for any of the four receptors.

IL-6R expression remained low for all experiments and became undetectable when treated with fMLP in two experiments. The downregulation of IL-6R by fMLP was not significant compared to unstimulated. Detection of gp130 expression was also low. CD16 and CD11b expression varied widely between experiments.



**Figure 3.1**: Geometric mean expression in log (with the isotype control deducted) of CD16 (A), CD11b (B), gp130 (C) and IL-6R (D) from five individual experiments. Five different coloured squares in each of the four column conditions represent an individual experiment. Identical coloured squares are data from the same experiment. Histopaque isolated neutrophils were left untreated or treated with 2ng/ml TNFα, 1μM fMLP or 2ng/ml TNFα and 1μM fMLP prior to staining with PE-conjugated antibodies for CD16, CD11b, gp130 and IL-6R. Receptor expression on neutrophils was obtained by flow cytometry analysis. Median receptor expression for each condition is indicated by a black line. Statistical analysis of was performed using one way ANOVA and Bonferroni post test, showed no significance in the data.

#### 3.3.1.3 Percentage difference in receptor expression

To compare data between experiments and to remove the impact of day to day variation in the data, the geometric mean from stimulated neutrophils was averaged and graphically presented as percentage difference against untreated neutrophils. Using percentage difference highlights changes in receptor expression in response to stimuli. Untreated neutrophils were set at 100%. The geometric mean values obtained for IL-6R were too low to be expressed as percentage difference. Figure 3.2 shows percentage difference in gp130, CD16 and CD11b expression.

#### 3.3.1.4 Gp130 expression

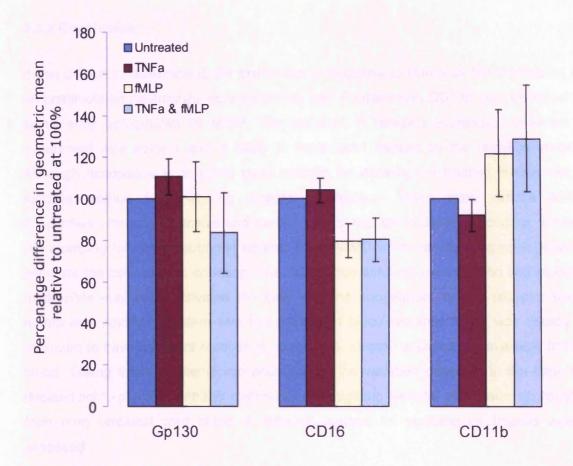
There was no significant difference in gp130 expression on untreated and treated neutrophils. TNF $\alpha$  upregulated gp130 expression (110%  $\pm$  8.6) but was not significant. Stimulation with fMLP had not altered expression but the combination of fMLP and TNF $\alpha$  had lowered gp130 expression (83%  $\pm$  19).

## 3.3.1.5 CD16 expression

There was no significant difference in CD16 expression between untreated and treated neutrophils. TNF $\alpha$  had no affect on CD16 (104%  $\pm$  5.7). FMLP reduced CD16 expression (79%  $\pm$  8.1). The addition of TNF $\alpha$  and fMLP had no significant difference on CD16 expression (80%  $\pm$  10.6) compared to untreated

#### 3.3.1.6 CD11b expression

There was no significant difference in CD11b expression between untreated and treated neutrophils. TNF $\alpha$  had no affect on CD11b (92%  $\pm$  7.6). FMLP reduced CD16 expression (79%  $\pm$  8.1). The addition of TNF $\alpha$  and fMLP had no significant difference on CD11b expression (128%  $\pm$  25.6) compared to untreated (figure 3.2).



**Figure 3.2**: Gp130, CD16 and CD11b expression on dextran and histopaque isolated neutrophils, stimulated with  $TNF\alpha$ , fMLP or both. Cells were stained with PE-conjugated antibodies and analysed by flow cytometry. Geometric means corresponding to fluorescence intensity and receptor expression were obtained. The data is expressed as percentage difference  $\pm$  SEM in geometric mean of treated neutrophils relative to the geometric mean of untreated neutrophils at 100%. Experiments are n = 5. Statistical analysis by non parametric ANOVA and Kruskal-Wallis with Dunn's multiple comparisons test.

#### 3.3.2 Conclusion

It was difficult to determine IL-6R expression in response to stimuli as the expression levels on unstimulated neutrophils were extremely low. Furthermore, CD11b was expected to be significantly upregulated by fMLP. The variation in receptor expression between each experiment was evident and is likely to have been caused by the isolation procedure. Although histopaque is a widely used method for isolating neutrophils, it may not be a suitable method for analysing receptor expression. There were various problems encountered despite extensive and careful efforts with the isolation procedure. There was cell clumping following neutrophil isolated from PBMC which required vigorous pipetting to separate the cells prior to counting. The histopaque solution, manipulation and pipetting of neutrophils may have activated the cells and the subsequent loss of receptor from the membrane. Another problem was the volume of blood required which was usually 10ml minimum to have sufficient number of neutrophils. Limited volumes are available from cord blood. Taking these problems into account and the variation observed in the data, it was decided not to proceed with this method for investigating receptor expression on neutrophils from term umbilical cord blood. A different method for purifying neutrophils would be assessed.

#### 3.3.3 Receptor expression on neutrophils isolated by dextran and percoll

#### 3.3.3.1 Methodology

Percoll is a widely used density medium for purifying neutrophils and the method used here was established by the Renal Immunobiology group, University of Birmingham (Williams, Pettitt et al. 2007). Purification of neutrophils by percoll gradients (described in section 2.4.2) was followed by treatment with 2ng/ml TNFα or 1μM fMLP or both prior to staining with PE-conjugated antibodies.

# 3.3.3.2 Receptor expression

The geometric mean corresponding to receptor expression was obtained and the individual data points from each experiment were plotted on log scatter graphs.

# 3.3.3.3 IL-6R expression

As shown in figure 3.3, IL-6R expression was slightly higher compared to IL-6R on histopaque isolated neutrophils. Furthermore, FMLP had significantly downregulated IL-6R, which had not been observed on histopaque isolated neutrophils.

#### 3.3.3.4 Gp130 expression

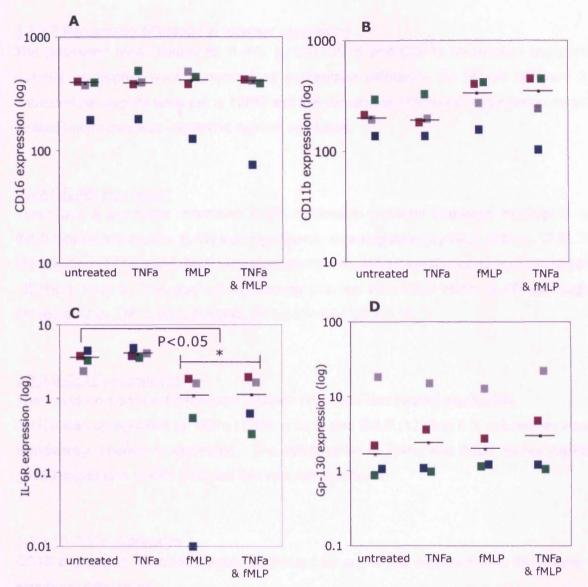
Gp130 expression was variable between experiments. There was no difference in gp130 expression between untreated and treated neutrophils (figure 3.3).

# 3.3.3.5 CD16 expression

CD16 expression was also variable between experiments. Stimulation with fMLP or TNF $\alpha$  had no significant effect on CD16 expression.

# 3.3.3.6 CD11b expression

CD11b was upregulated by fMLP but this was not significant. TNF $\alpha$  had no effect on CD11b expression.



**Figure 3.3**: The graphs show the geometric mean expression (isotype control deducted) of CD16 (A), CD11b (B), IL-6R (C) and gp-130 (D) of four individual experiments. Individual experiments are represented by four different coloured squares for each condition. Identical coloured squares represent data from the same experiment. The median receptor expression for each condition is shown by a black line. Dextran and percoll isolated neutrophils were untreated or treated with 2 ng/ml TNF $\alpha$ ,  $1 \mu$ M fMLP. Cells were stained with PE-conjugated antibodies and analysed by flow cytometry to determine receptor expression. Experiments are n = 4. Statistical analysis by non parametric ANOVA and Kruskal-Wallis with Dunn's multiple comparisons test.

#### 3.3.3.7 Percentage difference in receptor expression

The geometric mean values for IL-6R, gp130, CD16 and CD11b on dextran and percoll isolated neutrophils were presented as percentage difference. As shown in figure 3.4, untreated neutrophils were set at 100% and the percentage difference in geometric mean of treated neutrophils was calculated against untreated.

### 3.3.3.8 IL-6R expression

There was a significant difference IL-6R expression between untreated neutrophils and fMLP treated neutrophils. IL-6R was significantly downregulated by fMLP (44%  $\pm$  12.8). The combination of TNF $\alpha$  and fMLP also significantly reduced expression compared to untreated (35.9%  $\pm$  14.2) but this was not significantly different from fMLP alone. IL-6R was slightly upregulated by TNF $\alpha$  alone but was not significant (121%  $\pm$  16.7).

#### 3.3.3.9 Gp130 expression

There was no significant difference between untreated and treated neutrophils.

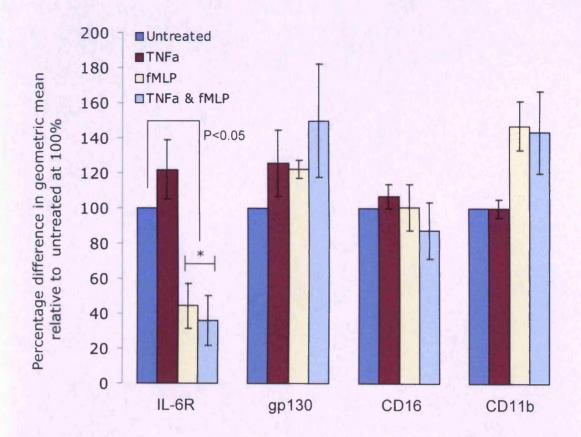
Gp130 was upregulated by TNF $\alpha$  (125%  $\pm$  18.7) and fMLP (122%  $\pm$  5.0) but neither were a significantly different to untreated. The combination of TNF $\alpha$  and fMLP further elevated gp130 expression (146%  $\pm$  32) but this was not significant.

#### 3.3.3.10 CD16 expression

CD16 expression remained largely unaffected by any of the treatments as there were no significant differences.

#### 3.3.3.11 CD11b expression

CD11b was unaffected by TNF $\alpha$  (99%.  $\pm$  5.3) but was upregulated by fMLP (147 %  $\pm$  13.9), although this was not significant (figure 3.4).



**Figure 3.4**: *IL-6R*, *gp130*, *CD16* and *CD11b* expressed as percentage difference  $\pm$  SEM to highlight differences in receptor expression on treated neutrophils compared to untreated neutrophils (set at 100%). Neutrophils isolated by dextran and percoll, treated with 2ng/ml TNF $\alpha$ , 1 $\mu$ M fMLP or both. Cells were stained with PE-conjugated antibodies and analysed by flow cytometry to determine receptor expression. Experiments are n=4. Statistical analysis by non parametric ANOVA and Kruskal-Wallis with Dunn's multiple comparisons test. Significance is represented by \* = p < 0.05.

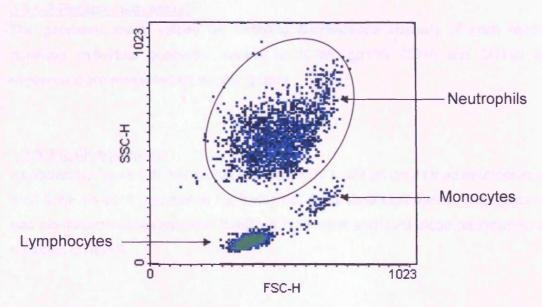
#### 3.3.4 Conclusion

Although IL-6R expression is slightly higher and perhaps less varied than observed in the histopaque method, it was still a concern that there may be loss of receptor as a result of the neutrophil isolation method. This may explain the variation seen in the individual data points, particularly for gp130 expression. In addition, the increase in CD11b in response to fMLP was not significantly different from unstimulated levels, against expectations. Similarly to the histopaque technique, at least 10ml volumes of blood from adults would be necessary to have sufficient numbers of neutrophils. As the volume of cord blood varies, this method would not be ideal. It was decided that other methods of isolating neutrophils would be explored to confirm if the receptor expression observed was a true reflection of the applied stimuli or was influenced by the histopaque and percoll separation techniques.

# 3.3.5 Receptor expression on neutrophils within a dextran-isolated leukocyte population

#### 3.3.5.1 Methodology

Isolating neutrophils by using histopaque or percoll may affect membrane receptors. As receptor expression was variable, it was decided to isolate leukocytes by using dextran only. The receptor expression on neutrophils was to be analysed within an isolated leukocyte population. Neutrophils can be identified from other cell populations based on their size and granularity as shown in the SSC-H/FSC-H graph in figure 3.5. The FSC-H is proportional to the diameter of the cell and the SSC-H is proportional to the granularity. The neutrophils are 'gated' to exclude the analysis of other cells, such as lymphocytes and monocytes, within the population.



**Figure 3.5**: An example of a dot plot obtained using WinMDI. FSC-H is forward scatter and represents the size of a cell. SSC-H is side scatter and represents cell granularity. The different population of cells are distinguished by their size and granularity.

Neutrophils from the cord blood of term infants were to be analysed for receptor expression along side neutrophils from adult blood.

Blood was obtained from healthy adult donors and from the umbilical cords of healthy term neonates. Both were treated with dextran and centrifuged as outlined in method section 2.4.3. Briefly, leukocytes were treated with 2ng/ml TNFα, 1μM fMLP or received both treatments. After staining with PE-conjugated antibodies, receptor expression on the neutrophils was analysed by flow cytometry and CellQuest analysis software package.

#### 3.3.5.2 Receptor expression

The geometric mean values representing fluorescence intensity of each receptor were obtained. Individual geometric means for IL-6R, gp130, CD16 and CD11b from each experiment are presented on scatter graphs.

# 3.3.5.3 IL-6R expression

As shown by figure 3.6, the overall expression of IL-6R on cord blood neutrophils was lower than IL-6R on adult neutrophils but it was not a significant difference. Stimulation with fMLP had significantly downregulated IL-6R on both adult and cord blood neutrophils. TNF $\alpha$  had no affect on IL-6R.

#### 3.3.5.4 gp130 expression

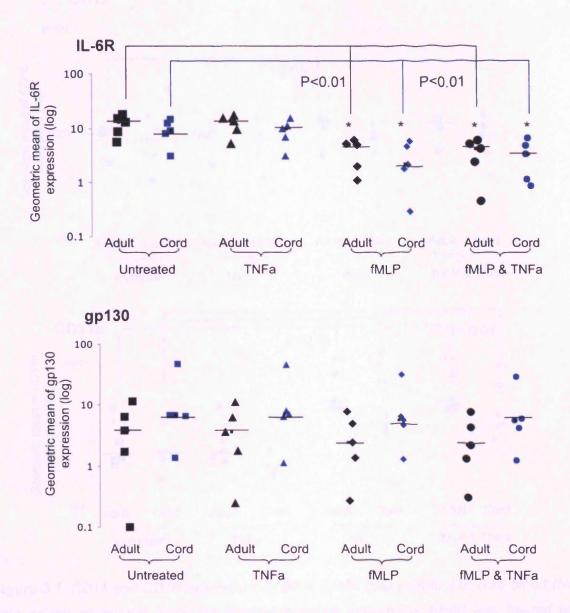
Cord blood neutrophils expressed more gp130 than adult PMN but this was not a significant difference. Expression of gp130 on cord blood and adult neutrophils varied widely between experiments. There was no significant difference between unstimulated and treated neutrophils (figure 3.6).

#### 3.3.5.5 CD16 expression

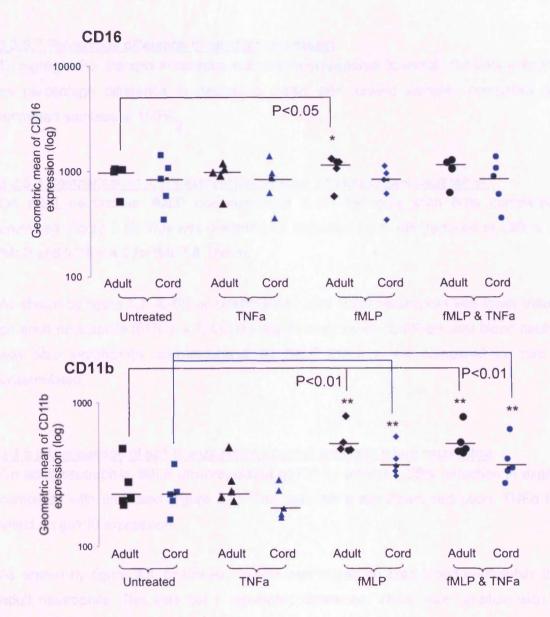
CD16 expression on cord blood neutrophils was lower than for adult neutrophils. This was not significant. FMLP had significantly elevated CD16 on adult neutrophils but had no affect on cord blood neutrophils. Receptor expression was not altered by TNFα (figure 3.7).

#### 3.3.5.6 CD11b expression

Receptor expression was similar between cord blood and adult neutrophils. CD11b was significantly elevated by fMLP. TNF $\alpha$  had no affect on receptor expression (figure 3.7).



**Figure 3.6**: IL-6R and gp130 expression on adult (black data points) and cord blood (blue data points) neutrophils within an isolated leukocyte population. Blood was treated with dextran and the isolated leukocytes were treated with 2ng/ml TNF $\alpha$  or 1 $\mu$ M fMLP or both combined. Cells were stained with PE-conjugated antibodies and analysed by flow cytometry to determine receptor expression. The median value for each is indicated by a black line. Experiments are n = 5. Statistical analysis was performed by one way ANOVA with Bonferroni post test.



**Figure 3.7**: CD16 and CD16 expression on adult (black data points) and cord blood (blue data points) neutrophils within an isolated leukocyte population. Blood was treated with dextran and the isolated leukocytes were treated with 2 ng/ml TNF $\alpha$  or  $1 \mu$ M fMLP or both combined. Cells were stained with PE-conjugated antibodies and analysed by flow cytometry to determine receptor expression. The median value for each is indicated by a black line. Experiments are n = 5. Statistical analysis was performed by one way ANOVA with Bonferroni post test.

#### 3.3.5.7 Percentage difference in receptor expression

To highlight the changes in receptor expression in response to stimuli, the data is expressed as percentage difference in geometric mean with treated samples compared against untreated samples at 100%.

# 3.3.5.8 Comparison of IL-6R expression on adult and term infant neutrophils

On adult neutrophils, fMLP downregulated IL-6R by more than 60% compared with untreated (figure 3.8). This was a significant reduction for IL-6R (reduced to 29%  $\pm$  3.1 for fMLP and 27%  $\pm$  4.0 for fMLP & TNF $\alpha$ ).

As shown by figure 3.7, IL-6R on unstimulated cord blood neutrophils was lower than IL-6R on adult neutrophils (61%  $\pm$  4.7) but not significantly lower. IL-6R on cord blood neutrophils was also significantly downregulated by fMLP (14%  $\pm$  4.4 compared to cord blood unstimulated).

#### 3.3.5.9 Comparison of gp130 expression on adult and term infant neutrophils

On adult neutrophils, fMLP downregulated gp130 by almost a 25% reduction in expression compared with untreated (figure 3.8). This was not a significant reduction. TNF $\alpha$  had no effect on gp130 expression.

As shown by figure 3.8, gp130 expression was higher on cord blood neutrophils than on adult neutrophils. This was not a significant difference. There was variation with gp130 expression between experiments. This variation in gp130 expression was not observed on adult neutrophils.

## 3.3.5.10 Comparison of CD16 expression on adult and term infant neutrophils

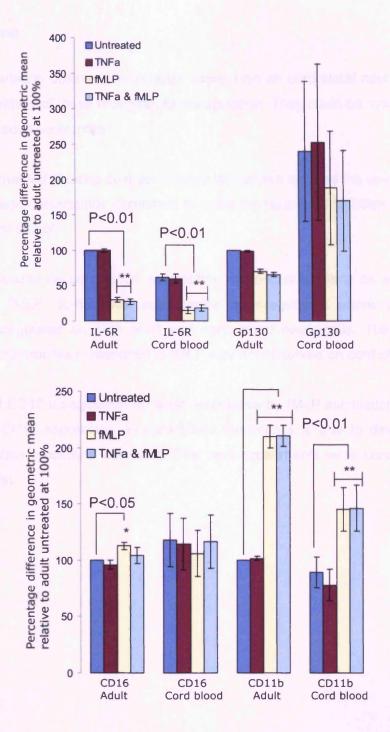
There was a minor but significant upregulation of CD16 expression in response to fMLP on adult neutrophils (112%  $\pm$  3.2). This was not observed on neutrophils isolated by histopaque or percoll. CD16 was not quite significantly altered by fMLP and TNF $\alpha$  combined or by TNF $\alpha$  alone (104%  $\pm$  7.1 and 95.7%  $\pm$  4 respectively). TNF- $\alpha$  had no significant affect on altering CD16 expression alone or combined with fMLP.

CD16 expression was not significantly different on cord blood neutrophils to adult neutrophils. However, there was variation in CD16 expression between experiments. This was not observed on adult neutrophils.

# 3.3.5.11 Comparison of CD11b expression on adult and term infant neutrophils

As shown in figure 3.8, CD11b on adult neutrophils was greatly upregulated by fMLP (209%  $\pm$  10) which was not observed on neutrophils isolated by percoll or histopaque. TNF- $\alpha$  had no significant affect on altering CD11b expression alone or combined with fMLP.

There were no significant difference in CD11b expression between untreated cord blood (88  $\pm$  13.7%) and adult neutrophils. Similarly to adult, CD11b on cord blood neutrophils also responded to fMLP by upregulating greatly to 145.1%  $\pm$  19.6.



**Figure 3.8**: Expression of CD16, CD11b, IL-6R and gp130 on adult and cord blood neutrophils from dextran isolated leukocytes. Data is shown as percentage difference in geometric mean  $\pm$  SEM set against adult untreated geometric mean (at 100%). Cells were stained with PE-conjugated antibodies and analysed by flow cytometry to determine receptor expression. Neutrophils were gated on the FSC-H/SSC-H analysis plot and the geometric mean value for fluorescence intensity of each receptor was obtained. Experiments are n = 5. Statistical analysis of percentage difference was performed using Kruskal-Wallis non parametric ANOVA test and Dunn's multiple comparisons post-test.

#### 3.3.6 Conclusion

Some of the variation observed in receptor expression on cord blood neutrophils could be due to the sensitivity of these receptors to manipulation. They could be more susceptible to activation than adult neutrophils.

Generally, the method of using dextran-isolated leukocytes reduced the variation of receptor expression on adult neutrophils, compared to using the neutrophil isolation techniques such as histopaque or percoll.

Receptor expression on cord blood neutrophils responded similarly as adult neutrophils, particularly to fMLP. IL-6R was significantly downregulated where as CD11b was significantly upregulated on both adult and cord blood neutrophils. The upregulation of CD16 on adult neutrophils in response to fMLP was not observed on cord blood neutrophils.

To determine if CD16 upregulation on adult neutrophils by fMLP stimulation or the variation in gp130 and CD16 expression on cord blood neutrophils is due to dextran, a different leukocyte isolation procedure was used. The next experiments were conducted on whole blood stimulation.

# 3.3.7 Receptor expression on neutrophils from ACK lysed whole blood isolation method

#### 3.3.7.1 Methodology

Variation in gp130 and CD16 expression was observed on cord blood neutrophils from dextran isolated leukocytes. In an attempt to further reduce the manipulation of cells and minimise the variation seen in cord blood and adult receptor expression, whole blood was treated with ACK lysis buffer instead of dextran. ACK is an ammonium-chloride-potassium solution that disrupts the erythrocyte membranes by hypotonic lysis. The mechanism of action is an accumulation of ACK within the cell, which increases the osmotic pressure resulting in the rupture of the erythrocyte membrane.

As described in method section 2.4.6, 2-4ml of whole blood was obtained from healthy adult donors and term neonates. The blood was treated with 2ng/ml TNF $\alpha$  or  $1\mu$ M fMLP or received both treatments. Erythrocytes were lysed with ACK solution. Cells were stained with PE-conjugated antibodies and analysed by flow cytometry analysis to determine the expression of IL-6R, gp130, CD16 and CD11b.

#### 3.3.7.2 Receptor expression

The geometric mean values representing fluorescence intensity of each receptor were obtained. Individual geometric means for IL-6R, gp130, CD16 and CD11b from each experiment are presented on scatter graphs.

#### 3.3.7.3 IL-6R expression

The overall expression of IL-6R on cord blood neutrophils was lower than IL-6R on adult neutrophils but it was not a significant difference (figure 3.9). Stimulation with fMLP had significantly downregulated IL-6R on both adult and cord blood neutrophils. TNF $\alpha$  had no affect on IL-6R.

# 3.3.7.4 gp130 expression

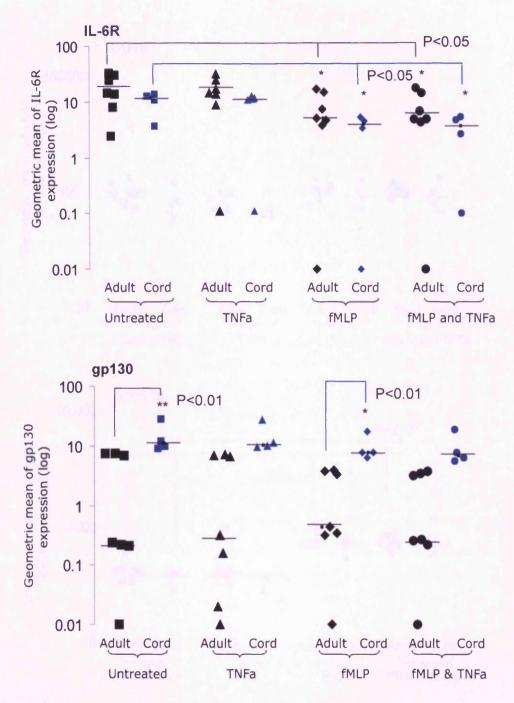
Cord blood neutrophils significantly expressed more gp130 than adult neutrophils. Gp130 expression on adult neutrophils varied widely between experiments. There was no significant difference between unstimulated and treated neutrophils (figure 3.9).

# 3.3.7.5 CD16 expression

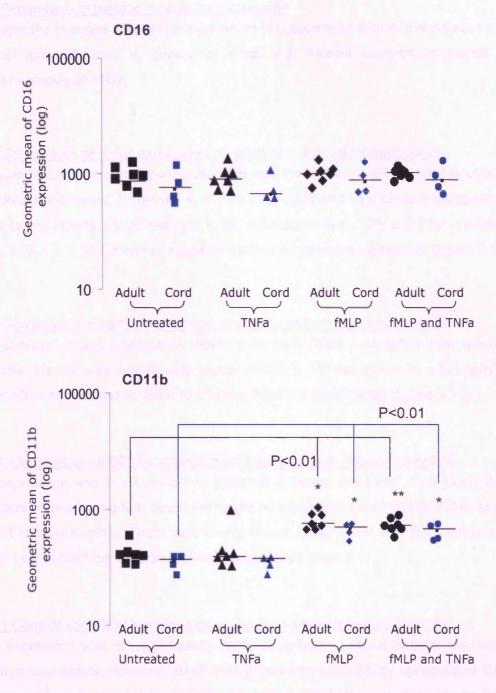
CD16 expression on cord blood and adult neutrophils were not significantly different. Stimulation with fMLP had no significant impact on adult or cord blood neutrophils. This is in contrast to dextran-isolated leukocytes in which an elevation of CD16 on adult neutrophils in response to fMLP was observed. Receptor expression was not altered by TNFα (figure 3.10).

# 3.3.7.6 CD11b expression

CD11b expression was similar between cord blood and adult neutrophils. FMLP significantly elevated CD11b on cord blood and adult neutrophils. TNF $\alpha$  had no affect on receptor expression (figure 3.10)



**Figure 3.9**: *IL-6R* and *gp130* expression on adult (black data points) and cord blood (blue data points) neutrophils within an isolated leukocyte population. Blood was lysed with ACK and the isolated leukocytes were treated with 2ng/ml TNF $\alpha$  or  $1\mu$ M fMLP or both combined. Cells were stained with PE-conjugated antibodies and analysed by flow cytometry to determine receptor expression. The median value for each is indicated by a black line. Experiments are n = 7 for adults and n = 4 for cord blood. Statistical analysis was performed by one way ANOVA with Bonferroni post test. For cord blood and adult comparisons, statistical analysis was performed by Kruskal-Wallis non parametric test.



**Figure 3.10**: CD16 and CD11b expression on adult (black data points) and cord blood (blue data points) neutrophils within an isolated leukocyte population. Blood was lysed with ACK and the isolated leukocytes were treated with 2ng/ml TNF $\alpha$  or  $1\mu$ M fMLP or both combined. Cells were stained with PE-conjugated antibodies and analysed by flow cytometry to determine receptor expression. The median value for each is indicated by a black line. Experiments are n = 7 for adults and n = 4 for cord blood. Statistical analysis was performed by one way ANOVA with Bonferroni post test. For cord blood and adult comparisons, statistical analysis was performed by Kruskal-Wallis non parametric test.

#### 3.3.7.7 Percentage difference in receptor expression

To highlight the changes in receptor expression in response to stimuli, the data is expressed as percentage difference in geometric mean with treated samples compared against untreated samples at 100%.

#### 3.3.7.8 Comparison of IL-6R expression on adult and term infant neutrophils

Expression of IL-6R on cord blood neutrophils was lower than IL-6R on adult neutrophils but not significantly different. Moreover, IL-6R on both adult and cord blood neutrophils reacted similarly to treatments. FMLP reduced IL-6R expression from 72%  $\pm$  9.7 for untreated cord blood to 25%  $\pm$  7.3. TNF $\alpha$  had no significant affect on receptor expression (figure 3.11).

#### 3.3.7.9 Comparison of gp130 expression on adult and term infant neutrophils

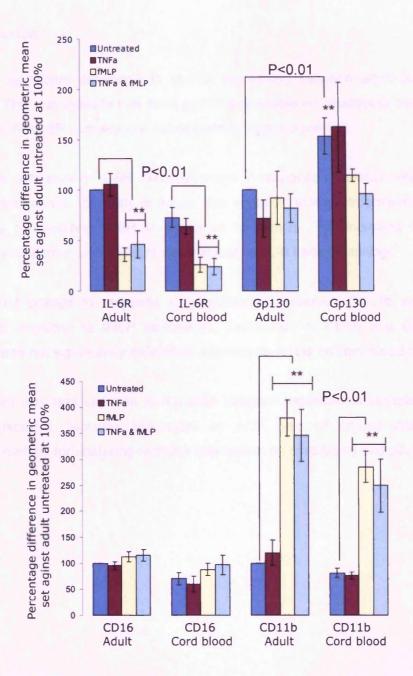
Gp130 expression was significantly different on cord blood neutrophils compared to adult neutrophils. Gp130 was significantly higher (153%  $\pm$  18) compared to adult gp130. Cord blood gp130 was reduced by fMLP to 114%  $\pm$  6 but not significantly (figure 3.11).

#### 3.3.7.10 Comparison of CD16 expression on adult and term infant neutrophils

CD16 expression was not significantly different between untreated cord blood and adult neutrophils. Similarly to adult, treatment made no significant difference to CD16 expression on cord blood neutrophils. There was no significant upregulation by fMLP, which was seen on adult neutrophils from dextran-isolated leukocytes (figure 3.11).

# 3.3.7.11 Comparison CD11b expression on adult and term infant neutrophils

CD11b expression was not significantly different between untreated adult neutrophils and cord blood neutrophils. However, fMLP stimulation had significantly upregulated CD11b on cord blood and adult neutrophils. TNF $\alpha$  had not altered CD11b expression (figure 3.11).



**Figure 3.11**: Percentage difference in geometric mean for CD16, CD11b, IL-6R, gp130 on adult and cord blood neutrophils within an ACK treated leukocyte population. Whole blood was lysed with ACK lysis buffer to isolate leukocytes. Following treatment and staining with PE-conjugated antibodies, neutrophils were gated on the FSC-H/SSC-H dot plot and analysed by flow cytometry. Receptor expression on adult treated, cord untreated and treated neutrophils are relative to adult untreated neutrophils (100%). Experiments are n = 7 for adult and n = 4 for cord. Statistical analysis of percentage difference was performed using Kruskal-Wallis non parametric ANOVA test and Dunn's multiple comparisons test.

#### 3.3.8 Conclusion

There is a significant difference in gp130 expression between adult and cord blood neutrophils. This may indicate that more gp130 is available on neutrophils from term infants, to bind the IL-6/sIL-6R complex and subsequently trigger signalling.

There is little difference in IL-6R expression and in response to stimuli between adult and cord blood neutrophils. Similarly to adults, this suggests that neutrophils from term infants are capable of shedding IL-6R in response to stimuli. This indicates that during an inflammatory response, sIL-6R could be available for IL-6 trans-signalling.

CD16 did not change in response to stimulations. However, CD11b was significantly increased in response to fMLP stimulation. Expression of CD16 and CD11b on adult neutrophils was not significantly different to expression levels on cord blood neutrophils.

Overall, there was less variation in the data between experiments compared to dextranisolated leukocytes. Isolating leukocytes by ACK lysis of erythrocytes is the most appropriate method for analysing receptor expression on cord blood and adult neutrophils.

# 3.3.9 Overall comparison of four methods for isolating neutrophils for investigating receptor expression

Receptor expression on untreated and on fMLP-stimulated neutrophils were graphically compared between four different isolation techniques. Figure 3.12 and 3.13 shows four graphs representing each of the four receptors.

# 3.3.9.1 Adult neutrophil receptor expression

IL-6R, CD16 and CD11b expression was significantly lower on histopaque or percoll isolated neutrophils compared to dextran-isolated leukocytes and ACK treated leukocytes.

Although gp130 expression was slightly altered between methods, there were no significant differences between the four isolation methods.

#### 3.3.9.2 Cord blood neutrophil receptor expression

There was no significant difference in receptor expression for cord blood neutrophils between dextran-isolated leukocytes and ACK-treated leukocytes.

The response of receptors on cord blood neutrophils to fMLP treatment was similar to the response observed with adult neutrophil receptors, regardless of isolation method.

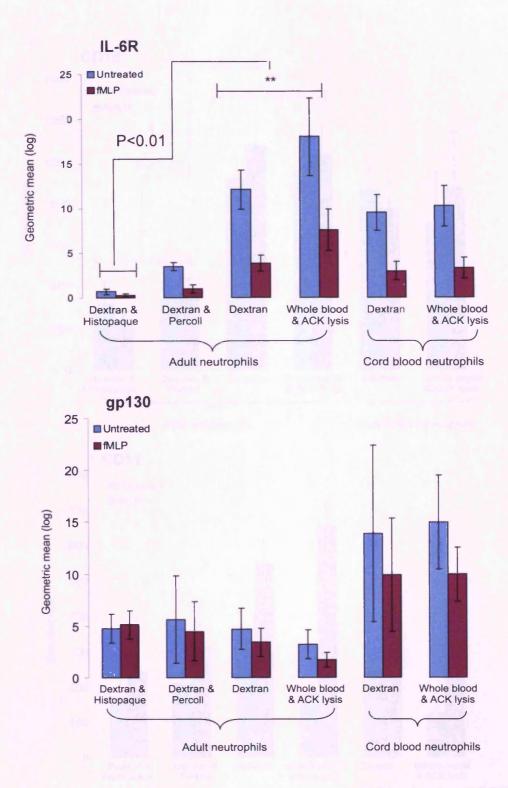


Figure 3.12: Comparison of receptor expression on untreated (blue bars) and fMLP-treated (purple bars) neutrophils from adult and cord blood isolated by four different methods: dextran and histopaque, dextran and percoll, dextran-isolated leukocytes and leukocytes from ACK lysed whole blood. Statistical analysis was performed by non-parametric one way ANOVA and Kruskal-Wallis test with Dunn's multiple comparisons' post test.

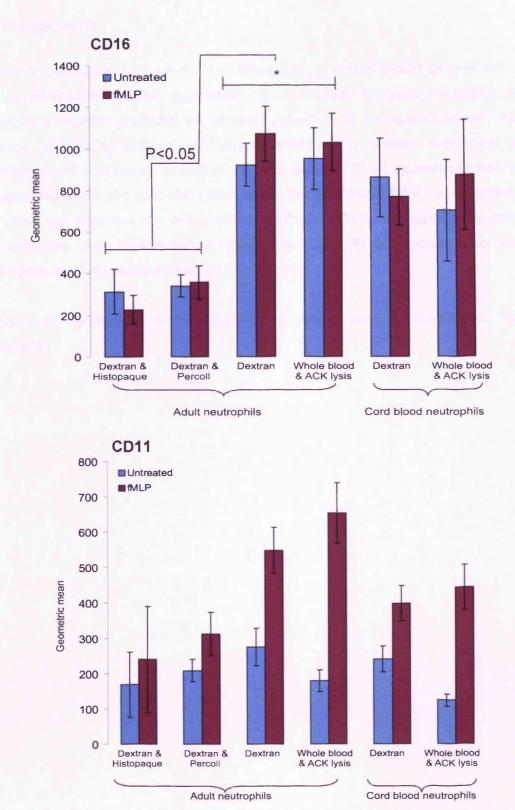


Figure 3.13: Comparison of receptor expression on untreated (blue bars) and fMLP-treated (purple bars) neutrophils from adult and cord blood isolated by four different methods: dextran and histopaque, dextran and percoll, dextran-isolated leukocytes and leukocytes from ACK lysed whole blood. Statistical analysis was performed by non-parametric one way ANOVA and Kruskal-Wallis test with Dunn's multiple comparisons' post test.

#### 3.3.10 Conclusion

Neutrophil purification methods such as histopaque or percoll should be used with caution for the analysis of receptor expression on neutrophils. Analysing neutrophils within a leukocyte population produced an accurate reflection of the applied stimuli. Using two methods of isolating leukocytes produced similarities in receptor expression on adult neutrophils and cord blood neutrophils. Similar levels of IL-6R appeared to be expressed on untreated adults and cord blood neutrophils. Treatment with fMLP downregulated IL-6R; this effect was observed with both isolation methods. CD11b was significantly upregulated on neutrophils using both leukocyte isolation methods. Gp130 expression on cord blood neutrophils was less variable using ACK lysed cells.

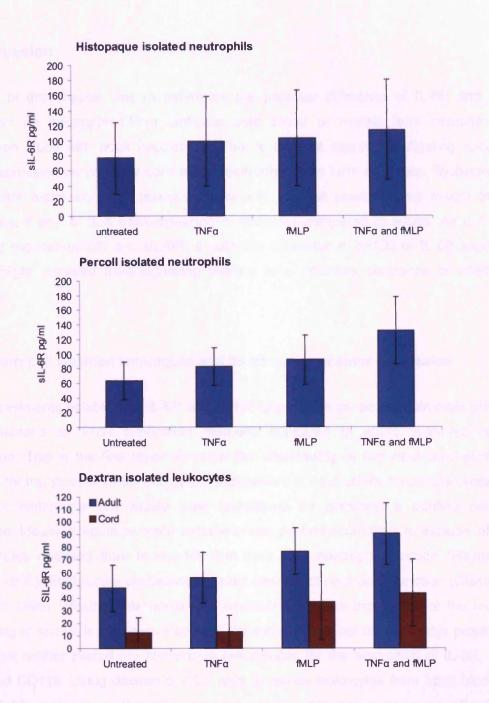
Overall, an appropriate methodology for analysing receptor expression has been established.

# 3.3.11 Do neutrophils and leukocytes produce sIL-6R and sgp130?

The airways of infants with CLD have an abundance of neutrophils and increased concentrations of pro-inflammatory cytokines, including IL-6 (Kotecha, Wilson et al. 1996). Unpublished observations in the Child Health group have observed an increase in sIL-6R in bronchoalveolar lavage fluid from infants with CLD compared to healthy infants. The increase in sIL-6R could be due to neutrophils shedding their IL-6R.

Supernatants were collected from histopaque and percoll isolated neutrophils (from adult blood only), and from dextran-isolated leukocytes (from adult and cord blood for the latter) following stimulation with 1μM fMLP or 2ng/ml TNFα or both treatments. An ELISA was used to measure slL-6R and sgp130 in the supernatants. Very low levels of slL-6R were detected, which remained below 200pg/ml, regardless of stimuli (figure 3.14). The data in this chapter have shown a variation in membrane expression of IL-6R in response to the different neutrophil purification methods. This indicates that much of IL-6R on the neutrophils was already shed and the slL-6R removed prior to treating the cells with stimuli. SlL-6R was expected to be present in the supernatants from dextran-isolated leukocytes following fMLP stimulation. As very low levels were detected, this may indicate that the 2 x 10<sup>6</sup>/ml of leukocytes used in these experiments were not enough to produce substantial amounts of slL-6R.

There was no sgp130 detected in the supernatants (data not shown as the values were below the level of detection of the ELISA). Shedding of gp130 from neutrophils has not been previously reported. The lack of sgp130 in the supernatants from dextran-isolated leukocytes may indicate that neutrophils do not shed gp130. However, using a small population of leukocytes may not have produced enough sgp130 to be detected.



**Figure 3.14:** Soluble IL-6R (sIL-6R) was measured in supernatants taken from isolated neutrophils and leukocytes following treatment with  $1\mu M$  fMLP or 2ng/ml TNF $\alpha$  or both treatments. Blood was obtained from healthy adult donors and the neutrophils were isolated by histopaque or percoll. Leukocytes were isolated from adult and cord blood by dextran.

#### 3.4 Discussion

The aim of this chapter was to determine the potential difference of IL-6R and gp130 expression on neutrophils from umbilical cord blood of normal term neonates in a comparison study with adult neutrophils. This is the first report investigating IL-6R and gp130 expression on umbilical cord blood neutrophils from term neonates. By determining the receptor expression and response to stimuli, this will provide some insight into the differences, if any, of IL-6 trans-signalling in neonates compared to adults. As IL-6 trans-signalling requires gp130 and sIL-6R, a potential difference in gp130 or IL-6R expression may highlight impaired trans-signalling leading to a defective clearance of infection in neonates.

# 3.4.1 Neutrophil isolation techniques and its affect on receptor expression

Initial experiments establishing IL-6R and gp130 expression on adult neutrophils proved to be problematic as PMN purification methods appeared to affect observed receptor expression. This is the first report to show the unsuitability of two neutrophil purification methods for the study of IL-6R and gp130 expression on neutrophils. Neutrophil isolation by percoll or histopaque are widely used techniques for obtaining a purified neutrophil population. Ideally, it would be more suitable to use purified neutrophils to exclude influence of molecules released from leukocytes that may alter neutrophil function (Haynes and Fletcher 1990). Erythrocyte contamination may also affect neutrophil function (Glasser and Fiederlein 1990). Whether this would alter neutrophil receptor expression or the receptors responding to stimuli is unknown. However, the experiments conducted in this project have shown that neither percoll nor histopaque are suitable for the evaluation of IL-6R, gp130, CD16 and CD11b. Using dextran or ACK lysis to isolate leukocytes from adult blood were more reliable methods as the data was less variable between experiments. Cord blood neutrophils are more sensitive to dextran isolation as gp130 and CD16 expression was quite variable between experiments. ACK lysis had reduced some of this variability.

It is known that neutrophils are sensitive, fragile and susceptible to activation, particularly during neutrophil isolation procedures, which can alter function and membrane integrity (Quinn, Deleo et al. 2007). Various studies, which will be discussed here, have used whole blood and leukocyte based methods to study cell surface markers to reduce cell manipulation and use smaller blood volumes.

Although dextran-isolated leukocytes and leukocytes from ACK lysed whole blood had reduced receptor loss compared to histopaque or percoll isolation methods, there were subtle differences between both methods. For example, there was a significant upregulation of CD16 in response to fMLP on adult neutrophils from dextran-isolated leukocytes but not on neutrophils from ACK lysed whole blood. It is possible that the dextran sedimentation may sensitise adult neutrophils to fMLP stimulation, triggering intracellular signalling and activating molecules involved in the upregulation of CD16. Furthermore, IL-6R expression was higher on neutrophils from ACK lysed whole blood than on neutrophils from dextran isolation. The most interesting difference is the gp130 expression on cord blood neutrophils was significantly higher compared to adult neutrophils. This was observed on ACK isolated neutrophils. Although gp130 expression was also high on cord blood neutrophils isolated by dextran but it was not significant.

Although these two methods had reduced receptor loss and reduced the variability of IL-6R, gp-130, CD16 and CD11b expression on adult neutrophils, these subtle differences may highlight the sensitivity of neutrophils and receptors to activation. This could indicate a potential methodological problem if minor manipulation of neutrophils alters their function and integrity. In light of this, interpretation of the data needs to be done with caution.

# 3.4.2 IL-6R and gp-130 expression

The findings from this chapter have shown baseline IL-6R was lower on adult neutrophils isolated by histopaque or percoll than on neutrophils from dextran isolated leukocytes or ACK lysed whole blood. In addition, a reduction of IL-6R by fMLP had been observed for all four methods. The assumption was that a loss of IL-6R would indicate shedding. As a result, the sIL-6R in supernatants would increase (Marin, Montero-Julian et al. 2002). Measuring sIL-6R in supernatants from isolated neutrophils revealed undetectable sIL-6R. This was likely to be due to the neutrophils losing their IL-6R in response to the percoll and histopaque purification methods. Soluble IL-6R was measured in supernatants from unstimulated leukocytes. Less than 200pg/ml was detected. Surprisingly, fMLP stimulation did not alter these levels. Marin et al had measured 500pg/ml in the supernatants from unstimulated neutrophils which increased to 1500 pg/ml in response to fMLP stimulation. They had suggested neutrophil degranulation as a result of stimulation with fMLP, may have caused the increase in sIL-6R (Marin, Montero-Julian et al. 2002). There is a possibility that the sIL-6R observed by Marin et al was actually produced from apoptotic neutrophils. By

using strong stimuli to evoke a response as Marin et al had used, it may have triggered apoptosis and subsequently the cleavage of IL-6R. Although earlier studies by Jones et al have shown fMLP to cause IL-6R shedding (Jones, Novick et al. 1999), more recent work by Chalaris et al has shown IL-6R is also naturally shed in response to neutrophil apoptosis. They reported the shedding of IL-6R can occur via PKC activation or independently of PKC by bacterial toxins or cholesterol depletion. In most cases, IL-6R is dependent on metalloproteinase ADAM 17 (Matthews, Schuster et al. 2003; Chalaris, Rabe et al. 2007) which in turn is dependent on caspase 3, 8 and 9 activation during apoptosis (Chalaris, Rabe et al. 2007). Possible explanation for lower levels of sIL-6R observed in this project would be i) the neutrophils were not apoptotic and a 15 min incubation with 1µM fMLP was not enough to evoke it, and therefore no IL-6R would be shed or ii) IL-6R had been shed but the neutrophils had degranulated, opening up the membrane and releasing proteolytic enzymes from intracellular granules causing degradation of sIL-6R. Performing experiments such as i) measuring CD63 expression by flow cytometry analysis would indicate degranulation, ii) adding protease inhibitors, would inhibit any potential sIL-6R digestion or iii) staining cells with annexin-V/topro 3 to measure apoptosis and necrosis. It is possible that fMLP or other bacterial-stimulating factors may not induce direct shedding but instead induce neutrophil apoptosis, presumably after phagocytosis under diseased states and inflammatory conditions. FMLP triggers IL-6R downregulation but not necessarily direct activation of ADAM 17 to cause shedding. Intracellular staining to highlight the potential internalisation of the receptor following downregulation could identify signalling molecules. This may clarify a signalling pathway used by both and establish if a link is present (Marin, Montero-Julian et al. 2002). The high sIL-6R levels detected in BALs and TAFs from premature infants with CLD may be due to the accumulating neutrophils undergoing apoptosis. They subsequently shed their IL-6R.

An interesting study by Dekkers *et al* using a bacterial component known as lipopolysaccharide (LPS) demonstrated decreased IL-6R on neutrophils from heparin treated whole blood, in a time dependent manner. There was no change in gp130 expression in response to LPS. Dekkers *et al* had also shown that without stimulus, gp130 expression naturally decreased after a four hour incubation at 37°C (Dekkers, Juffermans et al. 2000). Unfortunately, sIL-6R and sgp130 were not measured in the supernatants in their study. It is not known if gp130 was shed or internalised. In a similar study by Marsik *et al*, sIL-6R and sgp130 were measured in plasma taken from healthy male patients administered with LPS and found no difference from untreated blood. Marsik *et al* had reportedly not been able to reproduce the LPS downregulation of IL-6R observed in the

study by Dekkers *et al*, when using a lower concentration of 50pg/ml. In contrast a substantial increase in gp130 expression had been observed (Marsik, Halama et al. 2005). In the current project, stimulating neutrophils with a bacterial component fMLP had significantly reduced IL-6R. Furthermore, a reduction in gp130 on adult neutrophils was observed. Although it was not a significant reduction, it was observed on neutrophils from two leukocyte isolation methods. A reduction in gp130 expression in response to fMLP has not been previously reported.

Another study assessing IL-6R on neutrophils following treatment with a chemical that induces apoptosis showed no change in IL-6R levels. Jablonksi *et al* used N-nitrosodimethyloamine (NDMA) to trigger apoptosis of purified neutrophils which was measured by 'Cell death detection' ELISA assay. Although they claim to have seen increasing dose-dependent apoptosis in response to increasing NDMA concentration, flow cytometry analysis of PE-conjugated stained neutrophils showed a retained IL-6R. However, there are some flaws in their data analysis. The isotype control was not shown on the flow cytometry plots and background mean fluorescence was not stated. The mean fluorescence of unstimulated IL-6R on purified neutrophils was not stated and shows a potential problem in their methodology as well as interpretation of their published data. In addition, they had excluded data which showed variation in IL-6R in response to the higher doses of NDMA, which they refer to as 'ambigious' data and suggest the cells were necrotic (Jablonski, Jablonska et al. 1999).

To summarise my findings, IL-6R expression was slightly lower on cord blood neutrophils than on adult (but not significantly). It had responded to fMLP in the same manner as adult IL-6R, by significantly downregulating. On the other hand, gp130 expression was much higher on cord blood neutrophils than on adults. This was observed on neutrophils within a leukocyte population that had been isolated by two different methods. As a result, the differences observed in gp130 expression between adult and cord blood is unlikely to be an artefact of neutrophil manipulation and activation.

# 3.4.3 Findings from other researchers: CD16 and CD11b expression

There have been some studies on CD16 but most have investigated CD11b on leukocytes from adults and neonates.

CD16 is highly expressed on neutrophils, as shown by Middelhoven *et al* and the current findings in this project. However, in contrast, Middelhoven *et al* showed stimulation with fMLP caused shedding of CD16 (Middelhoven, Van Buul et al. 2001). They had isolated the neutrophils by percoll purification which may have sensitised the neutrophils and contributed to the subsequent loss of receptor. They reported a loss of CD16 expression occurred by the neutrophil undergoing apoptosis (Middelhoven, Van Buul et al. 2001).

Takahashi N et al used a whole blood method to analyse CD16 in healthy term neonates and found that untreated neutrophils expressed lower CD16 compared to adults. Interestingly, they had observed a significant increase in CD16 on neonate neutrophils after 48 hour incubation with IFN-y but found no upregulation on adults (Takahashi 1993).

Macey et al used four leukocyte isolation methods to investigate CD11b expression in adult neutrophils. Interestingly they stated that using sodium citrate (as used here), rather than using heparin or EDTA to anti-coagulate the blood, minimises the change in receptor expression when blood is at 37°C or 4°C. They showed that neutrophils that were isolated by dextran sedimentation and histopaque had increased CD11b expression compared to neutrophils in whole blood that had not received this treatment. An even higher upregulation of CD11b was observed on granulocytes that had been dextran-sedimented and then had erythrocytes lysed with water. Washing neutrophils in HBSS + Ca²+ + Mg²+ + Hepes following dextran sedimentation or histopaque isolation also upregulated CD11b compared to unwashed cells. They had reported that any stimulation of the neutrophil membrane may activate the translocation of CD11b from intracellular stores to the cell surface. However, similarly to the results in this project, they observed increased CD11b expression in response to fMLP incubation with neutrophils regardless of preparation (Macey, McCarthy et al. 1995).

Alvarez-Larrán et al assessed CD11b expression on unstimulated and phorbal myristate acetate (PMA)-stimulated neutrophils within a mixed leukocyte population. The leukocytes were isolated from whole blood by two different methods. In one method, the whole blood was lysed on ice and centrifuged following stimulation with PMA. The centrifuging and lysing

was excluded in the second method. In both methods, cells were incubated with the CD11b antibody and DRAQ-5. Nucleated cells are stained by DRAQ-5, which is a fluorescent dye. The main purpose of the dye is to distinguish the difference between erythrocytes and leukocytes during flow cytometry analysis, without physically removing the erythrocytes. Interestingly, CD11b on untreated cells was upregulated using the lysed and centrifuged method. It was also four fold higher after PMA treatment in comparison to the 'no-lyse, no-wash' method. They also noted a lower percentage of neutrophils remaining in the sample following PMA treatment using the lysed method (Alvarez-Larran, Toll et al. 2005). The whole blood was from 10 paediatric patients (age range was 3 months to 12 years, a mean age of 4 years) who had recurrent infections. The study assumed they had impaired neutrophil function but was not clinically diagnosed. It should be noted there was no healthy control group included in the study. Nevertheless, their study highlighted an important problem with neutrophil manipulation, artifactual activation and the possibility that lysing may cause priming.

Rebuck *et al* observed a reduction in CD11b expression on unstimulated neutrophils isolated by a density-gradient purification method (ficoll-hypaque) compared to whole blood in neonatal peripheral blood but not in healthy adults. In adults and neonates, fMLP significantly upregulated CD11b on neutrophils from whole blood but no effect was seen using the ficoll-hypaque isolation method. In some cases, using ficoll-hypaque resulted in undetectable CD11b expression on unstimulated neutrophils for both adult and neonates. They also stated that when there was expression, it was generally higher on neutrophils from term and preterm neonates than on adult neutrophils. Rebuck *et al* claim the differences seen was mainly due to the isolation method which may affect the cells ability to respond to fMLP, particularly in neonates (Rebuck, Gibson et al. 1995).

Previous studies have shown neonatal neutrophils respond differently than adult PMN to bacterial sepsis (Christensen 1989). Sepsis in neonates is associated with a positive bacterial blood culture, which can lead to neutropenia. Although diagnosis is still difficult to determine if blood cultures are negative when the neonates are symptomatic (Adib, Ostadi et al. 2007). This problem may be overcome by analysing PMN receptors, particularly CD11b as shown by Adib et al who determined CD11b sensitivity and specificity for the detection of sepsis in newborn infants at an early stage in the disease. This study highlighted the importance of measuring neutrophil receptors to aid early diagnosis of infections. A whole blood method was used to study neutrophil CD11b expression which excluded using density gradients for blood separation. Using blood isolation techniques that

do not harm cell surface markers by minimising cell manipulation would be critical for the diagnosis of infections (Adib, Ostadi et al. 2007).

#### 3.4.4 Conclusion

The findings in this chapter have highlighted methodological problems when assessing receptor expression on purified neutrophils. However in the current investigations, expression of IL-6R, CD11b and CD16 were similar between neutrophils from adults and term infant cord blood. A notable exception was gp130 expression which was significantly higher on cord blood neutrophils. With a high gp130 expression on cord blood neutrophils, this indicates that more gp130 molecules could be available to bind to sIL-6R/IL-6, which may also indicate a potential difference in IL-6 trans-signalling compared to adults. Furthermore, it is not known if the gp130 on cord blood neutrophils is functional for IL-6 trans-signalling.

Investigating receptor expression on neutrophils from premature infants with and without CLD would have been an ideal comparison study. Furthermore, investigating the effect of CLD on IL-6R and gp-130 expression would have provided more of an insight into the role and potential dysregulation of IL-6 trans-signalling in premature infants with CLD. Unfortunately for this study, cord blood from premature infants could not be obtained.

The next chapter will investigate IL-6 trans-signalling in adult neutrophils by determining the impact of IL-6 trans-signalling on neutrophil apoptosis.

# Chapter 4

The potential regulation of neutrophil apoptosis by IL-6 trans-signalling

#### 4.1 Introduction

Neutrophils are phagocytic granulocytes, produced in the bone marrow. In healthy adults, approximately 1011 mature neutrophils are produced daily and are released into the circulation, where they have a short half-life of 8-20 hours. They constitutively undergo a programmed cell death known as apoptosis. Apoptosis is characterised by cell shrinkage and is an essential process that prevents the release of the cells toxic anti-microbial contents to the surrounding tissues. It eliminates the neutrophils from areas of inflammation to prevent tissue damage. The apoptotic neutrophils are recognised by macrophages which remove the cells by phagocytosis (Kanduc, Mittelman et al. 2002; Akgul and Edwards 2003; Simon 2003; Scheel-Toellner, Wang et al. 2004). The life-span can be extended when the neutrophils leave the circulation and migrate to the site of infection or inflammation. Exposure of the cells to pro-inflammatory cytokines, such as granulocyte/macrophage colony stimulating factor (GM-CSF) and bacteria or bacterial components, such as lipopolysaccharides (LPS) significantly increases their survival (Akgul and Edwards 2003; Scheel-Toellner, Wang et al. 2004). Neutrophils have the ability to amplify the inflammatory response by releasing cytokines themselves (Simon 2003). Once the neutrophils have removed the pathogens, it is essential they undergo apoptosis and are safely eliminated. This process is vital for maintaining appropriate neutrophil numbers as well as ensuring the resolution of inflammation (Kanduc, Mittelman et al. 2002; Akgul and Edwards 2003; Simon 2003). When apoptosis fails, neutrophils can undergo another mode of death known as necrosis. With this process, neutrophils lose their membrane integrity and subsequently empty their harmful contents into the surrounding tissues leading to local tissue damage.

Delayed or otherwise dysregulated apoptosis can lead to the accumulation of aging neutrophils at sites of inflammation. This has been associated with many acute and chronic inflammatory diseases, such as acute respiratory distress syndrome (ARDS) and chronic lung disease (CLD) (Haslett 1999; Kotecha, Mildner et al. 2003). Infants with chronic lung disease have an abundance of neutrophils accumulating in the airways that fail to undergo apoptosis. The persistent presence of the neutrophils within the airways is possibly mediated by the increased numbers of chemokines and cytokines, such as IL-8, C5a and TNFα. This could be in response to continuous infection and inflammation in the airways of infants with CLD (Speer 1999; Koenig, Stegner et al. 2005).

One mechanism regulating neutrophil apoptosis *in vivo* is IL-6 trans-signalling activity (McLoughlin, Witowski et al. 2003). Using a murine model of acute peritoneal inflammation.

IFN-γ regulated IL-6 and sIL-6R to promote neutrophil apoptosis. In addition, IL-6 signalling through sIL-6R and gp130 has been shown to have a crucial role in leukocyte recruitment. IL-6 trans-signalling was shown to downregulate IL-8 and upregulate MCP-1. This change in chemokine release effectively removed the influx of neutrophils and triggered an influx of mononuclear cells to promote resolution of inflammation (Hurst, Wilkinson et al. 2001).

However, it is not known if IL-6 trans-signalling can regulate chemokine production and leukocyte recruitment in the airways. Furthermore, the ability of IL-6 to regulate neutrophil apoptosis through its sIL-6R has not been investigated. This chapter will investigate the potential role of IL-6 trans-signalling to regulate neutrophil apoptosis in adult neutrophils *in vitro*.

Earlier studies have investigated the role of IL-6 (without sIL-6R) on neutrophil apoptosis. Conflicting reports have shown IL-6 to promote neutrophil apoptosis (Afford, Pongracz et al. 1992) or to exert an inhibitory effect (Biffl, Moore et al. 1995; Biffl, Moore et al. 1996; Biffl, Moore et al. 1996; Asensi, Valle et al. 2004). The majority of studies have used purified neutrophils. However, with regards to the previous chapter, it was decided to use an isolated leukocyte population rather than purified neutrophils. As the previous chapter showed, isolating neutrophils by histopaque or percoll affected receptor expression. To minimise neutrophil manipulation, a mixed leukocyte population would be used.

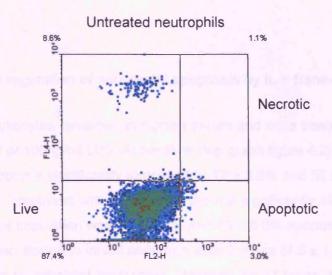
#### **4.2 Aims**

The aim of this chapter was to establish the role of IL-6 trans-signalling on neutrophil apoptosis. Flow cytometry will be used to assess the affect of IL-6 trans-signalling on neutrophil apoptosis using Annexin-V as a marker of apoptotic neutrophils. Apoptotic cells expose a phospholipid known as phosphatidylserine on their external surface. Cells can be labelled with Annexin-V which binds with high affinity to phosphatidylserine. Necrosis will also be measured by the staining of ToPro-3 dye which binds to the DNA in necrotic cells. ToPro-3 is unable to enter cells that have not necrosed and therefore allows apoptotic and necrotic cells to be distinguished.

# 4.3 Methodology

Leukocytes were isolated as outlined in the method section 2.5. Briefly, blood was obtained from healthy adult donors and anti-coagulated with sodium citrate. The erythrocytes were sedimented using dextran. In the initial experiments, isolated leukocytes remained in human serum as LPS requires an LPS binding protein (LBP) which is present in serum. In the proceeding experiments, human serum was removed by washing and centrifugation (see method section 2.5.3 for details). The isolated leukocytes were treated with 50ng/ml IL-6 and 50ng/ml sIL-6R or 100ng/ml LPS. LPS was included as a positive control as it is known to delay neutrophil apoptosis (Brach, deVos et al. 1992; Akgul, Moulding et al. 2001; Scheel-Toellner, Wang et al. 2004). LPS used at various doses from 10ng/ml to 1ug/ml in *vitro* have been shown to delay neutrophil apoptosis (Watson, Redmond et al. 1996; Watson, Rotstein et al. 1997). In light of this, 100ng/ml was the selected. At three time points (0h, 5h and 17h), leukocytes were prepared for staining with Annexin V and ToPro-3 to detect apoptotic and necrotic cells by flow cytometry. Supernatants from isolated and treated leukocytes were retained and stored at -80°C for future analysis.

Data analysis was performed by WinMDI. Leukocytes were viewed on the FSC-H/SSC-H plot and the neutrophils were gated to exclude all other leukocytes in the analysis. All neutrophils within this gated region were viewed on the FL2-H/FL4-H plot as shown in figure 4.1. A quadrant on the graph distinguished the status of the neutrophils, indicating whether the cells were live, apoptotic or necrotic. The data is expressed as mean percentage of apoptotic, necrotic or live neutrophils.



**Figure 4.1**: Neutrophils were gated on the FSC-H/SSC-H plot to exclude all other leukocytes in the flow cytometry analysis of neutrophil apoptosis and necrosis. A quadrant was placed on the graph to distinguish between live, apoptotic and necrotic cells. The lower left area of the quadrant represents live cells. The lower right represents apoptotic cells and the upper right represents necrotic cells. A percentage of apoptotic, necrotic or live neutrophils was obtained from each area of the quadrant.

Statistical analysis of experiments conducted in the presence of serum was performed using Kruskal-Wallis non parametric ANOVA test with Dunn's multiple comparisons post-test. Experiments conducted in the presence of serum were n = 4 for untreated and n = 3 for LPS or IL-6/sIL-6R treated. Due to the small sample size and the difference in sample number, a parametric one way ANOVA test could not be performed.

For experiments performed in the absence of serum, statistical analysis was performed using a one way ANOVA using Tukey-Kramer multiple comparisons test. Experiments were n = 9 for untreated, LPS-treated and IL-6/sIL-6R-treated, unless otherwise stated in the figure legends.

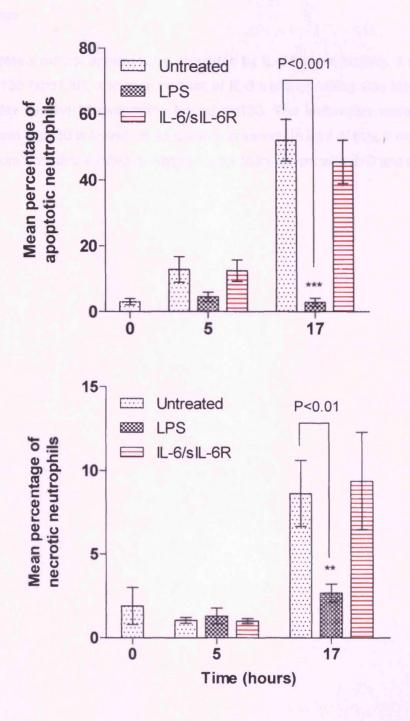
# 4.4 Results

# 4.4.1 The potential regulation of neutrophil apoptosis by IL-6 trans-signalling

Dextran isolated leukocytes remained in human serum and were treated with 50ng/ml IL-6 and 50ng/ml sIL-6R or 100ng/ml LPS. At baseline (top graph figure 4.2), 3.1% of neutrophils were apoptotic. Apoptosis significantly increased to  $12 \pm 3.8\%$  and  $52 \pm 6.3\%$  after a 5 and 17 hour incubation. Treatment with IL-6/sIL-6R did not significantly alter the profile of the cells. The neutrophil population was  $12 \pm 3.3\%$  and  $45 \pm 6.6\%$  apoptotic at 5 and 17 hours respectively. LPS had started to inhibit apoptosis after 5 hours  $(4.5 \pm 1.3\%)$  but this was not significantly different to untreated neutrophils. However, by 17 hours, LPS had significantly inhibited neutrophil apoptosis compared to untreated and IL-6/sIL-6R treated cells. As a result of LPS inhibiting apoptosis,  $2.8 \pm 1.2\%$  of the neutrophils were apoptotic.

# 4.4.1.1 Does IL-6 trans-signalling regulate neutrophil necrosis in vitro?

Very few neutrophils were necrotic (1.9  $\pm$  1.1% of the population) at baseline. After 5 hours, 1  $\pm$  0.1% of the population were necrotic. This increased to 8.6  $\pm$  1.9% after 17 hours. The percentage of necrotic neutrophils following five hours of incubation with LPS or IL-6/sIL-6R had not altered. However, after 17 hours, LPS significantly inhibited necrosis compared to untreated, as 2.6  $\pm$  0.5% of the population were necrotic. In contrast, IL-6/sIL-6R did not promote or inhibit necrosis as the population of necrotic neutrophils was 9.3  $\pm$  2.9% (figure 4.2).



**Figure 4.2**: The mean percentage of apoptotic (top) and necrotic neutrophils in the presence of human serum. Dextran treated leukocytes were incubated with and without treatment of 100 ng/ml LPS or 50 ng/ml IL-6 and sIL-6R for 5 or 17 hours at  $37^{\circ}\text{C}$ . Annexin-V PE and ToPro-3 stained cells were analysed by flow cytometry. Neutrophils were gated on the FSC/SSC plot. The percentage of apoptotic and necrotic neutrophils were obtained from the FL2-H/FL4-H quadrant plot. Data is expressed as mean  $\pm$  SEM. Untreated experiments are n = 4 and treated experiments are n = 3.

# 4.4.2 Conclusion

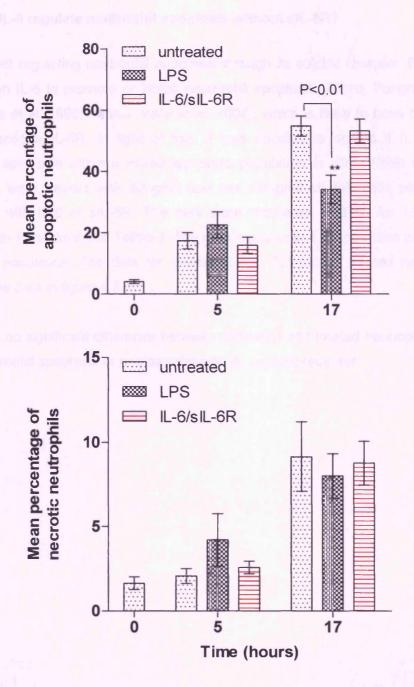
Neutrophil apoptosis did not appear to be regulated by IL-6 trans-signalling. It was possible that soluble gp130 (sgp130), a known inhibitor of IL-6 trans-signalling was blocking the IL-6/sIL-6R complex binding to membrane bound gp130. The leukocytes were exposed to human serum and sgp130 is known to be present in serum. In light of this, it was decided to remove the serum from the isolated leukocytes prior to treatment with IL-6 and sIL-6R.

# 4.4.3 The potential regulation of neutrophil apoptosis by IL-6 trans-signalling in the absence of serum

In the proceeding experiments, human serum was removed by washing and centrifugation (see method section 2.5.3 for details). The dextran isolated leukocytes were treated with 50ng/ml IL-6 and 50ng/ml sIL-6R or 100ng/ml LPS. The proportion of apoptotic neutrophils increased from a baseline of  $4.3 \pm 0.5\%$  to  $17.3 \pm 2.6\%$  after 5 hours and to  $54.7 \pm 3.3\%$  at 17 hours (figure 4.3) in the absence of any stimulus. LPS without the presence of serum did not notably inhibit apoptosis after 5 hours as  $22.5 \pm 4.2\%$  of the population were apoptotic. After 17 hours, LPS did exert a significant inhibition as the proportion of apoptotic neutrophils was 34.5%. IL-6/sIL-6R did not have a significant effect on neutrophil apoptosis compared to untreated. After 5 hours, neutrophils were  $15.8 \pm 2.6\%$  apoptotic and  $53 \pm 3.8\%$  after 17 hours.

# 4.4.3.1 Does IL-6 trans-signalling regulate neutrophil necrosis in vitro?

At baseline and after five hours, 1% of the population was necrotic. After 17 hours, only 8.6 ± 1.9% of the population were undergoing necrosis. Treatment with IL-6/sIL-6R had no affect on altering the necrotic population of neutrophils. Without serum, LPS did not significantly inhibit necrosis after 17 hours (figure 4.3), a contrast to the inhibition observed in figure 4.2.

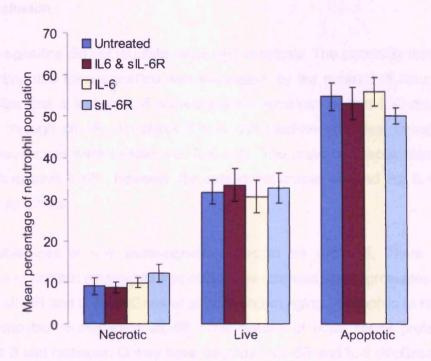


**Figure 4.3**: The mean percentage of apoptotic (top) and necrotic neutrophils in the absence of human serum. Dextran sedimented leukocytes were treated with 100ng/ml LPS or ng/ml IL-6 and sIL-6R up to 17 hours at 37°C. Annexin-V PE and t0Pro-3 stained cells were analysed by flow cytometry. Neutrophils were gated on the FSC/SSC plot. The percentage of apoptotic and necrotic neutrophils were obtained. Data is expressed as mean t1 SEM. Experiments are t2 for the five hour time point and the rest are all t3 statistical analysis was performed using a one way ANOVA using Tukey-Kramer multiple comparisons test.

# 4.4.4 Can IL-6 regulate neutrophil apoptosis without sIL-6R?

IL-6 was not regulating neutrophil apoptosis through its soluble receptor. Previous reports have shown IL-6 to promote or inhibit neutrophil apoptosis (Afford, Pongracz et al. 1992; Biffl, Moore et al. 1996; Asensi, Valle et al. 2004), which is likely to have been through its cognate receptor IL-6R. In light of this, it was decided to assess if IL-6 can regulate neutrophil apoptosis within a mixed leukocyte population *in vitro*. When dextran isolated leukocytes were treated with 50ng/ml IL-6 and 50ng/ml sIL-6R, cells were also treated separately with IL-6 or sIL-6R. The cells were incubated at 37°C for 17 hours prior to staining with Annexin-V and ToPro-3. The data is expressed as the mean percentage of the neutrophil population. The data for untreated and IL-6/sIL-6R treated neutrophils is the same as the data in figure 4.3.

There was no significant difference between untreated and treated neutrophils. IL-6 did not affect neutrophil apoptosis or necrosis through its cognate receptor.



**Figure 4.4** The mean percentage of necrotic, live and apoptotic neutrophils. Dextran sedimented leukocytes were treated with ng/ml IL-6 and sIL-6R separately or combined for 17 hours at 37°C. Annexin-V PE and ToPro-3 stained cells were analysed by flow cytometry. Neutrophils were gated on the FSC/SSC plot. The percentage of apoptotic and necrotic neutrophils were obtained. Data is expressed as mean  $\pm$  SEM. Experiments are n = 9 for the untreated and IL-6/sIL-6R combined. Treatment with IL-6 and sIL-6R separately are n = 5. Statistical analysis was performed using a one way ANOVA using Tukey-Kramer multiple comparisons test.

#### 4.4.5 Conclusion

IL-6 trans-signalling did not regulate neutrophil apoptosis. The possibility that sgp130 could be preventing IL-6 trans-signalling was eliminated, by the removal of serum. However, it was possible that a loss of IL-6 trans-signalling remained or that IL-6 may not regulate apoptosis through sIL-6R. To check if IL-6 can mediate apoptosis through its cognate receptor, leukocytes were treated with IL-6 only. The previous chapter demonstrated that neutrophils express IL-6R. However, the data in this chapter showed that IL-6 did not affect neutrophil apoptosis.

The possible loss of IL-6 trans-signalling was to be explored. There were a small percentage of necrotic neutrophils that could have released serine proteases that may have degraded sIL-6R and IL-6. McGreal *et al* have shown aging neutrophils to release elastase which is capable of degrading sIL-6R. The release of other serine proteases such as proteinase 3 and cathepsin G may have degraded sIL-6R and IL-6 (McGreal, Davies et al. 2010). To prevent any potential degradation of sIL-6R and IL-6, a serine protease inhibitor was included in the following experiments. Alpha-1 anti-trypsin (α1AT) is known to inhibit serine proteases and the degradation of both IL-6 and sIL-6R (McGreal, Davies et al. 2010)

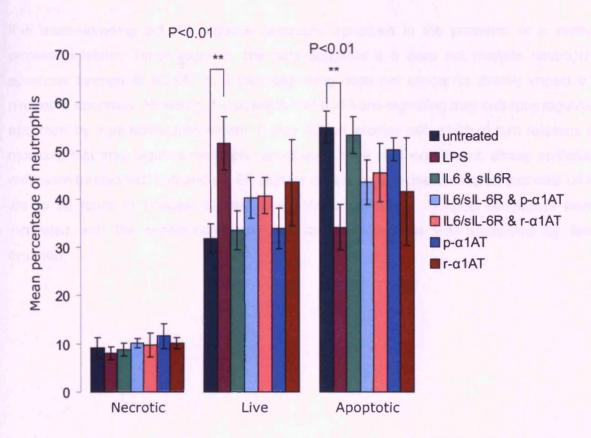
# 4.4.6 Can IL-6 trans-signalling regulate neutrophil apoptosis in the presence of a serine protease inhibitor?

The small percentage of necrotic neutrophils may release serine proteases which subsequently can degrade IL-6 and sIL-6R. As a result, IL-6 trans-signalling could be impaired. In light of this, dextran isolated and serum starved leukocytes were pre-treated with a serine protease inhibitor,  $\alpha$ 1AT prior to treatment with 50ng/ml IL-6 and 50ng/ml sIL-6R. Two derivates of  $\alpha$ 1AT were used; a plasma-derived (p $\alpha$ 1AT) and a recombinant form (r $\alpha$ 1AT) to compare the differences. Figure 4.5 shows the mean percentage of necrotic, live and apoptotic neutrophils after 17 hour incubation with 50µg/ml p $\alpha$ 1AT or r $\alpha$ 1AT with 50ng/ml IL-6 and sIL-6R. Leukocytes were also treated with 100ng/ml LPS without  $\alpha$ 1AT.

The proportion of untreated or IL-6/sIL-6R-treated neutrophils were 54  $\pm$  3.3% and 53  $\pm$  3.8% apoptotic, indicating that IL-6 trans-signalling had no affect on neutrophil apoptosis. The addition of p $\alpha$ 1AT or r $\alpha$ 1AT to IL-6/sIL-6R reduced apoptosis (43  $\pm$  4.6% and 45  $\pm$  6% respectively) but not significantly. Neither p $\alpha$ 1AT nor r $\alpha$ 1AT alone induced a significant effect on apoptosis.

As shown in figure 4.5, the survival of neutrophils was significantly enhanced by LPS.

None of the treatments promoted or inhibited neutrophil necrosis. Around 10% of the population were necrotic.



**Figure 4.5**: The mean percentage of neutrophils that were apoptotic, necrotic or live following 17 hour incubation at  $37^{\circ}$ C with and without treatment. Leukocytes were treated with 100ng/ml LPS, or 50ng/ml IL-6 and sIL-6R without or with plasma derived  $\alpha_1$ -antitrypsin ( $p\alpha1AT$ ) or recombinant derived  $\alpha_1$ -antitrypsin ( $r\alpha1AT$ ). Leukocytes were stained with Annexin-V/Topro-3 and analysed by flow cytometry. The neutrophil population was gated on the FSC/SSC plot and the percentage of apoptotic and necrotic neutrophils obtained. Data is expressed as mean  $\pm$  SEM. Experiments are n = 9 for untreated, LPS-treated, IL-6/sIL-6R treated; n = 7 for IL-6/sIL-6R/p $\alpha1AT$ ; n = 6 for  $p\alpha1AT$  and n = 3 for remaining experiments with  $r\alpha1AT$ . Statistical analysis was performed by non-parametric one way ANOVA Kruskal-Wallis test with Dunn's multiple comparisons test.

#### 4.4.7 Conclusion

IL-6 trans-signalling did not regulate neutrophil apoptosis in the presence of a serine protease inhibitor. Taken together, the data suggests IL-6 does not mediate neutrophil apoptosis through its sIL-6R. IL-6 trans-signalling does not appear to directly impact on neutrophil apoptosis. However, it is possible that IL-6 trans-signalling may indirectly regulate apoptosis by a paracrine mechanism. It may act on another cell, which in turn releases a molecule that may regulate neutrophil apoptosis. In the next experiment, airway epithelial cells were treated with IL-6 and sIL-6R (further details on cytokine stimulated epithelial cells are to be found in Chapter 5) and supernatants collected. Isolated neutrophils were incubated with the supernatants and the apoptosis/necrosis was measured by flow cytometry.

# 4.4.8 The potential for IL-6 trans-signalling to regulate neutrophil apoptosis by a paracrine mechanism

The data in this chapter suggests that IL-6 trans-signalling does not have direct impact on neutrophil apoptosis. The possibility of an indirect effect by a paracrine mechanism was investigated. IL-6 trans-signalling may act on other cell types, such as the airway epithelium. Molecules released from airway epithelial cells in response to IL-6 trans-signalling stimulation may in turn have an impact on neutrophil apoptosis.

An airway epithelial cell line, A549 were stimulated with 50ng/ml IL-6 and sIL-6R to induce IL-6 trans-signalling and chemokine release. Briefly, serum starved A549s were also treated with 2.5ng/ml IL-1 $\beta$  or a combination of IL-1 $\beta$  and IL-6/sIL-6R. IL-1 $\beta$  is a proinflammatory cytokine and was included as a control. This is discussed in more detail in chapter 5. The epithelial cells were incubated at 37°C for 24 hours and the supernatants collected. The supernatants were centrifuged to remove cell debris.

Neutrophils were isolated by histopaque to have a purified neutrophil population. This would eliminate the interference from other leukocytes. It was demonstrated in the previous chapter that gp130 expression may be slightly reduced on histopaque isolated neutrophils compared to neutrophils from dextran isolated leukocytes. This was not considered a problem as the *direct* impact of IL-6 trans-signalling on neutrophils was not being assessed. In addition, the isolated neutrophils were washed to ensure the removal of serum.

Neutrophils were incubated with the supernatants for 18 hours at 37°C. Isolated neutrophils without supernatant were also included as a control. The cells were washed and stained with Annexin-V PE and ToPro-3 as described in the method section 2.5.3. The neutrophils were analysed by flow cytometry and mean percentage of apoptotic, necrotic and live neutrophils was obtained. The data is expressed as percentage difference against the control which was set at 100%.

#### 4.4.8.1 Apoptotic neutrophils

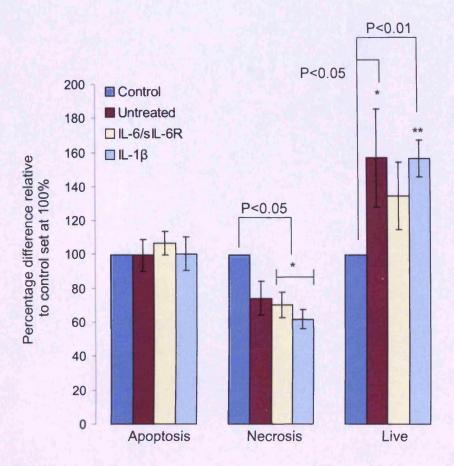
There was no significant difference in the percentage of apoptotic neutrophils, between the control and treatment with supernatant from IL-6/sIL-6R-stimulated A549s. This indicates that IL-6 trans-signalling had no paracrine effect on neutrophil apoptosis. Furthermore, supernatants from IL-1β-stimulated A549s or the combination of IL-6/sIL-6R and IL-1β-stimulated A549s, had no affect on apoptosis compared to the control (figure 4.6).

#### 4.4.8.2 Necrotic neutrophils

Neutrophils incubated with supernatants from stimulated-A549 cells had significantly less necrotic neutrophils than the control population. Supernatants from IL-6/sIL-6R stimulated A549 cells or from IL-1 $\beta$  stimulated A549 cells reduced the population of necrotic neutrophils to 70 ± 7.5% and 61 ± 5.4% respectively (figure 4.6)

#### 4.4.8.3 Live neutrophils

Overall, the A549 supernatants had increased the survival of neutrophils. There was a significant difference between the control and treatment with supernatant from unstimulated A549s (156  $\pm$  28% remained alive). Furthermore, supernatant from IL-1 $\beta$ -simulated A549s had also significantly increased the population of live neutrophils (156  $\pm$  10%) compared to the control. Although the population of live cells was increased, supernatants from IL-6/sIL-6R-stimulated A549s and IL-1 $\beta$ /IL-6/sIL-6R -simulated A549s had no significant impact (figure 4.6).



**Figure 4.6**: The indirect effect of IL-6 trans-signalling on neutrophil apoptosis and necrosis through a paracrine mechanism. Supernatants were obtained from A549 cells that were serum starved and either remained untreated, or were treated with 50ng/ml IL-6 and sIL-6R or 2.5ng/ml IL-1 $\beta$  or all combined, for 24 hours. Histopaque isolated neutrophils were incubated with the supernatants for 18 hours (in the absence of serum). Following staining with Annexin V-PE and ToPro-3, neutrophils were analysed by flow cytometry to determine apoptotic, necrotic and live cells. Data is expressed as percentage difference against the control. As a control, neutrophils were incubated without supernatant. Experiments are n = 4. Statistical analysis was performed by non-parametric one way ANOVA Kruskal-Wallis test and Dunn's multiple comparisons test.

#### 4.4.9 Conclusion

IL-6 trans-signalling does not indirectly regulate neutrophil apoptosis by a paracrine mechanism. It was interesting to observe the increased life-span of neutrophils caused by the stimulated A549 cells.

As IL-6 trans-signalling was having no impact on apoptosis, it was possible that IL-6 transsignalling was not occurring. The expression of gp130 was to be assessed in apoptotic neutrophils.

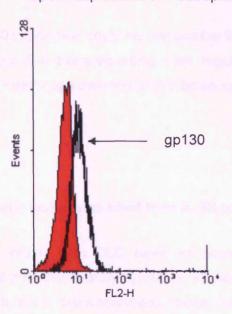
### 4.4.10 Is gp130 expression on neutrophils affected by apoptosis?

A possible loss in IL-6 trans-signalling could be responsible for the lack of IL-6/sIL-6R-regulation of neutrophil apoptosis. As IL-6 trans-signalling requires gp130, the expression of gp130 was assessed on neutrophils. Dextran isolated leukocytes were incubated for 17 hours at 37°C. The leukocytes were washed and labelled with a PE-conjugated antibody specific to gp130. The cells were also stained with a corresponding isotype control, IgG1, to measure background fluorescence.

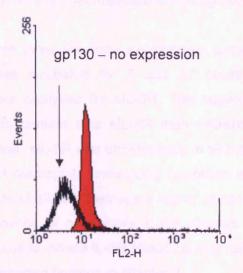
Data analysis was performed by WinMDI. Leukocytes were viewed on the FSC-H/SSC-H plot and the neutrophils were gated to exclude all other leukocytes in the analysis. A histogram in figure 4.7 shows the expression of gp130 on neutrophils at baseline and after 17 hours. The data is representative of three repeats.

There is gp130 expression on neutrophils at baseline. However, after 17 hour incubation at 37°C, there is a clear loss of gp130 expression.

Gp130 expression on neutrophils at baseline



Gp130 expression on neutrophils after 17 hours at 37°C



**Figure 4.7**: Gp130 expression on neutrophils at baseline and after 17 hours incubating at  $37^{\circ}$ C. Dextran isolated leukocytes were stained with CD130-PE; a PE-conjugated antibody specific to gp130. Leukocytes were stained with a corresponding isotype control, lgG1 (red peak). The black peak represents gp130 expression. Experiments are n = 3. The data is representative of three repeats.

#### 4.4.11 Conclusion

The loss of gp130 expression could be responsible for a lack of IL-6 trans-signalling. In turn this could prevent IL-6 trans-signalling from regulating neutrophil apoptosis. However, gp130 is present initially and therefore might be enough for signalling to occur.

# 4.4.12 Do apoptotic leukocytes shed their IL-6R to produce sIL-6R?

The airways of infants with CLD have an abundance of neutrophils and increased concentrations of pro-inflammatory cytokines, including IL-6 (Kotecha, Wilson et al. 1996). An increase in sIL-6R in bronchoalveolar lavage fluid from infants with CLD compared to healthy infants was observed (von Bismarck, Claass et al. 2008). This could be due to shedding of the IL-6R from neutrophils undergoing apoptosis (Chalaris, Rabe et al. 2007).

Supernatants from three separate experiments were collected from untreated and LPS-treated leukocytes incubated for 5 and 17 hours at 37°C. Using a sIL-6R ELISA, supernatants were analysed for sIL-6R. The supernatants from LPS-treated leukocytes were expected to contain less sIL-6R than untreated due to LPS reducing neutrophil apoptosis. However, sIL-6R was undetectable in all the supernatants. This may indicate that the population of neutrophils undergoing apoptosis was not dense enough to produce a detectable amount of sIL-6R. Perhaps a higher concentration of neutrophils was needed to reflect the abundance of neutrophils in the airways of infants with CLD. In addition, an appropriate stimulus to induce IL-6R shedding may have resulted in sIL-6R production. This is similar to the reported findings in chapter 3.

#### 4.5 Discussion

# 4.5.1 IL-6 trans-signalling does not regulate neutrophil apoptosis in vitro

This is the first report to investigate the role of IL-6 trans-signalling on neutrophil apoptosis within a mixed leukocyte population obtained from healthy adults. The data in this chapter show IL-6 and sIL-6R does not have a direct or indirect effect on neutrophil apoptosis. This was an unexpected result as it was hypothesised that IL-6 through its soluble receptor was capable of promoting neutrophil apoptosis. McLoughlin *et al* had shown IL-6 trans-signalling to be involved in the process of removing neutrophils by promoting apoptosis in a murine model of acute peritoneal inflammation (McLoughlin, Witowski et al. 2003). In contrast, the current findings were from *in vitro* experiments performed in the absence of an infection. Evidence from other researchers demonstrates IL-6 to regulate neutrophil apoptosis *in vitro* (Afford, Pongracz et al. 1992; Biffl, Moore et al. 1996; Asensi, Valle et al. 2004). However, these studies did not assess if IL-6 modulation of neutrophil apoptosis was through its cognate receptor or its soluble receptor. It is important to take sIL-6R into account when investigating a possible role for IL-6 in apoptosis.

The earlier studies by Biffl et al and Afford et al who were investigating a role for IL-6 (but not sIL-6R) in neutrophil apoptosis used purified neutrophils (Afford, Pongracz et al. 1992; Biffl, Moore et al. 1996). Data in chapter 3 has shown that purifying neutrophils by a percoll or histopaque method could alter receptor expression. Based on this observation, a mixed leukocyte population was used in this project to reduce neutrophil manipulation and maintain gp130 expression. The investigations by Biffl et al and Asensi et al incubated the purified neutrophils in media containing foetal calf serum (FCS). This was excluded from the current experiments as it was not known if the sgp130 present in FCS was capable of binding to the IL-6/sIL-6R complex. In light of this, leukocytes were incubated in media without FCS. However, in the initial experiments, the isolated leukocytes remained in human serum when treated with IL-6 and sIL-6R. As IL-6/sIL-6R was having no affect on apoptosis. it was assumed that the sgp130 present in human serum was binding to the IL-6/sIL-6R and preventing IL-6 trans-signalling. Human serum is known to contain sgp130 and is a known natural inhibitor of IL-6 trans-signalling (Narazaki, Yasukawa et al. 1993; Suzuki, Yasukawa et al. 1993; Jostock, Mullberg et al. 2001). Washing out the human serum and continuing to exclude FCS made little difference. IL-6/sIL-6R continued to have no regulatory affect on neutrophil apoptosis.

Neutrophil apoptosis was unaffected by IL-6 with or without sIL-6R in the presence of a mixed leukocyte population. It was possible that using a mixed leukocyte population could have influenced the outcome of the results. Lymphocytes and macrophages releasing cytokines or chemokines could potentially regulate the response of neutrophils to stimuli. For example, T cells are known to regulate neutrophils under inflammatory conditions (Keller, Spanou et al. 2005; McLoughlin, Lee et al. 2008). Moreover, macrophages may have an influential effect. They release pro-inflammatory cytokines such as IL-1β and TNFα during an infection, which can regulate neutrophil function (Janeway 2001). In turn, these pro-inflammatory cytokines could regulate other cytokines such as IL-6 and possibly sIL-6R. However, it is unknown if IL-6 or IL-6/sIL-6R and/or neutrophil apoptosis were being regulated by other cellular factors within the leukocyte population. Furthermore, there are no reports to suggest that neutrophil responses to stimuli are regulated by macrophages or T cells in normal healthy states. The leukocytes used in the current investigation were taken from healthy adult donors with no infections. It is possible that IL-6 trans-signalling may only regulate neutrophil apoptosis under inflammatory conditions and in the presence of an infection, and not in normal states.

Treating the leukocytes with a serine protease inhibitor such as plasma derived (p $\alpha$ 1AT) or recombinant (r $\alpha$ 1AT)  $\alpha_1$ -anti-trypsin combined with IL-6/sIL-6R did decrease neutrophil apoptosis. This was not a significant reduction. A recent study by McGreal *et al* highlighted degradation of IL-6 and sIL-6R by proteolytic enzymes released from neutrophils. Interestingly, neutrophils not only release serine proteinases when undergoing necrosis, but they can release elastase, cathepsin G and proteinase 3 under normal conditions (McGreal, Davies et al. 2010).

#### 4.5.2 Experimental factors affecting neutrophil apoptosis in vitro

Biffl *et al* investigated the impact of cell density on neutrophil apoptosis and found that the *in vitro* effects of IL-6 on neutrophil apoptosis varied with cell density in culture. It was also dependent on the concentration of IL-6. Neutrophils cultured at 1 x  $10^6$ /ml to 5 x  $10^6$ /ml for 24 hours were not prevented from undergoing apoptosis by IL-6 at 100, 10 or 1ng/ml. In the current experiments, 2 x  $10^6$ /ml leukocytes were treated with 50ng/ml IL-6. Furthermore, Biffl *et al* had shown that significantly more neutrophils survived when a high density of 10 to  $20 \times 10^6$ /ml were stimulated with 10ng/ml or 100ng/ml of IL-6 (Biffl, Moore et al. 1995). Similarly, there were inconsistent results observed in another study that was investigating

the affect of TNF- $\alpha$  on neutrophils apoptosis *in vitro* (Simon 2003). Low concentrations of TNF- $\alpha$  were shown to have an anti-apoptotic effect where as higher concentrations promoted neutrophil apoptosis (van den Berg, Weyer et al. 2001).

In a separate study, Hannah *et al* showed that cell density also had a major impact on neutrophil apoptosis *in vitro* and demonstrated that a density of 4 x 10<sup>6</sup> neutrophils/ml had significantly lower rate of apoptosis than a lower density of 0.5 x 10<sup>6</sup> neutrophils/ml. The rate of apoptosis was inversely proportional to the cell density. Neutrophil survival was also shown to be prolonged when incubated with FCS or BSA (Hannah, Nadra et al. 1998). The experiments conducted by Biffl *et al* in which IL-6 was delaying apoptosis, were performed on purified neutrophils that were incubated in media containing 10% FCS and bovine brain extracts. Both FCS and bovine brain extracts contain proteins for neutrophil survival (Biffl, Moore et al. 1995).

There are inconsistent reports regarding neutrophil apoptosis, which have been associated with different neutrophil purification methods. It is generally accepted that neutrophil apoptosis is delayed by LPS as shown in this project and by others (Sabroe, Jones et al. 2002) but there are reports that state LPS to have no effect (Dibbert, Weber et al. 1999). According to one group, LPS has no direct anti-apoptotic effect on neutrophils and can only delay neutrophil apoptosis via monocytes contaminating purified neutrophils (Sabroe, Jones et al. 2002). The current findings showed LPS inhibited neutrophil apoptosis within a mixed leukocyte population and in the presence of human serum. For LPS to exert a maximum inhibitory affect on apoptosis, it requires the LBP binding protein present in serum, TLR4, CD14 and MD-2 protein. With the removal of serum, it was surprising that LPS still had a partial inhibitory effect. Neutrophils express minimal CD14 and TLR4 but monocytes express high levels of CD14 (Sabroe, Jones et al. 2002). This may indicate that the monocytes present within the leukocyte population may have been responsible for the partial inhibition in response to LPS.

# 4.5.3 IL-6 and neutrophil apoptosis - in vitro findings from other researchers

Researchers investigating the effect of IL-6 on neutrophil apoptosis have conflicting reports.

An early investigation by Brach *et al* supports the observations in the current project, that IL-6 had no effect on neutrophil apoptosis. In their studies, heparin anti-coagulated blood was subjected to dextran sedimentation, ficoll-hypaque and percoll gradient centrifugation to obtain purified neutrophils. Neutrophils incubated in media containing 10% serum for 24 hours showed DNA fragmentation (cell death) after analysis of agarose gels. They demonstrated that IL-6 neither inhibited nor promoted apoptosis. In contrast to IL-6, neutrophils had survived up to 216 hours with GM-CSF, which was reversed by anti-GM-CSF (Brach, deVos et al. 1992).

In a conflicting report, Asensi et al claimed that IL-6 inhibited neutrophil apoptosis. Neutrophils were isolated from healthy adult volunteers by dextran and ficoll-hypaque before 12 hour incubation with recombinant IL-6 and control sera. Neutrophils were stained with propidium iodide which is commonly used to detect apoptotic cells by intercalating into the DNA and analysed by flow cytometry. Asensi stated that with increasing IL-6 concentrations from 0.0001ng/ml to 10ng/ml, a correlating decrease in neutrophil apoptosis from 70% to 55% respectively was observed. The group investigated osteomyelitis, a bone infection characterised by progressive inflammatory destruction of the infected bone and new bone. Increased levels of inflammatory cytokines, including IL-6 have been associated with osteomyelitis. They observed an inverse correlation between neutrophil apoptosis and levels of IL-6 in patient serum. Anti-IL-6 blocked neutrophil survival but the addition of recombinant IL-6 promoted neutrophil survival. A suggested mechanism for IL-6 promoted neutrophil survival was via platelet-activating factor (Biffl, Moore et al. 1996) and activation of MAPK, enhancing expression of anti-apoptotic protein Mcl-1, A1 and Bcl-X (Akgul, Moulding et al. 2001). Furthermore, they could not exclude a role for other cytokines such as IL-1β or growth factors such as GM-CSF or IFN-y in the increased survival of neutrophils (Asensi, Valle et al. 2004).

In the report by Biffl et al, dextran-treated and plasma-percoll isolated neutrophils were incubated in media containing 10% HI-FCS for 24 hours with and without IL-6. Apoptosis was measured by nuclear morphology after cells were stained with acridine orange and ethidium bromide staining to assess cellular viability. Neutrophil survival was calculated by dividing the number of viable cells without apoptotic nuclei by the total number of cells.

Using this method, they detected 29 ± 3% of untreated neutrophils survived after a 24 hour incubation compared to  $45 \pm 1$ ,  $51 \pm 6$  and  $63 \pm 6\%$  survival after incubation with 0.1, 1 and 10ng/ml IL-6 respectively. The nuclear morphology method can distinguish between apoptosis and necrosis. However, this was not stated in their report. Neutrophils undergoing either mode of death may have been counted together giving a false result. In addition, neutrophils were pre-treated with a platelet-activating factor (PAF) antagonist WEB 2170 before adding recombinant IL-6. As a result, IL-6 promoted neutrophil survival was significantly reduced. They had demonstrated IL-6 stimulated neutrophils produced PAF which in turn mediated the regulatory effect of IL-6 on neutrophil apoptosis. IL-6 and PAF may have caused increased necrosis rather than reduced apoptosis as they had shown neutrophils to have been more susceptible to oxidative burst. Furthermore, Biffl et al claim to have shown that IL-6 not only delays neutrophil apoptosis, but it primes the neutrophils for oxidative burst as the aging neutrophils are more sensitive to superoxide release following stimulation (Biffl, Moore et al. 1995; Biffl, Moore et al. 1996; Biffl, Moore et al. 1996). If IL-6 delayed neutrophil apoptosis, this would result in retained neutrophils at the site of infection. IL-6 may be contributing to the retained accumulation of neutrophils in the airways of infants with CLD. Soluble IL-6R may be required to regulate this process and prevent IL-6 inhibition of apoptosis during an inflammatory response.

Both Asensi *et al* and Biffl *et al* had used less than 10ng/ml of IL-6 to observe an inhibition of neutrophil apoptosis (Biffl, Moore et al. 1996; Asensi, Valle et al. 2004). A contrasting report by Afford *et al* claimed that IL-6 at 20ng/ml promoted neutrophil apoptosis after 24 hours. Apoptosis was assessed morphologically by trypan blue dye exclusion and confirmed by DNA fragmentation by gel electrophoresis (Afford, Pongracz et al. 1992). After 24 hours,  $10 \pm 2.9\%$  of the control neutrophils were apoptotic compared to  $15 \pm 2.3\%$  of neutrophils that were apoptotic following treatment with IL-6. They had stated that a relatively small proportion of mature neutrophils in culture were apoptosing at an increased rate in response to IL-6. This was not observed in the total population. Morphologically assessing apoptotic cells instead of using flow cytometry may not be accurate enough to detect all apoptotic cells and can exclude necrotic cells. Afford *et al* have suggested that the number of apoptotic cells may have been underestimated. The mechanism underlining the proapoptotic effect of IL-6 in this study was not investigated but they have shown bcl-2 not to be upregulated.

À more recent investigation by McNamee et al used whole blood to investigate the effect of pro-inflammatory cytokines on neutrophil apoptosis. Similar to the method used in this project, they had used ammonium chloride lysing solution to remove the erythrocytes. In

contrast, they had lysed the samples after each time point. Apoptotic and necrotic cells were analysed by flow cytometry following staining with Annexin-V and propidium iodide. Treatment with either 125pg/ml IL-6, 0.01pg/ml IL-1β or 1pg/ml TNF-α had inhibited neutrophil apoptosis after 24 and 48 hours. It was stated that they had used concentrations that were similar to those observed in patients with bacterial infections. However, a significant decrease in the inhibition of apoptosis was observed as the concentrations of each stimuli increased to 1000pg/ml. Treatment with IL-6 below 125pg/ml had no affect on neutrophil apoptosis but at 250pg/ml, there was significant reduction in apoptosis (McNamee, Bellier et al. 2005).

# 4.5.4 IL-6 trans-signalling and mechanisms involved in regulating neutrophil apoptosis

IL-6 signalling via its cognate or soluble receptor not only activates JAK pathway and STAT3, but also MAPK (Singh, Jayaraman et al. 2006). MAPK pathways have been suggested to be involved in the process of neutrophil apoptosis, particularly p38 MAPK which seems to be activated by stress to promote apoptosis. At least two pathways, a p38 MAPK- dependent and an independent pathway, have been shown to promote neutrophil apoptosis by Frasch et al. It was suggested that a stress-activated pathway was dependent on p38 MAPK activation but p38 MAPK was not required for anti-FAS induced apoptosis or spontaneous apoptosis (Frasch et al 1998). Studies by Alvarado-Kristensson et al conflict with this observation and reported that activation of p38 MAPK signals survival in neutrophils. They found that activation of caspase-3 and 8 coincided with a reduction of p38 MAPK activity in isolated neutrophils, with no involvement of survival-promoting molecules such as Akt, p21 ras or p42/p44 MAPK. Inhibition of p38 MAPK was associated with Fas-induced PI3-K activation and consequently led to proapoptotic signals in isolated neutrophils (Alvarado-Kristensson 2002). It has not been shown if IL-6 promotes or inhibits neutrophil apoptosis in vitro through MAPK signalling or through other signalling molecules such as PI3-K or NF-kB.

#### 4.5.5 Modulation of IL-6 activity during apoptosis in vivo

IFN-γ and IL-6 modulate leukocyte apoptosis *in vivo* during peritoneal inflammation. McLouglin *et al* used a murine model of acute peritoneal inflammation to show that IFN-γ regulates IL-6 expression which acts through sIL-6R to promote neutrophil apoptosis and clearance. Mice deficient in IFN-γ (IFN-γ<sup>-/-</sup>) or IL-6 (IL-6<sup>-/-</sup>) were treated with cell-free supernatant prepared from *Staphylococcus epidermidis* (SES) to induce inflammation in the peritoneal cavity. Annexin-V/PI staining showed neutrophil apoptosis was defective in both IFN-γ<sup>-/-</sup> and IL-6<sup>-/-</sup> mice. PMN apoptosis was restored by the recombinant IL-6/sIL-6R complex, hyper-IL-6 in IFN-γ<sup>-/-</sup> mice. They also showed wild type mice treated with sgp130 to inhibit IL-6 trans-signalling, had significantly diminished early PMN apoptosis and had reduced caspase 3 activation but late apoptosis/necrosis was not inhibited (McLoughlin, Lee et al. 2008).

# 4.5.6 Understanding the role of IL-6 in the inhibition of neutrophil apoptosis in disease pathogenesis

Lower respiratory tract disease in infants is commonly caused by Respiratory Syncytial virus (RSV) and is associated with numerous neutrophils present in the airways. Granulocytes incubated with RSV exhibited delayed apoptosis, which was found to be dependent on PI3-K and NF-kB but independent of p38 MAPK activation. In addition, RSV upregulated IL-6 production from neutrophils and this was mediated by NF-kB upregulation. It is speculated that RSV-induced IL-6 production may be responsible for extending the neutrophil lifespan as IL-6 was shown to contribute to the inhibition of neutrophil apoptosis by a PI3-K dependent mechanism. Both RSV and IL-6 activated the anti-apoptotic protein, McI-1, via PI3-K, which was suggested to be a possible cause of cell survival. Interestingly, heat-inactivated RSV was also able to inhibit neutrophil apoptosis, which has clinical implications as virus associated debris present in the airways is enough to retain the unwanted accumulation of neutrophils and the involvement of IL-6 contributing to the pathogenesis of the disease (Lindemans 2004).

In an attempt to understand the mechanism behind the delayed neutrophil apoptosis observed in patients with osteomyelitis, Ocaña et al demonstrated the *in vitro* protective properties of IL-6. Neutrophils stimulated with *Staphylococcus aureus* or *Escherichia coli* or

bacterial components LPS or LTA produced significantly higher levels of IL-6 as well as IL-1 $\beta$  and TNF- $\alpha$ . The secreted IL-6 in turn decreased the rate of apoptosis. Interestingly, the addition of a neutralizing antibody against IL-6 and TNF- $\alpha$  significantly removed the protective effect and the rate of apoptosis increased. They suggested a mechanism for the cytokine involvement in delaying neutrophil apoptosis was associated with the upregulation of anti-apoptotic protein Bcl- $x_L$  and the downregulation of pro-apoptotic protein Bax- $\alpha$ . They confirmed this observation by incubating neutrophils with recombinant IL-6 at 1ng/ml and 10ng/ml, which did significantly decrease neutrophil apoptosis compared to untreated via the same mechanism as the secreted IL-6 (Ocana, Asensi et al. 2008). For these experiments, they had used large volumes of neutrophils, 25 x 10<sup>6</sup>/ml and had incubated the purified cells with serum before measuring apoptosis. It is highly likely that cell density and growth factors would have contributed towards the IL-6 promoted survival.

#### 4.5.7 Conclusion

IL-6 signalling through its membrane receptor may regulate neutrophil apoptosis, although it is not clearly defined. In disease pathogenesis, there is evidence to suggest that IL-6 elicits an inhibition. According to this study, IL-6 bound to its soluble receptor does not appear to have a role in neutrophil apoptosis. In addition, there is little evidence in the literature to suggest otherwise. The idea that IL-6 and sIL-6R would delay apoptosis is questionable since the function of IL-6 trans-signalling has been shown to have an important role in the transitional change from innate to acquired immunity by promoting neutrophil resolution. A delay in neutrophil apoptosis would hinder this inflammatory process and the resolution of inflammation. It would be more plausible if IL-6 trans-signalling would promote apoptosis of maturing neutrophils during an infection, particularly in chronic lung disease where aging neutrophils that fail to undergo apoptosis are contributing to the severity of the disease. McLoughlin et al have indeed described such a pro-apoptotic effect (McLoughlin, Witowski et al. 2003). However, IL-6 does not participate in regulating human neutrophil apoptosis in vitro. This strongly suggests that the accumulation of aging neutrophils and their failure to undergo apoptosis in CLD may not be directly associated with IL-6 trans-signalling. On the other hand, the presence of an infection may have an important influence in the role of IL-6 and sIL-6R on neutrophil apoptosis. This can only be determined in an in vivo model.

The role of IL-6 trans-signalling in the airways will be investigated to determine if airway epithelial cells respond to IL-6/sIL-6R complex.

# Chapter 5

The role of IL-6 trans-signalling in regulating chemokine expression in transformed airway epithelial cells

#### 5.1 Introduction

The pathogenesis of CLD in preterm infants is characterised by a dominance of neutrophils. This has been associated with elevated levels of pro-inflammatory cytokines, such as IL-6, which may be involved in initiating the inflammatory response observed in the airways. Data obtained within the Child Health group, have shown elevated levels of IL-6, in BAF samples from preterm infants with CLD (Kotecha, Wilson et al. 1996). Elevated levels of sIL-6R in infants with CLD were also detected (McGreal, Davies et al.; von Bismarck, Claass et al. 2008; McGreal, Davies et al. 2010). The source of IL-6, sIL-6R and sgp130 is unknown, as is the reason for the sustained presence of neutrophils and pro-inflammatory cytokines. It is highly likely that an infection may play a crucial role by inappropriately activating a sustained influx of pro-inflammatory cytokines and neutrophils into the airways. The data in chapter three showed neutrophils expressed IL-6R, which was reduced in response to a bacterial stimulant. This may contribute to the increased sIL-6R observed in the airways. In a murine model of peritoneal inflammation, sIL-6R and IL-6 were important in resolving inflammation by stimulating MCP-1 release from mesothelial cells and downregulating IL-8 and IL-1βmediated IL-8 release. The same outcome was demonstrated in human peritoneal mesothelial cells (HPMC) in vitro (Hurst, Wilkinson et al. 2001); (Jones 2005). In light of this, the hypothesis was that IL-6 trans-signalling may have a similar important role in airway epithelial cells, which may be defective in preterm infants with CLD. This is a plausible explanation for the sustained inflammation and lack of mononuclear cell influx in the airways. Airway epithelial cells are known to express gp130 (Chattopadhyay. Tracy et al. 2007) but it is unknown if these cells are responsive to IL-6 trans-signalling.

A549 and BEAS2B cells are transformed airway epithelial cells and will be used to investigate the role of IL-6 trans-signalling in airway epithelial cells. The A549 cell line is a continuously cultured cell line derived from a human pulmonary adenocarcinoma and obtained from a 58-year-old Caucasian male. The cells have morphological and biochemical features similar to those of pulmonary alveolar type II cells (Smith 1977). BEAS2B cells were derived from normal bronchial epithelium, obtained from an autopsy of non-cancerous individuals. The cells were infected and immortalised with an adenovirus, SV40 (Reddel, De Silva et al. 1995) to generate a continuous culture.

#### **5.2 Aims**

IL-6 trans-signalling in adult and neonatal airways is poorly understood. The impact of IL-6/sIL-6R on transformed human airway epithelial cells A549s and BEAs2Bs will be investigated by measuring chemokine release. This will help provide an insight into the role of IL-6/sIL-6R as a potential regulator of leukocyte recruitment in the airways. In addition, the role of IL-6/sIL-6R during hyperoxia will also be investigated. Premature infants on mechanical ventilation receive oxygen at high concentrations, which subsequently damages the airway epithelial cells. Hyperoxia may alter the IL-6/sIL-6R regulation of chemokine production and leukocyte recruitment.

#### The aims of the chapter were to:

- 1. Establish if airway epithelial cells express gp130 and IL-6R
- 2. Determine if airway epithelial cells produce MCP-1 in response to IL-6 transsignalling
- 3. Evaluate the modulation of cellular responses to IL-6 trans-signalling by exposing the cells to high oxygen concentrations (hyperoxia) and proinflammatory cytokines, such as IL-1β.
- 4. Establish if airway epithelial cells produce IL-8 and whether IL-6 trans-signalling downregulates IL-8 and IL-1β-stimulated release of IL-8 in airway epithelial cells.

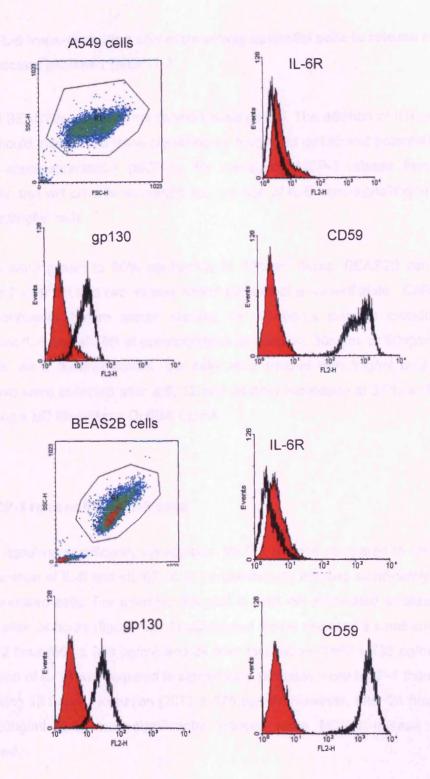
#### 5.3 Results

# 5.3.1 Receptor expression on transformed alveolar and bronchial airway epithelial cells

A549 and BEAS2Bs cells need to express membrane bound gp130 in order for the cells to respond to IL-6 trans-signalling. Furthermore, it was not known if these cells expressed IL-6R. One study has shown detectable membrane expression of IL-6R on primary bronchial epithelial cells (Gomez, Sokol et al. 2005). If IL-6R is absent on the cell surface, the cells are not able to respond to IL-6. If gp130 is present, the cells should respond to IL-6 in the presence of sIL-6R, making them a suitable model for IL-6 trans-signalling cellular responses.

A549 and BEAS2B cells were obtained from an internal cell bank at Cardiff University. Experiments were set up to analyse cell surface receptors gp130 and IL-6R by flow cytometry. Briefly, the A549 and BEAS2B cells were stained with PE-conjugated antibodies specific for IL-6R, gp130 or CD59. The latter was included as a positive control as this particular receptor is constitutively expressed on most cell types. As a negative control, cells were stained with an IgG1 or IgG2a antibody specific to the PE-conjugated antibodies. These are known as isotype controls (represented by the full red peaks in figure 5.1) and measure background fluorescence.

As shown in figure 5.1, A549 and BEAS2B cells were IL-6R negative, gp130 positive and CD59 positive (as illustrated by the black peaks). The cell density populations are shown in the FSC-H/SSC-H plots.



**Figure 5.1:** A549 and BEAS2B cells were prepared and stained with PE-conjugated antibodies prior to analysing membrane expression of IL-6R, gp130 and CD59 by flow cytometry. Red peaks represent the isotype control. Graphical analysis of the data was performed using WinMDI and graphs here represent data obtained from one experiment, which is representative of three repeats.

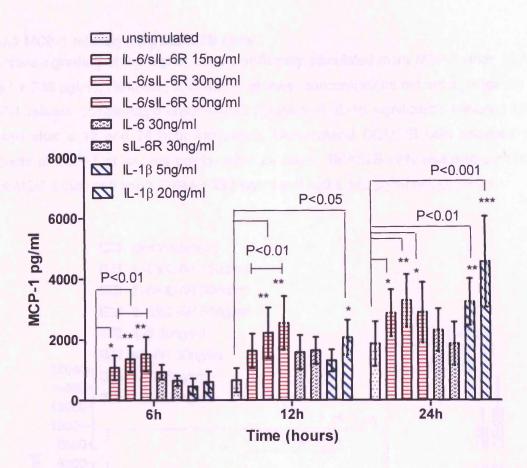
# 5.3.2 Can IL-6 trans-signalling stimulate airway epithelial cells to release monocyte chemoattractant protein-1 (MCP-1)?

A549s and BEAS2Bs express membrane bound gp130. The addition of IL-6 and sIL-6R to the cells should induce IL-6 trans-signalling by binding to gp130 and potentially upregulate monocytic chemoattractant-1 (MCP-1). By measuring MCP-1 release from IL-6/sIL-6R treated cells, this will provide an insight into the role of IL-6 trans-signalling in alveolar and bronchial epithelial cells.

A549 cells were grown to 80% confluency in T25cm<sup>2</sup> flasks. BEAS2B cell density was adjusted to 2 x 10<sup>5</sup>/ml and two ml was added per well of a six well plate. Cells were grown to 80% confluency before serum starving for 18 hours prior to cytokine treatment. Recombinant IL-6 and sIL-6R at concentrations of 15ng/ml, 30ng/ml or 50ng/ml were added to the cells. As a positive control, the cells were treated with 5ng/ml or 20ng/ml IL-1β. Supernatants were collected after a 6, 12 and 24 hour incubation at 37°C and analysed for MCP-1 using a BD Bioscience OptEIA ELISA.

#### 5.3.2.1 MCP-1 release from A549 cells

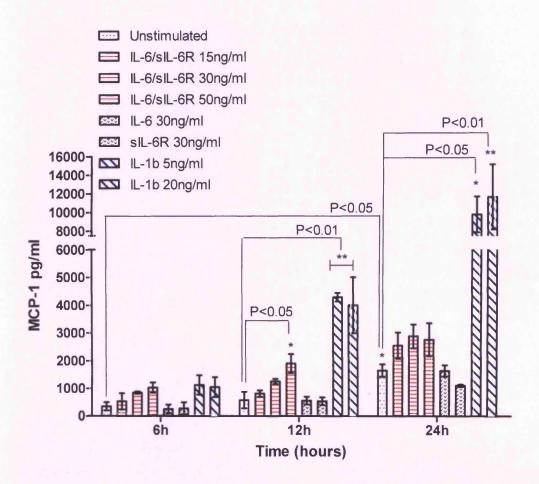
IL-6 trans-signalling significantly upregulated MCP-1 release compared to untreated levels. The combination of IL-6 and sIL-6R at all concentrations induced significantly more MCP-1 than unstimulated cells. For example 30ng/ml IL-6/sIL-6R stimulated a release of 3286  $\pm$  853 pg/ml after 24 hours (figure 5.2). Unstimulated A549s released a small amount of MCP-1 after a 12 hour (660  $\pm$  398 pg/ml) and 24 hour incubation (1862  $\pm$  735 pg/ml). The higher concentration of IL-1 $\beta$  was required to significantly stimulate more MCP-1 than unstimulated cells following 12 hours incubation (2073  $\pm$  576 pg/ml). However, after 24 hours, 5ng/ml as well as 20ng/ml IL-1 $\beta$  had significantly induced more MCP-1 release compared to unstimulated.



**Figure 5.2:** MCP-1 was measured in supernatants taken from A549 cells at various time points following cytokine treatment. The cells were grown in  $T25cm^2$  flasks to 80% confluency before serum starving for 18 hours. Cells were then treated with 15ng/ml, 30ng/ml or 50ng/ml of IL-6 and sIL-6R combined. As a positive control, the cells were treated with 5ng/ml or 20ng/ml IL-1 $\beta$ . Following incubation at 6, 12 or 24 hour at 37°C, supernatants were harvested and analysed for MCP-1 using a BD Bioscience OptEIA ELISA kit. Experiments are n = 4 and statistical analysis was performed by using the repeated measures ANOVA and Tukey-Kramer comparison test.

#### 5.3.2.2 MCP-1 release from BEAS2B cells

IL-6 trans-signalling at the higher dose significantly stimulated more MCP-1 after 12 hours (1897  $\pm$  338 pg/ml). However, IL-6/sIL-6R at lower concentrations did not significantly alter MCP-1 release compared to unstimulated (figure 5.3). IL-1β significantly elevated MCP-1 release after a 12 and 24 hour incubation. Unstimulated BEAS2B cells released small amounts of MCP-1 at all time points. After 24 hours, BEAS2B cells released significantly more MCP-1 than at 6 hours (1644  $\pm$  223 pg/ml and 350  $\pm$  146 pg/ml respectively).



**Figure 5.3:** MCP-1 was measured in supernatants taken from BEAS2B cells at various time points following cytokine treatment. Cells were seeded at  $2 \times 10^5$ /ml per well and two ml were added per well of a 6 well plate. The cells were grown to 80% confluency before serum starving for 18 hours. Cells were then treated with 15ng/ml, 30ng/ml or 50ng/ml of IL-6 and sIL-6R combined. As a positive control, the cells were treated with 5ng/ml or 20ng/ml IL-1 $\beta$ . Following incubation at 6, 12 or 24 hour at 37°C, supernatants were harvested and analysed for MCP-1 using a BD Bioscience OptEIA ELISA kit. Experiments are n = 3 and statistical analysis was performed by using the repeated measures ANOVA and the non-parametric Friedman comparison test

### 5.3.2.3 Conclusion

It has been established that transformed airway epithelial cells, A549s and BEAS2Bs respond to IL-6 trans-signalling. IL-6/sIL-6R at all concentrations used here significantly increased MCP-1 release from A549s at all time points. For the BEAS2B cells, a higher dose of 50ng/ml of IL-6/sIL-6R was required to increase significant amounts of MCP-1 release. The next step was to investigate if MCP-1 release in response to IL-6 transsignalling could be altered by exposing the cells to a high oxygen concentration (hyperoxia).

# 5.3.3 Can hyperoxia modulate IL-6 trans-signalling responses in airway epithelial cells?

The airways of ventilated preterm infants are exposed to hyperoxia, which could be a contributing factor to the development of CLD. The impact of hyperoxia on IL-6 transsignalling is unknown. If IL-6 trans-signalling induced MCP-1 becomes altered by hyperoxia *in vitro*, this may indicate a dysregulation in IL-6 trans-signalling in ventilated premature infants and provide some insight into the understanding of CLD development. A549 cells were more responsive to IL-6 trans-signalling than BEAS2B cells. In light of this, A549 cells were stimulated with IL-6/sIL-6R or IL-1β and exposed to hyperoxia. After specified incubation times, the viability of the cells was assessed by the MTS assay. In addition, supernatants were collected and an ELISA determined the MCP-1 concentration.

## 5.3.3.1 Measuring cell viability using the MTS assay

A viability study using an MTS assay was performed on A549s to compare the effect of normoxia (21% O<sub>2</sub> also normal air) and hyperoxia (80% O<sub>2</sub>) with and without cytokine treatment. An MTS assay is a colourimetric method for determining the number of viable cells.

A549s were seeded at 7 x  $10^4$ /ml and  $100\mu$ l was added per well of a 96 well plate. The cells were grown to 80% confluency. Cells were serum starved for 18 hours and treated with 30ng/ml IL-6 and sIL-6R or 20ng/ml IL-1 $\beta$ . The cells were exposed to normoxia or hyperoxia for 12, 24 and 48 hours at 37 °C and 5% CO<sub>2</sub>. After each specified time point, the cells were treated with 20 $\mu$ l MTS solution and incubated for a further 2 hours at 21% O<sub>2</sub> (as outlined in methodology section 2.11). Absorbance values for untreated and treated cells were obtained at 490nm on a spectrophotometer. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. When cells are non-viable, the formazan product is not produced. The absorbance readings were set against the baseline (at 100%) to calculate percentage difference in cell viability compared to baseline viability. The baseline (which is also control at 0h) was determined as the mean absorbance value of unstimulated A549s cultured in normoxia prior to the addition of treatments or exposure to hyperoxia for 48 hours.

### 5.3.3.1.1 Cell viability under normoxic conditions with and without cytokine treatment

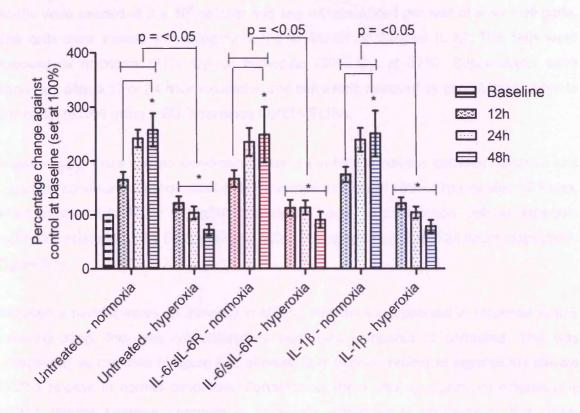
As shown in figure 5.4, cell proliferation increased over the 48 hours in culture. After 12 and 24 hours, viability of cells increased to  $165 \pm 14\%$  and  $241 \pm 16\%$  respectively. After 48 hours, the number of viable cells significantly increased to  $257 \pm 30\%$ , compared to the number of viable cells at 12 hours. This is likely to reflect proliferation of cells over this period.

Cell viability was not significantly altered by IL-6 trans-signalling nor by IL-1β compared to untreated.

# 5.3.3.1.2 Cell viability under hyperoxic conditions with and without cytokine treatment

Cell viability was significantly different between normal and hyperoxic conditions. The proliferation of cells appeared to be greatly reduced following exposure to 80%  $O_2$ . As shown in figure 5.4, the viability of untreated cells was 121  $\pm$  12% following a 12 hour exposure to hyperoxia. Viability decreased to 103  $\pm$  11% at 24 hours and 72  $\pm$  10% after 48 hours.

Cell viability was not significantly altered by IL-6 trans-signalling nor IL-1β compared to untreated cells under hyperoxic conditions.



**Figure 5.4:** Percentage change in cell viability as determined by the MTS assay. Untreated or cytokine treated A549s were exposed to hyperoxia (80%  $O_2$ ) or normoxia (21%  $O_2$ ) for 12, 24 and 48 hours. At each time point, MTS solution was added to each well and incubated for a further 2 hours before obtaining an absorbance value at 490nm wavelength. The data is shown as percentage difference against baseline of unstimulated A549 cells (set at 100%). Experiments are n = 4. Statistical analysis was performed by the Friedman non-parametric repeated measures ANOVA test with Dunn's multiple comparison post-test.

#### 5.3.3.1.3 Conclusion

In an *in vitro* experiment, alveolar epithelial cells exposed to 80%  $O_2$  had a significantly reduced number of viable cells over time compared to normoxia. Neither IL-6 transsignalling nor IL-1 $\beta$  had altered cell viability under normoxia or hyperoxia. As cell viability was reduced in response to hyperoxic conditions, a reduction in chemokine release would be expected. MCP-1 was measured in supernatants from untreated and cytokine treated A549s under hyperoxic and normoxic conditions.

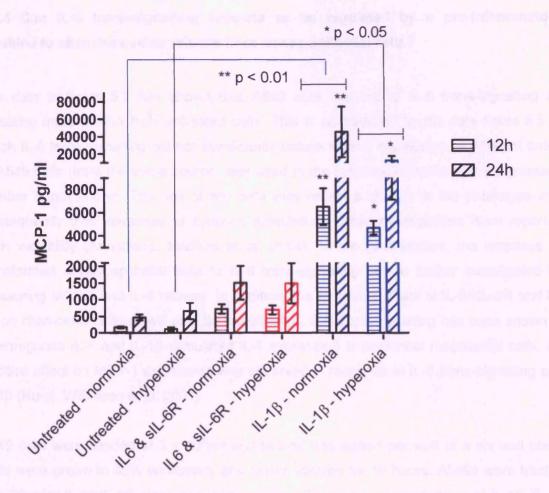
# 5.3.3.2 MCP-1 release from A549s exposed to hyperoxia

A549s were seeded at 3 x 10<sup>5</sup> cells/ml and two ml was added per well of a six well plate. The cells were treated with 30ng/ml IL-6 and sIL-6R or 20ng/ml IL-1β. The cells were exposed to normoxia (21% O<sub>2</sub>) or hyperoxia (80% O<sub>2</sub>) at 37°C. Supernatants were harvested after a 12 or 24 hour incubation and cell debris removed by centrifugation prior to testing for MCP-1 using a BD Bioscience OptEIA ELISA.

Unexpectedly, there was no significant difference in MCP-1 release between normoxic and hyperoxic conditions. Unstimulated cells in normoxia released  $169 \pm 27 pg/ml$  after 12 hours, which increased to  $470 \pm 87$  pg/ml following 24 hours. In comparison, cells in hyperoxic conditions released  $116 \pm 58.5 pg/ml$  and  $660 \pm 244$  pg/ml after 12 and 24 hours respectively (figure 5.5).

Although a trend towards an increase in MCP-1 release was observed in response to IL-6 trans-signalling, this was not statistically significant compared to untreated. This was unexpected as the data in figure 5.2, showed IL-6 trans-signalling to significantly elevate MCP-1 release in normal conditions. Furthermore, there was no significant difference in MCP-1 release between normoxia or hyperoxia conditions in response to IL-6 trans-signalling.

A549s responded well to IL-1 $\beta$  by significantly releasing 6526  $\pm$  1584 pg/ml (at 12 hours) and 45391  $\pm$  29109 pg/ml of MCP-1 (at 24 hours). This was higher than previously shown in figure 5.2. Furthermore, hyperoxia did not impair IL-1 $\beta$ -stimulated MCP-1. Despite the exposure to hyperoxia, the cells maintained a significant release of MCP-1 in response to IL-1 $\beta$ , compared to unstimulated cells. However, these levels were not significantly different to IL-1 $\beta$ -stimulated MCP-1 in normoxia.



**Figure 5.5:** MCP-1 measured in supernatants obtained from untreated or treated A549 cells exposed to 21% or 80% oxygen. Initially, cells were seeded in six well plates, serum starved for 18 hours and treated with 30ng/ml IL-6 and sIL-6R or 20ng/ml IL-1 $\beta$ . After a 12 or 24-hour incubation and exposure to normoxia or hyperoxia, the supernatants were harvested. An ELISA was used to determine the MCP-1 concentration. Experiments are n = 4. Statistical analysis was performed by the Friedman non-parametric repeated measures ANOVA test with Dunn's multiple comparison post-test.

#### 5.3.3.3 Conclusion

Cell viability was significantly reduced by hyperoxia but there was no significant difference in MCP-1 release compared to the normal condition. Surprisingly, the reduced number of viable cells maintained MCP-1 levels. Hyperoxia may trigger a stress response that increases MCP-1 release from the remaining viable cells. In addition, stimulating cells with IL-6/sIL-6R of IL-1β did not alter MCP-1 release in response to hyperoxia.

# 5.3.4 Can IL-6 trans-signalling regulate or be regulated by a pro-inflammatory cytokine to alter chemokine release from airway epithelial cells?

The data in figure 5.2 has shown that A549 cells respond to IL-6 trans-signalling by releasing more MCP-1 than untreated cells. This is contradicted by the data figure 5.5 in which IL-6 trans-signalling did not significantly induce MCP-1 expression. A different batch of A549 cells (from the same source) was used in the initial experiments and the passage number is not known. The age of the cells may reflect a change in the phenotype and subsequently their response to cytokine stimulation. Other investigations have reported such variability (Armstrong, Medford et al. 2004). In the next section, the response of transformed airway epithelial cells to IL-6 trans-signalling will be further investigated by measuring MCP-1 and IL-8 release. In addition, the combined effect of IL-6/sIL-6R and IL-1β on chemokine release will also be investigated. IL-6 trans-signalling has been shown to downregulate IL-8 and IL-1β-stimulated IL-8 expression in peritoneal mesothelial cells. An additive affect on MCP-1 expression was observed in response to IL-6 trans-signalling and IL-1β (Hurst, Wilkinson et al. 2001).

A549 cells were seeded at 3 x 10<sup>5</sup>/ml and two ml was added per well of a six well plate. Cells were grown to 80% confluency and serum starved for 18 hours. A549s were treated with 30ng/ml IL-6/sIL-6R alone or in combination with varying concentrations of IL-1β. IL-1β ranging in concentration from 0.3 to 10ng/ml was used to stimulate A549s with and without 30ng/ml IL-6/sIL-6R. Following 18 hours incubation at 37°C, supernatants were harvested, pelleted and diluted accordingly.

### 5.3.4.1 MCP-1 release from IL-6/sIL-6R and IL-1β- stimulated A549s

Unstimulated A549 released 445  $\pm$  91 pg/ml of MCP-1. IL-6/sIL-6R did not induce significantly more MCP-1 than unstimulated. IL-1 $\beta$  elevated MCP-1 but was not statistically significant to unstimulated levels (figure 5.6). This was in contrast to the series of experiments presented in figure 5.2 in which IL-6 trans-signalling and IL-1 $\beta$  had significantly stimulated more MCP-1 release than unstimulated

Interestingly, the combination of 10ng/ml IL-1 $\beta$  and IL-6/sIL-6R significantly upregulated MCP-1 (14340  $\pm$  1625 pg/ml) compared to 10ng/ml IL-1 $\beta$  alone (3107  $\pm$  745 pg/ml). Although the combination of 5ng/ml IL-1 $\beta$  and the soluble receptor had elevated MCP-1, it

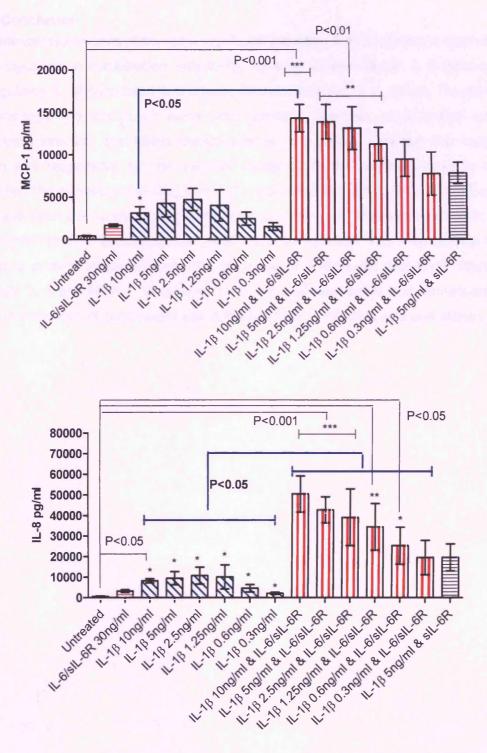
was not significantly more from 5ng/ml IL-1 $\beta$  alone or significantly different from IL-1 $\beta$  and IL-6/sIL-6R (figure 5.6).

# 5.3.4.2 IL-8 release from IL-6/sIL-6R and IL-1β- stimulated A549s

IL-6/sIL-6R had greatly elevated IL-8 release (3256  $\pm$  662 pg/ml) compared to unstimulated (648  $\pm$  228 pg/ml) but this was not a significant difference. IL-1 $\beta$  alone had also elevated IL-8 release, in which 10ng/ml had significantly induced 8233  $\pm$  1009 pg/ml compared to unstimulated cells.

Interestingly, IL-1 $\beta$  in combination with IL-6/sIL-6R had significantly elevated IL-8 release compared to IL-1 $\beta$  alone. This was observed at all concentrations of IL-1 $\beta$ . For example, using a higher dose IL-1 $\beta$  at 10ng/ml in combination with 30ng/ml IL-6/sIL-6R had significantly elevated IL-8 release to 42722  $\pm$  6306 pg/ml. In addition, stimulating the cells with the lower dose IL-1 $\beta$  at 0.3ng/ml plus 30ng/ml IL-6/sIL-6R had significantly increased MCP-1 release to 7754  $\pm$  2553 pg/ml, compared to 0.3ng/ml IL-1 $\beta$  alone (1511  $\pm$  446pg/ml).

IL-8 release in response to 5 ng/ml IL-1 $\beta$  and the soluble receptor was not significantly different from 5 ng/ml IL-1 $\beta$  alone or IL-1 $\beta$  and IL-6/sIL-6R (figure 5.6).



**Figure 5.6:** MCP-1 and IL-8 release from A549s. Cells were seeded onto to six well plates and allowed to reach 80% confluency prior to serum starvation for 18 hours. Cells were treated with 30ng/ml IL-6/sIL-6R or IL-1 $\beta$  at various concentrations or all stimuli combined. Following an 18 hour incubation at 37°C, the supernatants were collected and analysed for MCP-1 and IL-8 using an ELISA kit. Experiments are n = 4.

Statistical analysis was performed by Kruskal-Wallis non parametric ANOVA test with Dunn's multiple comparison post-test.

#### 5.3.4.3 Conclusion

It was unexpected to have observed a significant elevation in IL-8 release in response to IL-6 trans-signalling in combination with IL-1β. Hurst *et al.* had shown IL-6 trans-signalling downregulates IL-1β stimulated IL-8 release (Hurst, Wilkinson et al. 2001). The response to IL-6 trans-signalling and IL-1β alone were variable. However, in a routine testing for *mycoplasma* infection, the A549s tested positive. It is highly likely that the *mycoplasma* infection was responsible for the variation observed in the cell responses to cytokine-stimulation. The elevation in MCP-1 and IL-8 in response to IL-6/sIL-6R and IL-1β combined could have been due to the *mycoplasma* infection. However, it was interesting to observe such an inflammatory response in the presence of an infection. This may indicate that if an infection is present in the airways, the presence of IL-6/sIL-6R and IL-1β could greatly upregulate IL-8 and MCP-1, causing a major influx of neutrophils and mononuclear cells. Further investigation of such responses in the absence of infection was undertaken.

# 5.3.5 Chemokine release from mycoplasma-free A549s in response to IL-6 transsignalling

The responses of mycoplasma-infected A549 cells were to be compared with *mycoplasma*-free A549 cells. A549 cells were obtained from the European collection of cell cultures (ECACC). These cells were of a low passage number and free of infection. A549 cells were seeded at 3 x 10<sup>5</sup>/ml and two ml was added per well of a six well plate. Cells were grown to 80% confluency and serum starved for 18 hours. A549s were treated with 30ng/ml IL-6/sIL-6R alone or in combination with varying concentrations of IL-1β. IL-1β ranging in concentration from 0.3 to 10ng/ml was used to stimulate A549s with and without 30ng/ml IL-6/sIL-6R. Following 18 hours incubation at 37°C, supernatants were harvested, pelleted and diluted accordingly.

5.3.5.1 MCP-1 release from IL-6/sIL-6R and IL-1 $\beta$ - stimulated mycoplasma-free A549s Unstimulated cells did not produce detectable MCP-1 nor did they produce MCP-1 in response to IL-6/sIL-6R. IL-1 $\beta$  at 10ng/ml significantly increased MCP-1 release (167  $\pm$  55pg/ml). The cells had responded to a range of lower IL-1 $\beta$  concentrations by releasing small amounts of MCP-1 but it was not significantly different to unstimulated.

Interestingly, the combination of IL-6/sIL-6R and IL-1 $\beta$  had significantly elevated MCP-1 compared to unstimulated. However, this was not significantly different to IL-1 $\beta$ -stimulated MCP-1.

The combination of  $5 \text{ng/ml IL-} 1\beta$  and the soluble receptor had not significantly altered MCP-1 release compared to  $5 \text{ng/ml IL-} 1\beta$  alone or IL-1 $\beta$  and IL-6/sIL-6R (figure 5.7).

**5.3.5.2 IL-8 release from IL-6/sIL-6R and IL-1β- stimulated mycoplasma-free A549s** Unstimulated cells did not produce detectable IL-8 nor did they produce IL-8 in response to IL-6/sIL-6R. IL-1β at all concentrations elevated IL-8 release compared to unstimulated. This was a significant upregulation in response to 10ng/ml (5629  $\pm$  2004 pg/ml), 5ng/ml (3327  $\pm$  2378 pg/ml), 2.5ng/ml (3647  $\pm$  2611 pg/ml) and 1.25ng/ml (3949  $\pm$  2031 pg/ml) compared to unstimulated cells.

Although the combination of IL-6/sIL-6R and IL-1 $\beta$  significantly increased IL-8 release compared to unstimulated, it was *not* significantly different from the IL-8 release in response to IL-1 $\beta$  alone.

IL-8 release in response to  $5 \text{ng/ml IL-1}\beta$  and the soluble receptor was not significantly different from IL-1 $\beta$  alone or IL-1 $\beta$  and IL-6/sIL-6R (figure 5.7).

#### 5.3.5.3 Conclusion

IL-6 trans-signalling did not induce MCP-1 or downregulate IL-1 $\beta$ -stimulated IL-8 release. An increase in both MCP-1 and IL-8 in response to the combined stimuli of IL-6/sIL-6R and IL-1 $\beta$  was observed but this was not significantly different from IL-1 $\beta$ -stimulated chemokine release. The cells did not express basal MCP-1 or IL-8.

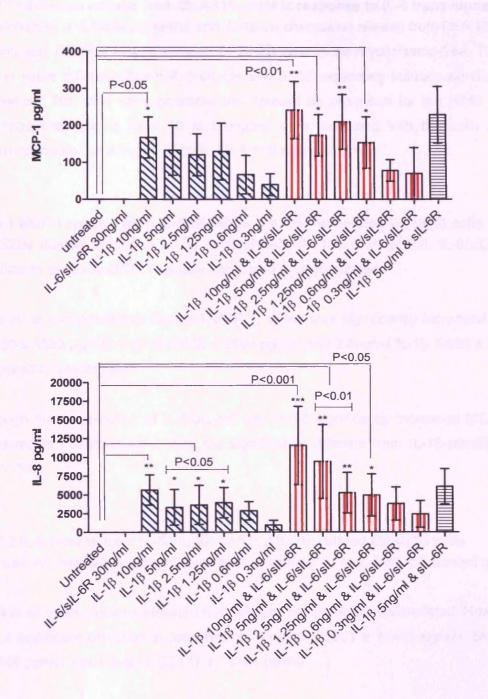


Figure 5.7: MCP-1 and IL-8 release following 18 hours stimulation with IL-1 $\beta$  at varying concentrations, with and without 30ng/ml IL-6 and sIL-6R. Mycoplasma-free and low passaged A549s were seeded in six well plates. Upon reaching 80% confluency, the cells were serum starved for 18 hours prior to treatments. After 18 hours at 37°C, supernatants were harvested and analysed for MCP-1 and IL-8 by an ELISA (eBioscience and R&D respectively). Experiments are n = 3. Statistical analysis was performed by Kruskal-Wallis non parametric ANOVA test with Dunn's multiple comparison post-test.

# 5.3.6 Chemokine release from BEAS2B cells in response to IL-6 trans-signalling

The impact of IL-6 trans-signalling and IL-1 $\beta$  on chemokine release from BEAS2B cells was investigated. Routine testing showed BEAS2B cells to be mycoplasma-free. The previous data in figure 5.3 had shown IL-6/sIL-6R and IL-1 $\beta$  separately induces significant MCP-1 expression. The cells were prepared and treated as described for the A549 cells. IL-1 $\beta$  concentrations ranging from 10 to 0.6ng/ml were incubated with the cells alone or in combination with the 30ng/ml IL-6/sIL-6R for 18 hours at 37°C.

## 5.3.6.1 MCP-1 release from IL-6/sIL-6R and IL-1β- stimulated BEAS2B cells

BEAS2Bs basally produced a low level of MCP-1 (140  $\pm$  39 pg/ml). IL-6/sIL-6R did not significantly increase MCP-1 release compared to unstimulated.

IL-1 $\beta$  at all concentrations elevated MCP-1, which was significantly increased by 10ng/ml (11596 ± 1585 pg/ml), 5ng/ml (9442 ± 2604 pg/ml) and 2.5ng/ml IL-1 $\beta$  (9668 ± 3551 pg/ml) compared to unstimulated.

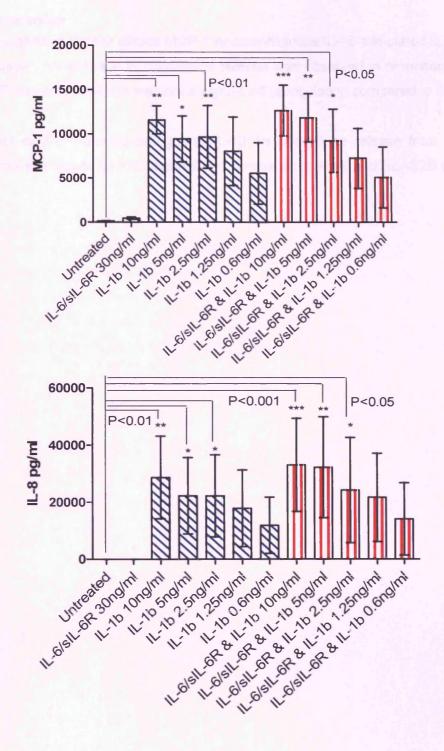
Although the combination of IL-6/sIL-6R and IL-1 $\beta$  significantly increased MCP-1 release compared to unstimulated, it was not significantly different from IL-1 $\beta$ -stimulated MCP-1 (figure 5.8).

### 5.3.6.2 IL-8 release from IL-6/sIL-6R and IL-1β- stimulated BEAS2B cells

IL-8 was not detected in supernatants from unstimulated or IL-6/sIL-6R treated cells.

IL-1β at all concentrations elevated IL-8 release compared to unstimulated. However, it was only a significant elevation in response to 10ng/ml (28631  $\pm$  14451 pg/ml), 5ng/ml (22241  $\pm$ 13345 pg/ml) and 2.5ng/ml (22177  $\pm$  14334 pg/ml).

The combination of IL-6/sIL-6R and IL-1 $\beta$  did not significantly increase IL-8 release compared to IL-1 $\beta$ -stimulated IL-8 (figure 5.8).



**Figure 5.8:** MCP-1 and IL-8 release following 18 hours stimulation with IL-1 $\beta$  at varying concentrations with and without 30ng/ml IL-6 and sIL-6R. BEAS2Bs were seeded into six well plates and allowed to reach 80% confluency prior to serum starvation for 18 hours. Following treatment for a further 18 hours at 37°C, the supernatants were collected and analysed for MCP-1 and IL-8 by an ELISA (eBioscience and R&D respectively). Experiments are n = 3.

# 5.3.6.3 Conclusion

IL-6 trans-signalling did not induce MCP-1 or downregulate IL-1 $\beta$ -stimulated IL-8 in BEAS2B cells. However, an increase in chemokine release was observed in response to IL-1 $\beta$  and IL-6/sIL-6R combined but this was not a significant upregulation compared to IL-1 $\beta$  alone.

The impact of IL-6 trans-signalling and IL-1β on chemokine release from a non-airway epithelial cell line was to be investigated and compared to A549 and BEAS2B cells.

# 5.3.7 The impact of IL-6 trans-signalling in a non-airway epithelial cell line

In two transformed airway epithelial cell lines, IL-6 trans-signalling did not significantly elevate MCP-1 release or downregulate IL-8 release. In comparison, the responses from a transformed cervical epithelial cell line known as Hela cells were assessed to determine if the apparent non-responsiveness of A549s and BEAS2B cells are unique to airway epithelial cells.

## 5.3.7.1 Expression of gp130 on Hela cells

Prior to measuring chemokine release from IL-6/sIL-6R and IL-1β stimulated Hela cells, initial experiments were set up to determine the presence of gp130 expression. The IL-6/sIL-6R complex requires gp130 for trans-signalling to occur. The Hela cells were stained with PE-conjugated antibodies specific for IL-6R, gp130 or CD59. The latter was included as a positive control as this particular receptor is constitutively expressed at high levels on most cells. The cells were analysed by flow cytometry to identify cell surface receptor expression

Figure 5.9 shows Hela cells to be IL-6R negative, gp130 positive and CD59 positive (the black open peaks). As a negative control, cells were stained with an IgG1 or IgG2a antibody specific to the PE-conjugated antibodies, known as isotype controls (represented by the full red peaks)

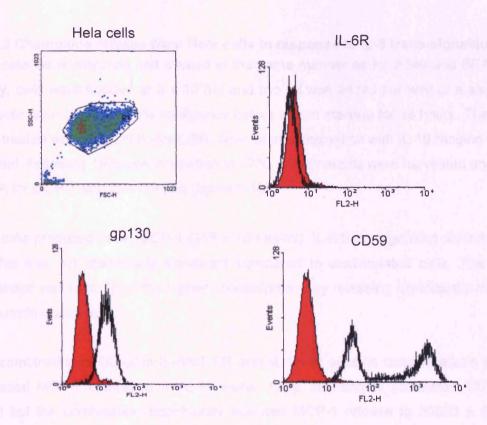


Figure 5.9: Hela cells were prepared and stained to determine receptor expression by flow cytometry. Graphical analysis was performed using WinMDI. Red peaks represent the isotype control. The graphs represent data obtained from one experiment, which is representative of three repeats.

### 5.3.7.2 Chemokine release from Hela cells in response to IL-6 trans-signalling

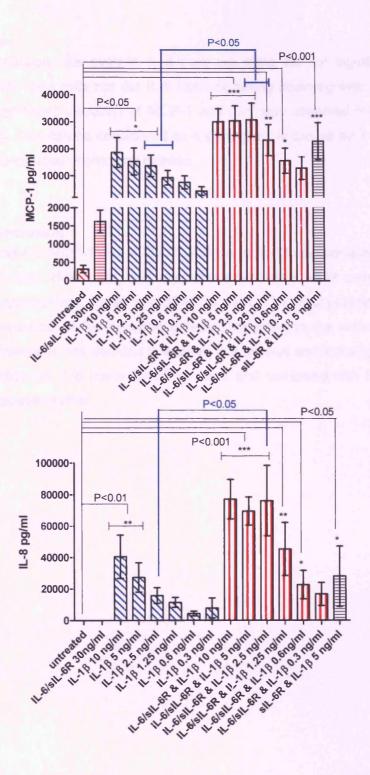
Hela cells were prepared and treated in the same manner as for A549 and BEAS2B cells. Briefly, cells were seeded at 3 x  $10^5$ /ml and two ml was added per well of a six well plate. The cells were grown to 80% confluency before serum starving for 18 hours. The Hela cells were treated with 30ng/ml IL-6/sIL-6R alone or in combination with IL-1 $\beta$  ranging from 0.3 to 10ng/ml. Following 18 hours incubation at 37°C, supernatants were harvested and tested by ELISA for MCP-1 and IL-8 release (figure 5.10).

Hela cells produced basal MCP-1 (318  $\pm$  100 pg/ml). IL-6 trans-signalling did induce MCP-1 but this was not statistically significant compared to unstimulated cells. The Hela cells responded well to IL-1 $\beta$  at the higher concentrations by releasing significantly more MCP-1 than unstimulated cells.

The combination of 30ng/ml IL-6/sIL-6R and IL-1 $\beta$  at specific concentrations significantly increased MCP-1 compared to IL-1 $\beta$  alone. IL-1 $\beta$  at 2.5ng/ml stimulated 13732  $\pm$  3884 pg/ml but the combination significantly elevated MCP-1 release to 30803  $\pm$  6163 pg/ml. There was also a significant difference in MCP-1 release in response to 1.25ng/IL-1 $\beta$  and IL-6/sIL-6R compared to 1.25ng/ml IL-1 $\beta$  alone.

IL-8 release was not detected in supernatants from unstimulated cells or in response to IL-6/sIL-6R. The titration of IL- $1\beta$  stimulated an IL-8 dose release but the higher concentrations of IL- $1\beta$  were required to induce significantly more IL-8 than unstimulated.

The combination of 30ng/ml IL-6/sIL-6R and 2.5ng/ml IL-1β significantly increased IL-8 release in comparison to 2.5ng/ml IL-1β alone (figure 5.10).



**Figure 5.10:** MCP-1 and IL-8 release following 18 hours stimulation with IL-1 $\beta$  at varying concentrations with and without 30ng/ml IL-6 and sIL-6R. Hela cells were seeded into six well plates and allowed to reach 80% confluency prior serum starvation for 18 hours. Following cytokine treatment for 18 hours at 37°C, supernatants were harvested and analysed MCP-1 and IL-8 by an ELISA. Experiments are n = 4.

#### 5.3.7.3 Conclusion

Although some induction was evident, IL-6 trans-signalling did not significantly stimulate MCP-1 release from Hela cells nor did IL-6 trans-signalling downregulate IL-1 $\beta$  stimulated IL-8. Instead, a significant elevation of MCP-1 and IL-8 was observed in response to IL-6/sIL-6R and IL-1 $\beta$ . This can be considered as a synergistic response as it was significantly different from IL-1 $\beta$ -induced chemokine release.

### 5.3.7.4 Overall conclusion

A synergistic increase in MCP-1 and IL-8 in response to IL-6 trans-signalling and IL-1 $\beta$  had been observed in both Hela cells and mycoplasma-infected A549 cells. This was not observed in mycoplasma-free A549s nor BEAS2B cells. The mycoplasma-infected A549s and the mycoplasma-free A549s provided different insights into the activity of IL-6 transsignalling in the airways. It was decided to explore this avenue and investigate the apparent impact of an infection on IL-6 trans-signalling alone and combined with IL-1 $\beta$  in terms of MCP-1 and IL-8 release *in vitro*.

# 5.3.8 The impact of *Ureaplasma Parvum* on IL-6 trans-signalling in airway epithelial cells

#### 5.3.8.1 Introduction

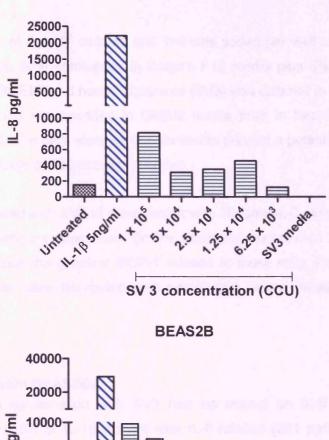
Ureaplasma is a member of the mycoplasma family and have been strongly associated with the development of CLD (Schelonka and Waites 2007). They are mucosal pathogens lacking a cell wall (Viscardi, Kaplan et al. 2002). One type of *Ureaplasma parvum* is serovar 3 (SV3). It is the most common isolate obtained from preterm infants with CLD (Beeton, Chalker et al. 2009). *Ureaplasma* may contribute to the neonatal lung injury by augmenting the inflammatory response within the airways (Viscardi, Kaplan et al. 2002). Given the observations in mycoplasma infected A549s, transformed airway epithelial cells were treated with heat-killed SV3 and chemokine release was measured.

# 5.3.8.2 IL-8 release from airway epithelial cells in response to *U.parvum*

A549s and BEAS2Bs were seeded at 7 x 10<sup>4</sup> cells/ml and 100µl was added per well of a 96 well plate. At 70% confluency, cells were treated with a titration of heat-killed *U.parvum* (more detail on preparation in method section 2.14) in Kaign's F12 media containing 5% FCS and incubated for 24 hours at 37°C. Supernatants were harvested and centrifuged to remove debris. An ELISA was used to determine the IL-8 concentration.

An initial titration (figure 5.11) showed BEAS2Bs to be more responsive than A549s to SV 3. At 1 x  $10^5$  colour-changing units (CCU), 9717 pg/ml of IL-8 was stimulated from BEAS2Bs compared to 813 pg/ml release from A549s. Figure 5.11 shows a dose-response of IL-8 release from BEAS2Bs in response to SV3. The CCU corresponds to the number of viable cells. As SV3 stimulated more IL-8 release from BEAS2Bs than A549s, BEAS2B cells were assessed for chemokine release in response to SV3 and IL-6/sIL-6R or IL-1 $\beta$  separately, or all stimuli combined.





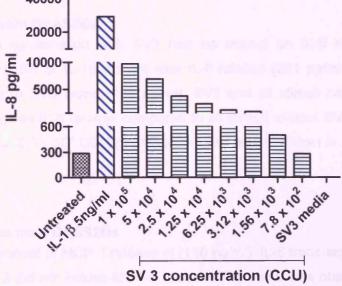


Figure 5.11: A549 and BEAS2B cells were treated with a titration of Ureaplasma Parvum (SV3) for 18 hours at 37°C. An ELISA was used to determine the IL-8 concentration in the supernatants. The data is from a single experiment to initially test the cells response to heat-killed U.parvum.

# 5.3.8.3 The impact of *U.Parvum* on chemokine release in response to IL-6 transsignalling BEAS2B cells.

BEAS2Bs were seeded at 6 x 10<sup>4</sup> cells/ml and 1ml was added per well of a 24 well plate. The cells were grown to 80% confluency in Kaign's F12 media plus 5% FCS. BEAS2Bs were serum starved in DMEM for 6 hours. *U.parvum* (SV3) was cultured to a defined density determined by CCUs and resuspended in DMEM media prior to heat-kill treatment (as outlined in section 2.14). The cells were serum starved to prevent a potential inhibition of IL-6 trans-signalling by soluble gp130 present in serum.

BEAS2B cells were treated with SV3 in combination with 30ng/ml IL-6 plus 100ng/ml sIL-6R or 5ng/ml IL-1β. A preliminary experiment (in the appendix) had shown 30ng/ml IL-6 and 100ng/ml sIL-6R to induce the greatest MCP-1 release in these cells. Following 18 hours incubation, supernatants were harvested and centrifuged before measuring chemokine release (figure 5.12).

#### 5.3.8.3.1 IL-8 release from BEAS2Bs

IL-6 trans-signalling in combination with SV3 had no impact on IL-8 release. SV3 in combination with IL-6/sIL-6R or IL-1 $\beta$  did not alter IL-8 release (201 pg/ml and 307 pg/ml respectively) compared to SV3 alone (161 pg/ml). SV3 and all stimuli combined did not have a significant impact on IL-8 release compared to all stimuli without SV3 (1557 pg/ml). In serum free conditions, 2.5 x 10<sup>4</sup> CCU of SV3 stimulated less IL-8 than in the presence of serum (figure 5.11).

#### 5.3.5.3.2 MCP-1 release from BEAS2Bs

SV3 stimulated a low amount of MCP-1 release of (110 pg/ml). IL-6 trans-signalling alone or in combination with SV3 did not induce MCP-1 release. The same was observed for IL-1β. The combined stimuli of IL-6/sIL-6R and IL-1β did stimulate MCP-1 release (1347 pg/ml). This was a slight increase in MCP-1 in response to IL-6/sIL-6R/IL-1β and SV3 (1547 pg/ml), reflecting an additive effect (figure 5.12).

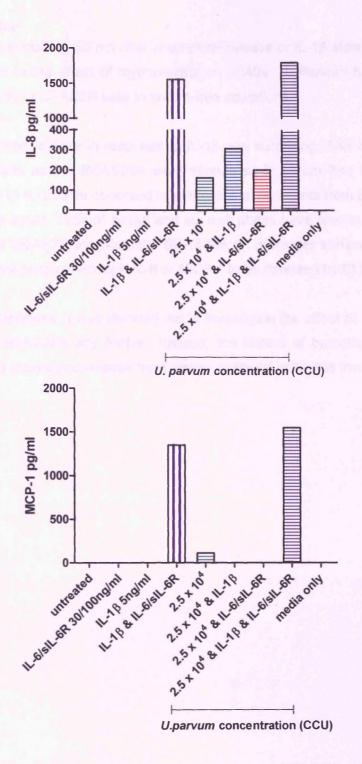


Figure 5.12: IL-8 and MCP-1 release from serum starved BEAS2B cells in response to U.parvum (SV3) stimulation, with and without cytokines. BEAS2Bs were seeded 6 x  $10^4$ /ml in a 24 well plate and grown to 80% confluency in Kaign's F12 media with 5% FCS. Cells were serum starved for six hours in DMEM media. Cells in the presence and absence of heat-killed U.parvum were treated with 30ng/ml IL-6/sIL-6R or 5ng/ml IL-1 $\beta$  or all stimuli combined. Experiment is n = 1.

#### **5.3.8.3.3 Conclusion**

SV3 and IL-6 trans-signalling did not alter chemokine release or IL-1β-stimulated chemokine release. In contrast to the affect of mycoplasma on A549s, *U. Parvum* had little effect on chemokine release from BEAS2B cells in serum-free conditions.

The lack of chemokine release in response to IL-1 $\beta$  was surprising. This could be due to a lower density of cells as the BEAS2Bs were stimulated in serum free media in 24 well plates. A response to IL-1 $\beta$  was observed in numerous experiments from BEAS2Bs seeded onto larger surface areas. T25cm² flasks and six well plates have previously been used in all IL-1 $\beta$ -stimulated BEAS2B experiments. BEAS2Bs on a smaller surface area such as a 24 well plate may not produce enough IL-8 or MCP-1 to be detected by ELISA.

Due to the poor responses, it was decided not to investigate the effect of *U.parvum* on IL-6 trans-signalling in BEAS2Bs any further. Instead, the impact of bacterial components on cytokine-stimulated chemokine release from airway epithelial cells was investigated.

# 5.3.9 The impact of bacterial components and Escherichia coli on chemokine release from cytokine-stimulated airway epithelial cells

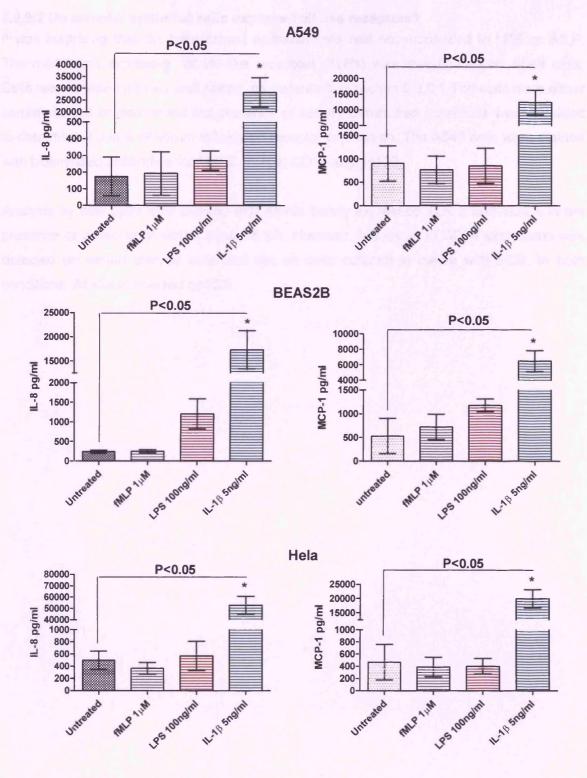
In the presence of a mycoplasma infection, a synergistic increase in MCP-1 and IL-8 was observed in A549s treated with IL-6/sIL-6R and IL-1 $\beta$ . In contrast, BEAS2Bs stimulated with IL-6/sIL-6R and IL-1 $\beta$  in the presence of a heat-killed mycoplasma-related organism, Ureaplasma parvum, did not synergistically induce MCP-1 or IL-8 release.

The effect of bacterial components and *E.coli* on chemokine release from cytokine stimulated A549s and BEAS2Bs was investigated. Hela cells were included in the initial experiments as a control cell line. MCP-1 and IL-8 release was measured to assess the potential influence of infection on IL-6 trans-signalling *in vitro*.

# 5.3.9.1 Can bacterial components stimulate chemokine release from transformed epithelial cells?

Cells were seeded at 3-4 x  $10^5$  cells/ml. Two ml was added per well of a six well plate. A549 and Hela cells were cultured in DMEM containing 5% FCS, where as BEAS2B cells were cultured in Kaign's F12 with 5% FCS. Cells were grown to 50-60% confluency prior to treatment. All three cell lines received fresh media containing 5% FCS without treatment or with 100ng/ml LPS or  $1\mu$ M/ml fMLP or 5ng/ml IL-1 $\beta$ . After 18 hours incubation, the supernatants were collected and cell debris removed prior to testing the supernatants for MCP-1 and IL-8 by an ELISA.

Chemokine release was not significantly elevated in response to LPS or fMLP in any of the cell lines. Although MCP-1 and IL-8 were elevated in BEAS2Bs in response to LPS (1179  $\pm$  133 and 1204  $\pm$  383 pg/ml respectively) it was not significantly different from untreated (MCP-1: 528  $\pm$  370; IL-8: 238  $\pm$  38 pg/ml). All cell lines released significantly more MCP-1 and IL-8 in response to 5ng/ml IL-1 $\beta$  than unstimulated (figure 5.13).



**Figure 5.13:** IL-8 and MCP-1 release measured in supernatants from A549, BEAS2B or Hela cells stimulated with  $l\mu M$  fMLP, 100ng/ml LPS or 5ng/ml IL-1 $\beta$  for 18 hours. Experiments are n=3. Statistical analysis was performed by the Friedman non-parametric repeated measures ANOVA test with Dunn's multiple comparison post-test.

#### 5.3.9.2 Do alveolar epithelial cells express Toll-like receptors?

It was surprising that the transformed epithelial cells had not responded to LPS or fMLP. The membrane expression of toll-like receptors (TLRs) was investigated on A549 cells. Cells were seeded into six well plates, as described in section 5.3.6.1. The cells were either serum starved or maintained the presence of serum. Serum-free conditions were included to determine if a lack of serum influenced receptor expression. The A549 cells were stained with biotinylated antibodies for TLR 2, TLR 4, CD14 and gp130.

Analysis by flow cytometry showed that A549s barely expressed TLR 2 and TLR 4 in the presence or absence of serum (figure 5.14). However, low levels of CD14 expression was detected on serum starved cells and not on cells cultured in media with FCS. In both conditions, A549s expressed gp130.

# A549 in DMEM with 5% FCS TLR-2 TLR-4 CD14 gp130 A549s in DMEM media without FCS TLR 4 TLR 2 CD14 gp130 Events

**Figure 5.14:** Receptor expression on A549 cells in the presence and absence of serum, as determined by flow cytometry. A549s were prepared and stained with biotinylated antibodies specific for TLR 2, TLR 4, CD14 and gp130.

10<sup>2</sup> FL3-H 10<sup>2</sup> FL3-H 10³

#### 5.3.9.3 The response of BEAS2Bs to heat-killed E.coli

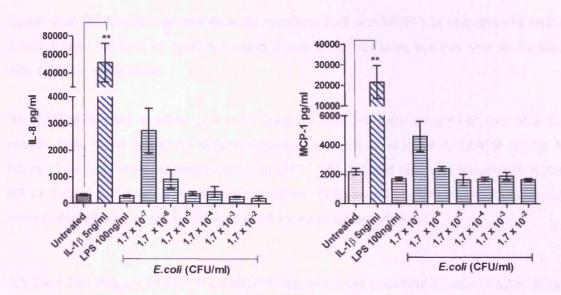
In previous experiments, the transformed airway epithelial cell lines had not significantly released IL-8 or MCP-1 in response to LPS. Using heat-killed *E.coli* may induce a better response. It was decided to proceed with the BEAS2Bs as they were more responsive to *U.Parvum* than A549s, as they had released more IL-8.

BEAS2B cells were seeded at  $4 \times 10^5$  cells/ml and 2ml was added per well to six well plates. Cells were maintained in serum conditions (Kaign's F12 media with 5% FCS) or were cultured to 80% confluency before serum starving in DMEM media. In both conditions, cells were treated with titrating concentrations of *E.coli* for 24 hours at 37°C. Supernatants were collected and analysed by an ELISA for IL-8 and MCP-1.

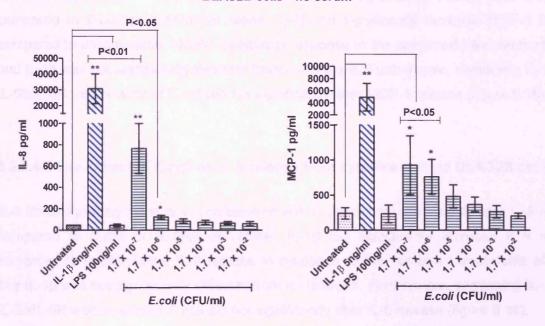
*E.coli* at 1.7 x  $10^{-7}$  CFU/ml induced significantly more MCP-1 and IL-8 release than unstimulated cells in serum free conditions. This significant upregulation was not observed in the presence of serum. This could be due to a high basal release of chemokines in the presence of serum. BEAS2B cells in serum released more basal MCP-1 (2186  $\pm$  221 pg/ml) and IL-8 (323  $\pm$  34 pg/ml) than in serum free conditions (238  $\pm$  80 pg/ml MCP-1 and 45  $\pm$  1 pg/ml IL-8).

IL-1 $\beta$  significantly elevated MCP-1 and IL-8 from both serum starved and serum treated BEAS2Bs (figure 5.15).

#### BEAS2B cells - in serum



#### BEAS2B cells - no serum



**Figure 5.15**: BEAS2Bs were cultured on six well plates and either serum starved in DMEM or kept in Kaign's F12 media containing 5% FCS. E.coli was prepared and the BEAS2Bs were incubated with E.coli titrations for 24 hours at  $37^{\circ}$ C. Supernatants were analysed for IL-8 and MCP-1 by ELISA. Experiments are n = 3. Statistical analysis was performed by non parametric repeated measures ANOVA and the Dunn's multiple comparisons test.

#### 5.3.9.4 Chemokine release from cytokine-treated and E.coli-stimulated BEAS2Bs

Serum-free BEAS2Bs had significantly released IL-8 and MCP-1 in response to heat-killed E.coli. It was decided to continue using E.coli at  $10^{-7}$  CFU/ml but this time in combination with cytokine stimulation.

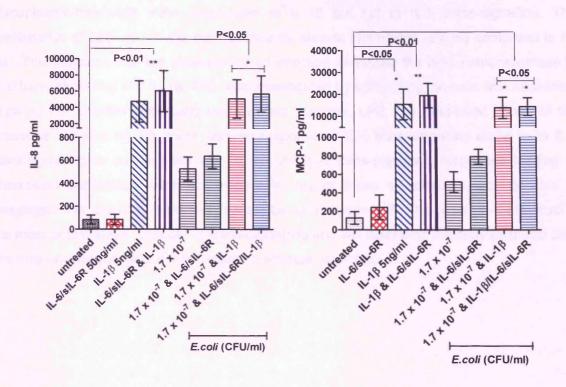
BEAS2B cells were seeded at 4 x  $10^5$  cells/ml and 2ml were added per well of a six well plates. Cells were grown to 80% confluency and serum-starved in DMEM media for 18 hours. The cells were treated with *E.coli* at  $10^{-7}$  CFU/ml and received 50 ng/ml IL-6 and sIL-6R or 5 ng/ml IL-1 $\beta$  or all cytokines combined. Following 24-hour incubation, supernatants were collected and analysed by an ELISA for chemokine release.

#### 5.3.9.4.1 The impact of E.coli on MCP-1 release from cytokine-treated BEAS2B cells

IL-6 trans-signalling in combination with E.coli did not significantly elevate MCP-1 release compared to E.coli or IL-6/sIL-6R alone. IL-1 $\beta$  did significantly increase MCP-1 release compared to unstimulated. MCP-1 release in response to the combined treatments of E.coli and IL-1 $\beta$  was not significantly different from IL-1 $\beta$  alone. Furthermore, combining IL-1 $\beta$  and IL-6/sIL-6R with or without E.coli did not significantly alter MCP-1 release (figure 5.16).

#### 5.3.9.4.2 The impact of E.coli on IL-8 release from cytokine-treated BEAS2B cells

IL-6 trans-signalling alone or in combination with E.coli did not significantly alter IL-8 release compared to E.coli or IL-6/sIL-6R alone. IL-1 $\beta$  did significantly increase IL-8 release compared to unstimulated. IL-8 release in response to the combined treatments of E.coli and IL-1 $\beta$  was not significantly different from IL-1 $\beta$  alone. Furthermore, combining IL-1 $\beta$  and IL-6/sIL-6R with or without E.coli did not significantly alter IL-8 release (figure 5.16).



**Figure 5.16**: *IL-8* and *MCP-1* release from cytokine-stimulated BEAS2Bs in response to heat-killed E.coli. BEAS2Bs seeded onto six well plate, grown to 80% confluency and serum starved. E.coli was prepared and heat-killed as outlined in methodology section 2.13. The BEAS2Bs were incubated with heat-killed E.coli titrations for 24 hours at 37°C with or without *IL-6/sIL-6R*, *IL-1\beta* or *IL-6/sIL-6R* plus *IL-1\beta*. Supernatants were analysed for *IL-8* and *MCP-1* by ELISA. Experiments are n = 3. Statistical analysis was performed by the Friedman non-parametric repeated measures ANOVA test with Dunn's multiple comparison post-test.

#### **5.3.9.4.3 Conclusion**

Heat-killed *E.coli* did not induce a response to IL-6 trans-signalling, IL-1β or all stimuli combined.

#### 5.3.9.5 Overall conclusion

The response to IL-6 trans-signalling in the presence of IL-1β was evident in *mycoplasma*-infected cells as chemokine release was significantly elevated compared to IL-1β alone. Mycoplasma-free cells were responsive to IL-1β but not to IL-6 trans-signalling. The combination of both stimuli did not significantly elevate chemokine release compared to IL-1β. This indicates that the presence of an infection enhanced the cells responsiveness to IL-6 trans-signalling and IL-1β. This was investigated by stimulating the cells with heat-killed *U.parvum* or another potentially more potent stimulus, LPS and heat-killed *E.coli*. In the presence of these stimuli, there was no response to IL-6 trans-signalling alone or to IL-6 trans-signalling in combination with IL-1β. As IL-6 trans-signalling responses leading to chemokine expression were not evident in any of these situations, it was decided to investigate the role of IL-6 trans-signalling by using hyper IL-6. The hyper IL-6 molecule is the most potent known inducer of trans-signalling and will determine if airway epithelial cells are truly un-responsive or if they require a stronger stimulus.

#### 5.3.10 Hyper IL-6 regulation of chemokine release from airway epithelial cells

Airway epithelial cells appear to be unresponsive to IL-6 trans-signalling alone but in the presence of an infection and IL-1β, the cells are responsive. The cells may require a stronger stimulus, such as hyper IL-6 for the induction of trans-signalling. The hyper IL-6 molecule consists of IL-6 covalently linked to sIL-6R. When bound to gp130, hyper IL-6 induces trans-signalling responses which are more potent than those achieved by IL-6/sIL-6R alone. Hyper IL-6 is active on gp130 expressing cells *in vitro* and *in vivo* (Peters, Blinn et al. 1998).

Chemokine release in response to hyper IL-6 was to be investigated in A549 and BEAS2B cells, to determine if these cells are non-responsive to IL-6 trans-signalling.

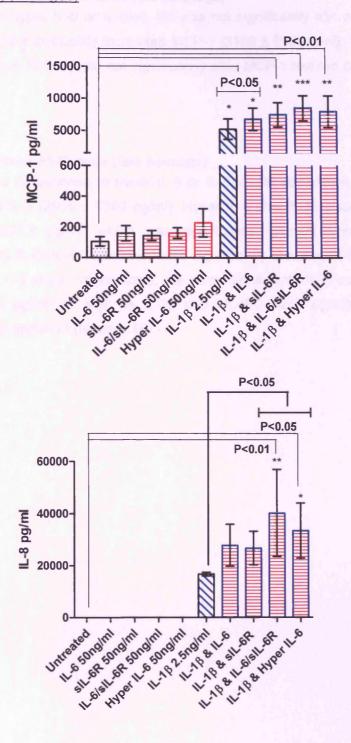
A549 cells that were mycoplasma-free (both the low passaged and the extensively passaged were used) and BEAS2B cells were seeded onto six well plates, allowed to adhere overnight before serum starving. Cells were treated with 50ng/ml of Hyper IL-6 or IL-6/sIL-6R or IL-6 or sIL-6R separately. IL-1 $\beta$  at 2.5ng/ml was used to stimulate cells as a control. Cells were also treated with a combination of IL-1 $\beta$  and Hyper IL-6 or with IL-6/sIL-6R or IL-6 and sIL-6R separately. After 24-hour incubation, supernatants were collected and analysed by ELISA for IL-8 or MCP-1.

#### 5.3.10.1 MCP-1 release from A549 cells (extensively passaged)

The cells basally produced MCP-1. Hyper IL-6 did not significantly induce more MCP-1 than unstimulated or IL-6/sIL-6R-treated cells (figure 5.17). IL-1 $\beta$  significantly enhanced MCP-1 (5250 ± 1580 pg/ml). The combination of IL-1 $\beta$  and IL-6/sIL-6R or IL-1 $\beta$  and hyper IL-6 did not significantly increase MCP-1 release, compared to IL-1 $\beta$  alone.

#### 5.3.10.2 IL-8 release from A549 cells (extensively passaged)

The cells did not produce basal IL-8 nor could it be induced by hyper IL-6 or IL-6/sIL-6R treatment. IL-1 $\beta$  significantly elevated IL-8 levels (16674  $\pm$  693 pg/ml). The combination of IL-1 $\beta$  and IL-6/sIL-6R significantly increased IL-8 (40197  $\pm$  16711 pg/ml) as did IL-1 $\beta$  plus hyper IL-6 (33414  $\pm$  10606 pg/ml) compared to IL-1 $\beta$  alone (figure 5.17). This indicates that a synergistic increase of IL-8 had occurred.



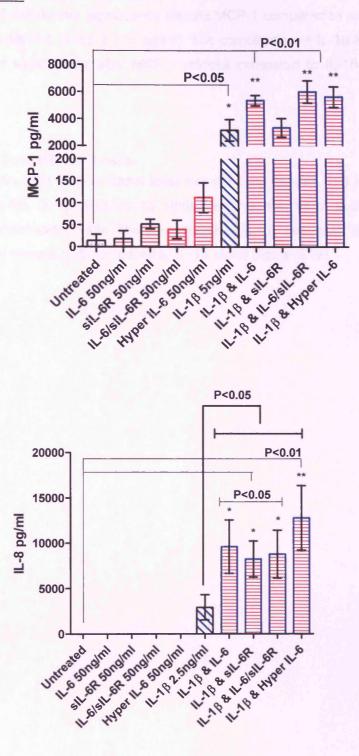
**Figure 5.17:** MCP-1 and IL-8 were measured in supernatants by ELISA, harvested from cytokine-treated A549s after 24 hours incubation. Experiments are n = 3. Statistical analysis was performed by the Friedman non-parametric repeated measures ANOVA test with Dunn's multiple comparison post-test.

#### 5.3.10.3 MCP-1 release from A549 cells (low passage)

MCP-1 in response to hyper IL-6 or IL-6/sIL-6R was not significantly altered compared to unstimulated cells. IL-1 $\beta$  significantly increased MCP-1 (3168 ± 794 pg/ml). The addition of IL-6/sIL-6R or hyper IL-6 to IL-1 $\beta$  did not significantly alter MCP-1 release compared to IL-1 $\beta$  alone (figure 5.18).

#### 5.3.10.4 IL-8 release from A549 cells (low passage)

IL-8 was not produced in response to hyper IL-6 or IL-6/sIL-6R stimulation. IL-1 $\beta$  induced significant amounts of IL-8 (2915 ± 1390 pg/ml). Hyper IL-6 plus IL-1 $\beta$  also increased IL-8 release (12786 ± 3567.8 pg/ml) which was significantly different from IL-1 $\beta$  alone. Interestingly, IL-1 $\beta$  and IL-6/sIL-6R combined also significantly elevated IL-8 release (8789 pg/ml) compared to IL-1 $\beta$  alone. Interestingly, the same significant effect was observed for IL-1 $\beta$  and IL-6 (9605 pg/ml) as well as IL-1 $\beta$  and sIL-6R (8235 pg/ml). A synergistic response was certainly apparent (figure 5.18).



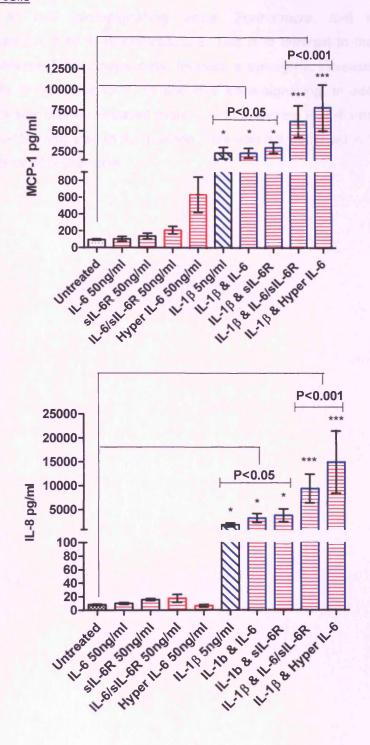
**Figure 5.18:** MCP-1 and IL-8 were measured in supernatants by ELISA. The supernatants were harvested from cytokine-treated A549s after 24 hours incubation. Experiments are n = 3. Statistical analysis was performed by the Friedman non-parametric repeated measures ANOVA test with Dunn's multiple comparison post-test.

#### 5.3.10.5 MCP-1 release from BEAS2B cells

Hyper IL-6 and IL-6/sIL-6R did not significantly elevate MCP-1 compared to untreated. IL-1 $\beta$  significantly increased MCP-1 (2385 ± 711 pg/ml). The combination of IL-1 $\beta$  and hyper IL-6 or IL-6/sIL-6R did not significantly alter MCP-1 release compared to IL-1 $\beta$  alone (figure 5.19).

#### 5.3.10.6 IL-8 release from BEAS2B cells

BEAS2Bs released very little IL-8 at basal level nor did they release IL-8 in response to hyper IL-6 or IL-6/sIL-6R. IL-1 $\beta$  significantly stimulated the release of IL-8 (1815 ± 391 pg/ml) compared to unstimulated cells. The combination of IL-1 $\beta$  plus hyper IL-6 or IL-6/sIL-6R did not significantly elevate IL-8 compared to IL-1 $\beta$  alone (figure 5.19)



**Figure 5.19:** MCP-1 and IL-8 were measured in supernatants by ELISA, harvested from cytokine-treated BEAS2Bs after 24 hours incubation. Experiments are n = 4. Statistical analysis was performed by the Friedman non-parametric repeated measures ANOVA test with Dunn's multiple comparison post-test.

#### 5.3.10.7 Conclusion

Using hyper IL-6 showed that transformed airway epithelial cells did not significantly respond to IL-6 trans-signalling alone. Furthermore, IL-6 trans-signalling did not downregulate IL-8 or IL-1 $\beta$ -induced IL-8. This is in contrast to the responses observed in human peritoneal mesothelial cells. Instead, a synergistic increase in IL-8 was observed in A549 cells in response to IL-1 $\beta$  and IL-6 trans-signalling. In addition, the low passaged A549 cells significantly released more IL-8 in response to IL-6 and IL-1 $\beta$  as well as to sIL-6R and IL-1 $\beta$ , compared to IL-1 $\beta$  alone. This was not observed in the extensively passaged A549 cells or BEAS2B cells.

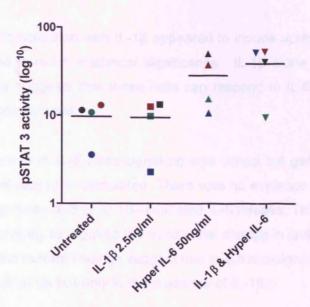
#### 5.3.11 Can IL-6 trans-signalling activate phospho-STAT3 in airway epithelial cells?

In human peritoneal mesothelial cells, the upregulation of pSTAT3 was observed in response to IL-6 trans-signalling and IL-6 trans-signalling mediated IL-1β-induced chemokine release (Hurst, Wilkinson et al. 2001). The airway epithelial cells did not incur chemokine expression in response to hyper IL-6. STAT3 phosphorylation in response to hyper IL-6 was to be investigated to determine if IL-6 trans-signalling actually occurs in these cells. Trans-signalling may occur but does not regulate chemokine expression. STAT3 is a universal downstream molecule that is activated by gp130. Therefore, if IL-6 trans-signalling occurs, STAT3 phosphorylation should be observed.

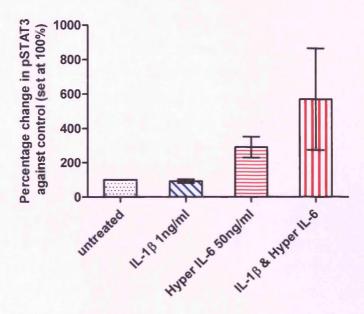
#### 5.3.11.1 Can hyper IL-6 induce pSTAT3 upregulation?

A549s were seeded at 3 x 10<sup>5</sup> cells/ml and two ml was added per well of a six well plate. The cells were grown to 80% confluency and serum starved. Cells were stimulated for 30 minutes with 50ng/ml hyper IL-6, 2.5ng/ml IL-1β, hyper IL-6 & IL-1β. As outlined in the method section 2.15, cells were fixed in 2% PFA and permeabilised by 90% methanol. This allowed the intracellular staining with pSTAT3 antibodies. Cells were analysed by flow cytometry. The data points on the scatter graph in figure 5.20 represent individual experiments. A different colour represents a different experiment but the same colour data point represents the same experiment. The data is also expressed as percentage difference against untreated to reduce the variability between experiments (figure 5.21).

As figure 5.20 shows A549s constitutively express low activity of pSTAT at basal level, which was not significantly altered by IL-1 $\beta$ . Hyper IL-6 alone or in combination with IL-1 $\beta$  did induce notable upregulation of pSTAT3 but it was variable and not significantly different to untreated cells in this series of experiments (figure 5.21).



**Figure 5.20**: Activity of pSTAT 3 (log) in unstimulated A549s or stimulated with 2.5ng/ml IL-1 $\beta$ , 50ng/ml hyper IL-6 or 2.5ng/ml IL-1 $\beta$  plus 50ng/ml hyper IL-6. Experiments are n=3.



**Figure 5.21**: The percentage difference in pSTAT3 activity. A549s were stimulated with 2.5ng/ml IL-1 $\beta$ , 50ng/ml hyper IL-6 or 2.5ng/ml IL-1 $\beta$  plus 50ng/ml hyper IL-6 and compared to unstimulated (set to 100%). Experiments are n=3. Statistical analysis was performed by the Friedman non-parametric repeated measures ANOVA test with Dunn's multiple comparison post-test.

#### **5.3.11.2 Conclusion**

Hyper IL-6 alone or in combination with IL-1 $\beta$  appeared to induce upregulation of pSTAT3, although this data failed to reach statistical significance. IL-1 $\beta$  alone did not significantly alter pSTAT3. This data suggests that these cells can respond to IL-6 trans-signalling but such responses are relatively weak.

MCP-1 release in response to IL-6 trans-signalling was varied but generally the response was weak and not significant to unstimulated. There was no evidence to suggest that IL-6 trans-signalling downregulates IL-8 or IL-1 $\beta$ -stimulated IL-8 release. This could indicate that IL-6 trans-signalling is unlikely to regulate the transitional change in leukocyte recruitment in the airways. However, the current findings suggest that IL-6 trans-signalling may have a role in the initial influx of neutrophils but only in the presence of IL-1 $\beta$ .

Overall the response of airway epithelial cells was inconsistent, suggesting that they are a poor model of airway epithelial responses to IL-6 trans-signalling. In the next chapter, the impact of IL-6 trans-signalling and IL-1 $\beta$  will be investigated in primary airway epithelial cells.

#### 5.4 Discussion

#### 5.4.1 Chemokine release in response to IL-6 trans-signalling

Transformed airway epithelial cells were used to investigate the ability of IL-6 transsignalling to regulate chemokine production as seen in other cell types.

The initial experiments (figure 5.2 and figure 5.3) showed a significant release of MCP-1 in response to IL-6 trans-signalling in A549 and BEAS2B cells. This was contradicted by the data in figure 5.5 and 5.18, which showed IL-6 trans-signalling did not significantly induce MCP-1 in A549 or BEAS2B cell. Although the A549 and BEAS2B cells were obtained from the same source, a different batch was used in some of the experiments. The different batches of cells responded differently to IL-6 trans-signalling. This could be due to the age and passage number of the cells. For example, the age of the 'extensively passaged' A549s is not known were but it is apparent these transformed cells may characteristically change and behave functionally different from less passaged A549s. It was interesting that Armstrong et al. (2004) had stated that A549s cultured for many generations 'poorly reflect the phenotype of the parent primary cell' indicating that this had been the reason for the discrepancy observed in their results when comparing alveolar type II cell response with that of A549s. In addition, the presence of an infection in one batch of A549s and BEAS2Bs used in the experiments for figure 5.2 and 5.3 could be responsible for the significant release of MCP-1 in response to IL-6 trans-signalling. However, specific experimental conditions could have had an influence. In the initial experiments to test if A549s and BEAS2Bs were IL-6/sIL-6R responsive, the cells had been seeded into T25cm<sup>2</sup> flasks. All subsequent experiments were performed in six well plates or smaller. It was possible that seeding the cells at a lower density might have affected the outcome, as there were fewer cells and consequently a lower response. For example, cells seeded onto 24 well plates for the E.coli and U.Parvum experiments meant there were was less MCP-1 in the supernatant or the ELISA may not have been sensitive enough to detect it. It was certainly 'hit-and- miss' whether the cells released MCP-1 in response to IL-6 trans-signalling.

Although IL-6 trans-signalling responses were variable and weak, IL-1β-stimulated cells maintained a good response in all experiments by releasing significant amounts of MCP-1 and IL-8, which confirms that the variation in response to IL-6 trans-signalling is not due to methodological problems. More than likely, IL-6 trans-signalling does not have an important role in normal healthy airway epithelial cells. Interestingly, Van Der Velden stated that

bronchial epithelial-derived MCP-1 is not involved in the recruitment of leukocytes under healthy conditions (van der Velden, Verheggen et al. 1998), and the outcome of this project supports that finding. Furthermore, they had shown IFNγ, produced in response to viral or antigen stimulus induced copious amounts of MCP-1 but had no effect on IL-8. The suggested mechanism was activation of the JAK/STAT pathway (van der Velden, Verheggen et al. 1998). IL-6 trans-signalling is known to signal through the JAK/STAT pathway. Although the current findings have shown some activation of pSTAT3 in response to IL-6 trans-signalling in A549 cells under normal conditions but was not statistically significant.

#### 5.4.2 Mycoplasma infection in airway epithelial cells

It was unfortunate that a batch of A549s used had a Mycoplasma infection. However, it revealed a potentially new and interesting role for IL-6 trans-signalling during an infection. There are over 100 species of the genus Mycoplasma that vary in their pathogenicity (Yang, Hooper et al. 2002). Interestingly, Mycoplasma pneumoniae is a frequent cause of upper and lower respiratory tract infections, and shown to contribute to pulmonary fibrosis and the development of asthma (Chmura, Bai et al. 2008). Genital Mycoplasmas including the Ureaplasma parvum strain used here, have been isolated from the trachea of small preterm neonates, and are thought to increase the risk of developing CLD (Baier and Kruger 2000). M pneumoniae interaction with airway epithelial cells including A549s and BEAS2B stimulate the release of IL-8 (Yang, Hooper et al. 2002; Chmura, Bai et al. 2008). Another species of Mycoplasma known as Mycoplasma hominis (Mh) induced MCP-1 expression in A549s. Both live and heat-inactivated Mh stimulated a dose and time dependent release of MCP-1 but did not appear to involve IL-1β, as it was not detected in Mh-stimulated A549s (Baier and Kruger 2000). There are no reports of the effects of Mycoplasma on cytokineinduced chemokine release. Yang et al. (2002) investigated cytokine and chemokine expression as (including IL-8) from M.pneumoniae-infected A549s. The cells treated with M.pneumoniae for 24 hours had 20-30% decreased cell viability but RT-PCR showed elevated expression of IL-1β and IL-8. Supernatants analysed by an ELISA revealed IL-8 had been released in response to *M.pneumoniae*. In addition, IL-1β mRNA production had been elevated but did not result in the release of IL-1β into the supernatant (Yang, Hooper et al. 2002). The mechanism involved in Mycoplasma-induced chemokine or cytokine production/release is not known.

## 5.4.3 The response of airway epithelial cells to IL-6 trans-signalling and *U.parvum*, fMLP, LPS and *E.coli* stimulation

In an attempt to explore the influence of mycoplasma infection on IL-8 and MCP-1 expression in response to IL-6 trans-signalling, transformed airway epithelial cells were treated with a series of pathogen derived inflammatory stimuli (*U.parvum*, fMLP, LPS or *E.coli*).

Stimulating BEAS2B cells with heat-killed *U.parvum* or *E.coli* had no impact on chemokine release in response to IL-6 trans-signalling. Using a heat-killed pathogen may not have been a strong enough stimulus to evoke a response to IL-6 trans-signalling, as observed in the *mycoplasma*-infected cells.

However, the response of airway epithelial cells to pathogen derived inflammatory stimuli was varied and generally weak. None of the cell lines responded to fMLP, although A549s are known to have a functional formyl peptide receptor required for fMLP-induced activation (Rescher, Danielczyk et al. 2002). However, we did not test the A549s used in this study for fMLP receptor expression.

Although not statistically significant, BEAS2Bs released more IL-8 in response to LPS than unstimulated cells. BEAS2Bs had responded significantly better to *E.coli* than to LPS but it was variable. Unexpectedly, A549s and Hela cells barely responded to LPS as MCP-1 and IL-8 expression did not significantly alter.

Similarly, Standiford *et al.* (1991) did not detect MCP-1 expression in A549s treated with LPS at concentrations ranging from 1pg/ml to 10µg/ml. Data from Shulz *et al* (2002) support this lack of responsiveness to LPS in airway epithelial cells. Their study showed BEAS2Bs and A549s to have the mRNA for TLR 1-6 but not 7-9, so they should in theory respond to LPS. However, they did not show if either cell line express these receptors at the cell surface. Data in the current project has shown that A549s did not express TLR 2 or TLR 4 on the cell membrane. TLR 4 is the key receptor in LPS-induced activation and shares a similar signalling pathway with that of IL-1β-stimulated pathways (Schulz, Farkas et al. 2002). Zhang *et al* also reported undetectable TLR 2 and 4 on unstimulated A549s or on A549s exposed to fungal spores of *Aspergillus fumigatus* (Zhang, Liu et al. 2005).

In a separate investigation, Shulz *et al* cultured BEAS2B cells in RPMI media and A549s in Ham's F12 media, both of which contained 10% FCS. Both cell lines treated with 10ng/ml LPS did not significantly secrete IL-8 compared to untreated. However, in the presence of 10% heat inactivated human serum from a healthy individual; IL-8 release was significantly elevated. This suggested LPS-induced activation of alveolar and bronchial epithelial cells require components in human serum (Schulz, Farkas et al. 2002). This is in contrast to a study by Sorrentino *et al* who demonstrated that A549s release substantial amounts of IL-8 following stimulation with LPS or heat-killed *E.coli*. LPS at a concentration of 1µg/ml significantly induced IL-8 as did heat-killed *E.coli* at 10<sup>-7</sup> and 10<sup>-8</sup> CFU/ml after 24 hours incubation. A549s also responded to gram-positive bacterium *S.aureus* by significantly releasing IL-8. Interestingly, IL-8 gene expression was synergistically enhanced when the cells were treated with gram-negative and gram-positive bacteria combined (Sorrentino, de Souza et al. 2008).

This is the first report to investigate the impact of pathogen derived inflammatory stimuli on chemokine expression in response to IL-6 trans-signalling in airway epithelial cells. However, the outcome of the results showing the weak response of A549 cells to fMLP, LPS, heat-killed *E.coli* and *U.parvum* suggests that this cell line may not be the ideal model for investigating airway epithelial responses to pathogen-derived stimuli. A549 cells have been described as a poor model by other researchers, such as O'Brien *et al.* When assessing cytotoxicity in A549, O'Brien *et al.* found the cells had lost their differentiated cellular function and failed to mimic the *in vivo* situation (O'Brien, Smith et al. 1987). This emphasises the fact that care must be taken when using transformed cell lines as they may respond differently to cells *in vivo*.

#### 5.4.4 The effect of hyperoxia on IL-6 trans-signalling and epithelial cells

IL-6 trans-signalling did not alter cell viability or MCP-1 expression in response to hyperoxia. Exposing airway epithelial cells to 80% O<sub>2</sub> reduced epithelial cell viability, which is likely to reflect stunted cell growth and subsequent cell death, as dead cells do not metabolise MTS. The assumption was that a decrease in cell density would in turn decrease the expression and release of MCP-1 but this was not the case. It is possible that the hyperoxic environment triggered a stress response in the remaining cells to maintain MCP-1 levels in an attempt to resolve inflammation. However, MCP-1 may have a protective affect. According to Okuma *et al*, MCP-1 signalling has the capability of exerting a protective affect

against hyperoxia-induced lung injury by suppressing the production of reactive oxygen species *in vivo* (Okuma, Terasaki et al. 2006). This may be the case *in vitro* and could be investigated by the MTS assay or LDH assay to assess cell viability when raising the toxicity levels (by exposure to hydrogen peroxide or hyperoxia) and treating the cells with MCP-1. Another *in vitro* experiment could look at the impact of MCP-1 on superoxide release from neutrophils. This would be highly relevant as the abundance of neutrophils failing to undergo apoptosis within the airways of premature infants with CLD, have the potential to cause damage to the epithelial cells by releasing the toxic contents. Bacterial peptide fMLP has been shown to induce superoxide release from neutrophils (Ben-Smith, Dove et al. 2001). The cells could be pre-treated with MCP-1 before exposure to fMLP and then superoxide production can be measured by the superoxide dismutase inhibitable reduction of ferricytochrome C to compare the presence and absence of MCP-1 on superoxide release. A reduction in superoxide production in the presence of MCP-1 would indicate its protective affect and the ability of MCP-1 to suppress the production and release of reactive oxygen species *in vitro*.

It was interesting that IL-1β induced MCP-1 expression was maintained, indicating that hyperoxia had not inhibited the IL-1β signalling pathway, nor the pathways involved in MCP-1 production. If this were to reflect a true scenario in adult human airways receiving mechanical ventilation and exposed to high O<sub>2</sub>, hyperoxia may not affect cytokine-stimulated pathways or MCP-1 release. Interestingly, BAL samples obtained from neonates and tested in the Child health department have shown MCP-1 is present (unpublished). This indicates that neonates on mechanical ventilation are capable of producing MCP-1. However, the immature airway of pre-term neonates may display altered expression of MCP-1. The current findings from this project suggest that MCP-1 does not appear to be regulated by IL-6 trans-signalling in normal conditions or hyperoxic conditions.

This project demonstrated that hyperoxia reduced proliferation of airway epithelial cells *in vitro*, which is in support of findings from *in vivo* studies. Neither IL-6 trans-signalling nor IL- $1\beta$  exerted a protective effect on A549s by encouraging cell proliferation when exposed to hyperoxia. Geiser *et al* demonstrated that IL- $1\beta$  did not promote cell proliferation. Instead, it promotes cell spreading and migration (Geiser, Atabai et al. 2001). In light of the findings from Geiser *et al*, the IL- $1\beta$ -induced MCP-1 levels could have been maintained by IL- $1\beta$  promoting alveolar repair, by encouraging cell migration and spreading.

Furthermore, there are conflicting reports regarding the role of IL-6 as to whether it protects cells from hyperoxic-induced injury. None of which have investigated the involvement of sIL-

6R. However, it has been suggested that IL-6 activates the JAK/STAT pathway, ERK pathway and PI3-K/Akt pathway (Kolliputi and Waxman 2009). The latter two pathways have been implemented in the protection of cells from hyperoxic injury (Truong, Monick et al. 2004; Kolliputi and Waxman 2009). Kolliputi et al showed IL-6 via PI3-K/Akt activation increased the survival of cultured HUVEC and SAEC *in vitro* when treated with 100ng/ml IL-6 and exposed to H<sub>2</sub>O<sub>2</sub>. In contrast, A549 cells exposed to 95% O<sub>2</sub> have downregulate PI3-K/Akt activation and loss of protein. Truong et al have shown A549s to have activated ERK which apparently compensated for the loss of PI3-K/Akt. Although there are conflicting reports *in vitro* and *in vivo* on survival pathways in response to hyperoxia and oxidant exposure, the consensus is that IL-6 has a role in protection. The current project is the first to investigate the impact of both IL-6 and sIL-6R in hyperoxia-exposed A549s. It is the generally assumed that sIL-6R acts as an agonist but it may have antagonistic properties (Knupfer and Preiss 2008), which may explain IL-6 trans-signalling having no affect on cell survival in the current project.

Chemokine upregulation from airway epithelial cells exposed to hyperoxia may be regulated in a paracrine fashion. In a study by Wendel *et al*, A549s were either exposed to 85% O<sub>2</sub> or to the supernatants aspirated from endothelial cells treated with IL-1β. Supernatant-treated A549 cells without hyperoxia exposure had upregulated IL-8 and MCP-1 expression, which was not observed in hyperoxia-exposed A549s without supernatants. A549s exposed to 85% O<sub>2</sub> for 12-hours did not have elevated IL-8 or MCP-1 expression and the addition of supernatant under normoxic conditions did not enhance chemokine release. Interestingly, supernatant-induced chemokine expression was reported to be independent of the IL-1 pathway as inhibiting the IL-1R did not suppress chemokine release. However, this was modified by hyperoxia (Wendel, Giessmann et al. 2008). This study was in contrast to the findings in this project as well as Allen *et al* who observed an increase in IL-8 following treatment of A549s with TNF-α and 95% O<sub>2</sub> (Allen, Menendez et al. 2000).

## 5.4.5 The synergistic increase of IL-8 in A549 cells in response to IL-1 $\beta$ and IL-6 transsignalling

This is the first report to demonstrate a synergistic increase of IL-8 in response to IL-1 $\beta$  and IL-6 trans-signalling in A549 cells in normal conditions. The *mycoplasma* infection may be responsible for the synergistic upregulation of MCP-1 in response to IL-6 trans-signalling and IL-1 $\beta$ , as this was not observed in cells without infection. In addition, chemokine release

from the BEAS2B cells was not a synergistic response to the combined stimulation of IL-1 $\beta$  and IL-6 trans-signalling. Two different batches of A549 cells were compared. Although A549s that are extensively passaged produce more MCP-1 and IL-8 in response to stimulation than low passaged A549s, their relative responses to stimuli were the same (figure 5.17 and 5.18). However, the low passaged cells had responded to IL-6 and sIL-6R separately in the presence of IL-1 $\beta$  by significantly increasing IL-8 release. This was not observed in extensively passaged A549 cells. This response has not been previously reported.

A synergistic increase of IL-8 release from A549s has been previously reported in a different study. Thomas *et al* reported a synergistic increase of IL-8 secretion from A549s after exposure to respiratory syncytial virus (RSV)-infected monocytes. They had reported that monocytic release of IL-1β was essential for this synergistic affect but interestingly, IL-6 was not required (Thomas, Wickremasinghe et al. 2000).

The synergistic upregulation of IL-8 in response to IL-6 trans-signalling and IL-1 $\beta$  observed in some situations is in contrast to the current understanding of IL-6 trans-signalling. It is responsible for the downregulation of IL-8 and IL-1 $\beta$ -induced IL-8 in peritoneal mesothelial cells (Hurst, Wilkinson et al. 2001). However, this downregulatory role by IL-6 transsignalling may be cell-specific.

The combination of IL-1β and IL-6 trans-signalling induced IL-8 release indicates a shared signalling molecule/pathway that is activated by IL-1β and IL-6 intracellular signalling. This may be partly mediated by hyper IL-6-induced pSTAT3 activity as it pSTAT3 was upregulated in response to hyper IL-6 and IL-1β. However, the variation in pSTAT3 activity in response to hyper IL-6 in these series of experiments was surprising IL-6 trans-signalling has been known to activate STAT3 in liver cells (Rakemann, Niehof et al. 1999) and murine embryonic stem cells (Jones and Rose-John 2002). IL-6 through its cognate receptor IL-6R activated STAT3 in murine lung epithelium via LPS-stimulated production of IL-6 from the epithelium and endothelium *in vivo* (Haddad, Birrell et al. 2001). In the same study, IL-1β had not activated pSTAT3, which is consistent with the findings from the A549s in the current project. Moreover, IL-1β-induction of MCP-1 and IL-8 does not appear to be via STAT3 activation. The mechanism responsible for the synergistic upregulation of IL-8 release is more likely to be a shared signalling molecule upstream of pSTAT3 activation.

In a study by Yang *et al*, Hela cells released IL-8 and IL-6 in response to IL-1 $\beta$  stimulation, which involves p38 MAPK and ERK1/ERK2 upregulation (Yang, Cohen et al. 2008). This is

interesting as IL-6 intracellular signalling activates p38 MAPK and ERK. This is a potentially shared signalling pathway with IL-1 $\beta$ . Had more time been available, p38 MAPK and ERK activity in response to the combined stimulation of IL-1 $\beta$  and IL-6 trans-signalling would have been investigated. In addition, NF-kB and PI3-K activity would have been investigated, as both molecules are known to be activated by IL-1 $\beta$  and IL-6 trans-signalling separately.

#### 5.4.6 Conclusion

The transformed epithelial cells have provided some insight into the role of IL-6 transsignalling in airway epithelial cells. They barely release basal level MCP-1 nor do they significantly release MCP-1 in response to IL-6 trans-signalling. It is possible that IL-6 transsignalling may not play an important role in the airway epithelium in a normal healthy state. However, the presence of an infection could have a major influence. IL-6 trans-signalling does not appear to downregulate IL-8 and instead may be a regulator, or be regulated by other pro-inflammatory cytokines, such as IL-1β, to elevate IL-8 and MCP-1. All cell lines maintained a good response to the positive control, IL-1β which appears to be a major inducer of MCP-1 and IL-8 in airway epithelial cells. It was interesting that Armstrong *et al*, had stated that A549 cells cultured for many generations 'poorly reflect the phenotype of the parent primary cell' indicating that this could be a reason for the discrepancy observed in their results when comparing primary alveolar type II cell response with that of A549 cells.

In the next chapter, the impact of IL-6 trans-signalling on chemokine expression will be investigated in primary airway epithelial cells.

### **Chapter 6**

Investigating the impact of IL-6 trans-signalling in regulating chemokine expression in primary airway epithelial cells

#### **6.1 Introduction**

Transformed airway epithelial cells have been used to investigate the role of IL-6 transsignalling in the lungs. However, inconsistency and variation in response to cytokine stimulation questions their suitability as an *in vitro* model of lung epithelial cell responses. Overall, the results from transformed airway epithelial cells appear to suggest that IL-6 trans-signalling does not stimulate MCP-1 release nor downregulate IL-8 or IL-1β-induced IL-8 under normal conditions. Instead, a synergistic increase in IL-8 was observed when stimulating cells with IL-1β and hyper IL-6 or IL-6/sIL-6R. In the next chapter, the role of IL-6 trans-signalling shall be investigated further using primary airway epithelial cells as they are likely to reflect the phenotype of airway epithelial cells.

There have been few studies investigating IL-6 trans-signalling in such cells. Gomez *et al* used primary bronchial epithelial cells to demonstrate that epithelial cells produce IL-6 and shed IL-6R; both of which bind gp130. This led to the upregulation of MCP-1 in response to IL-6 trans-signalling (Gomez, Sokol et al. 2005). Human primary mesothelial cells do not express IL-6R but recombinant IL-6/sIL-6R induced MCP-1 upregulation via gp130 (Hurst, Wilkinson et al. 2001).

Primary small airway epithelial cells (SAEC) were purchased from Lonza and cultured according to Lonza's guidelines (method section 2.7.2.2). In addition, human peritoneal mesothelial cells (HPMC) were kindly donated by Dr. C Fielding and Miss C Colmont, to be used in comparison to the SAEC.

Hyper IL-6 will be used because the hyper IL-6 molecule is the most potent known inducer of trans-signalling and will determine if airway epithelial cells are truly un-responsive or if they require a stronger stimulus. Hyper IL-6 consists of IL-6 covalently linked to sIL-6R. When bound to gp130, hyper IL-6 induces trans-signalling responses which are more potent than those achieved by IL-6/sIL-6R alone. Hyper IL-6 is active on gp130 expressing cells *in vitro* and *in vivo* (Peters, Blinn et al. 1998).

Chemokine release in response to hyper IL-6 was to be investigated in SAEC, to determine if primary airway epithelial cells are capable of responding to IL-6 trans-signalling.

#### 6.2 Aims

The role of IL-6 trans-signalling in primary airway epithelial cells SAEC will be determined to establish the validity of the results obtained from the transformed epithelial cell lines. The hypothesis is that SAEC cells are trans-signalling competent as they closely resemble normal airway cells

The specific aims of this chapter were to:

- 1. Establish if primary airway epithelial cells express membrane IL-6R and gp130
- 2. Determine if the primary airway epithelial cells produce MCP-1 in response to IL-6 trans-signalling
- 3. Determine if IL-6 trans-signalling can downregulate IL-8 and IL-1β-induced IL-8 in primary airway epithelial cells.
- 4. Compare and contrast the response from SAEC with a positive control line human peritoneal mesothelial cells (HPMC).

#### 6.3 Results

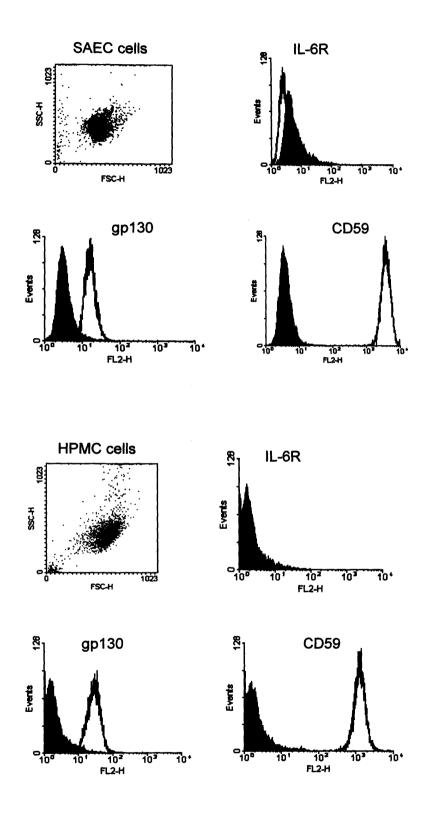
#### 6.3.1 Gp130 and IL-6R receptor expression

Gp130 is required for IL-6 trans-signalling. The SAEC cell membrane were analysed by flow cytometry to check for gp130 expression. In addition, IL-6R expression was determined. The previous chapter showed transformed airway epithelial cells do not express IL-6R. However, Gomez *et al* demonstrated IL-6R expression on primary bronchial epithelial cells (Gomez, Sokol et al. 2005). This is surprising as IL-6R expression is thought to be restricted to leukocytes and hepatocytes. Furthermore, HPMC were previously shown to express gp130 but not IL-6R (Hurst, Wilkinson et al. 2001).

To analyse cell surface receptors, SAEC and HPMC were stained with PE-conjugated antibodies specific for gp130, IL-6R and CD59. The latter was included as a positive control as it is constitutively expressed by both cell lines.

The data obtained by flow cytometry were analysed by WinMDI. The SSC-H/FSC-H graphs in figures 6.1 represent the cell density population obtained. The histograms demonstrate receptor expression of IL-6R, gp130 and CD59 (the black peaks) where as the red peaks represent the isotype control.

As shown in figure 6.1, both SAEC and HPMC were positive for gp130 and CD59 but were negative for IL-6R.



**Figure 6.1:** Cell surface expression of IL-6R, gp130 and CD59 on SAEC and HPMC. Cells were prepared for flow cytometry and stained with PE-conjugated antibodies specific for IL-6R, gp-130 and CD59. The data was analysed by WinMDI and graphically represents one of three experimental repeats. The red peaks are the isotype control.

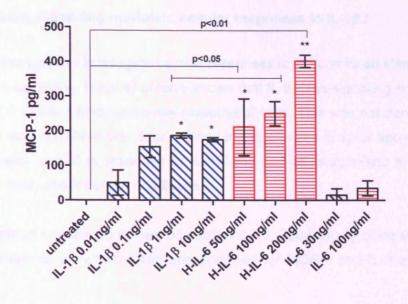
#### 6.3.2 Do SAEC release IL-8 or MCP-1 in response to cytokine stimulation?

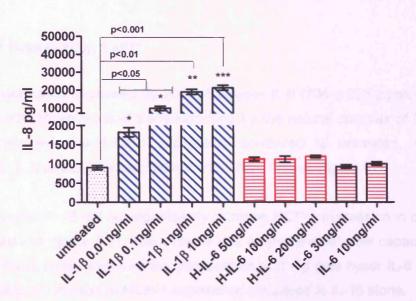
Primary airway epithelial cells, SAEC were shown to express cell surface gp130 which is essential for IL-6 trans-signalling. The next experiments were to analyse cell responses to cytokine stimulation by measuring MCP-1 and IL-8 in the supernatants.

SAEC were prepared as outlined in method section 2.7.2 and incubated for 24 hours with different concentrations of IL-1 $\beta$ , hyper IL-6 or IL-6. Supernatants were harvested and analysed for MCP-1 and IL-8 by an ELISA.

SAEC constitutively express low levels of IL-8 but do not basally express MCP-1. Hyper IL-6 significantly increased MCP-1 release but had no effect on IL-8 expression.

The cells responded to IL-1β concentrations as low as 0.01ng/ml by significantly releasing more IL-8 than unstimulated. However, a higher concentration of 1ng/ml was required to induce a significant release of MCP-1 above unstimulated levels, although MCP-1 expression was generally much lower than that observed in transformed epithelial cells. Chemokine levels were not significantly altered by IL-6 (figure 6.2), reflecting the absence of IL-6R on these cells.





**Figure 6.2:** MCP-1 and IL-8 release from SAEC. SAEC were seeded onto 12 well plates and grown to 50% confluency prior to stimulation. Following 24 hours incubation, supernatants were harvested, pelleted and stored at -80°C. Supernatants were analysed by ELISA for MCP-1 and IL-8. Statistical analysis was performed by the non parametric repeated measures ANOVA test and the Dunn's multiple comparison test. Experiments are n = 3.

#### 6.3.3 Can IL-6 trans-signalling modulate cellular responses to IL-1β?

The next experiment was to investigate cellular responses to the combined stimulation of IL-1β and IL-6 trans-signalling. Hurst *et al* have shown that IL-6 trans-signalling downregulates IL-1β-induced IL-8 release from peritoneal mesothelial cells. This was not demonstrated in airway epithelial cells. Furthermore, data outlined in the previous chapter appear to show a synergistic increase of IL-8 in response to IL-1β and IL-6 trans-signalling in transformed airway epithelial cells, under certain conditions.

SAEC were prepared as outlined in method section 2.7.2. Following cytokine stimulation for 24 hours, supernatants were harvested and analysed for MCP-1 and IL-8 expression by ELISA.

#### 6.3.3.1 MCP-1 release from SAEC

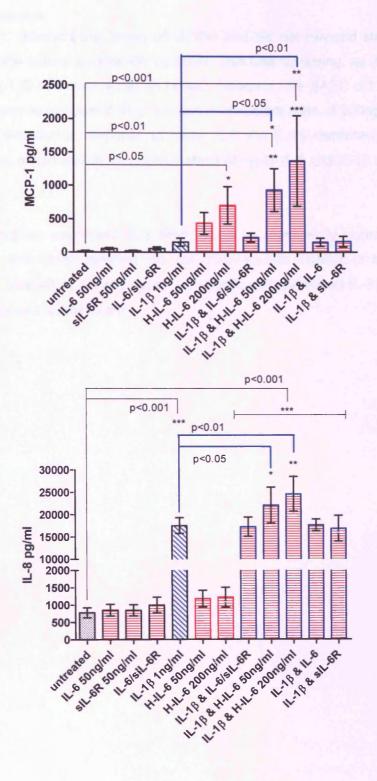
MCP-1 was significantly increased by 200ng/ml hyper IL-6 ( $704 \pm 278$  pg/ml) compared to unstimulated cells. However, IL-6 trans-signalling by the natural complex of IL-6/sIL-6R at 50ng/ml did not enhance MCP-1 expression compared to untreated. As expected, stimulation with IL-6 or sIL-6R alone did not alter MCP-1 levels (figure 6.3).

Surprisingly,  $1 \text{ng/ml IL-1}\beta$  did not significantly increase MCP-1 expression in contrast to the previous experiment (figure 6.2). This may be due to the relatively low capacity for MCP-1 expression in these cells. However, the combination of IL-1 $\beta$  plus hyper IL-6 at 50 ng/ml or 200 ng/ml significantly increased MCP-1 expression compared to IL-1 $\beta$  alone.

#### 6.3.3.2 IL-8 release from SAEC

SAEC constitutively released basal IL-8 (778  $\pm$  144 pg/ml). This was not significantly altered by IL-6 or sIL-6R separately or in combination (figure 6.3) nor did Hyper IL-6 significantly enhance IL-8 release.

IL-1β induced a substantial elevation of IL-8 release (17467 $\pm$  1756) as observed in the previous experiment. However, the addition of IL-6/sIL-6R did not alter IL-1β induced IL-8 release. Interestingly, IL-1β with 50ng/ml or 200ng/ml hyper IL-6 significantly increased IL-8 to 22061  $\pm$  4043 pg/ml and 24577  $\pm$  3884 pg/ml respectively, compared to IL-1β alone.



**Figure 6.3:** SAEC were seeded into 12 well plates and grown to 50% confluency prior to stimulation. Following 24 hour incubation, supernatants were harvested, pelleted and stored at -80°C. Supernatants were checked by ELISA for MCP-1 and IL-8. Statistical analysis was performed by ANOVA repeated measured and the Bonferroni multiple comparisons test. Experiments are n = 5.

#### 6.3.3.3 Conclusion

Overall, SAEC released low levels of MCP-1 and did not respond strongly to IL-6 transsignalling by the natural IL-6/sIL-6R complex. This was surprising, as the cells were shown to express gp130 at similar levels to HPMC. However, the SAEC did significantly release MCP-1 in response to hyper IL-6 at a much more potent dose of 200ng/ml. There was also a significant elevation in response to hyper IL-6 and IL-1 $\beta$  combined, compared to IL-1 $\beta$  alone. Hurst *et al* had shown an additive affect of hyper IL-6 and IL-1 $\beta$  on MCP-1 release in HPMC.

SAEC constitutively expressed IL-8 which could be significantly increased by IL-1 $\beta$ . More interestingly, IL-6 trans-signalling did not downregulate basal IL-8 expression or IL-1 $\beta$ -induced IL-8. Instead, IL-1 $\beta$  and hyper IL-6 significantly increased IL-8 expression, similarly to the observations in A549 cells.

# 6.3.4 Human peritoneal mesothelial cells (HPMC) and chemokine stimulated release in response to IL-6 trans-signalling and IL-1 $\beta$

The cellular responses observed in SAEC were similar to those observed in A549s in chapter 5. This is in contrast to the observations by Hurst *et al.* As a comparison to SAEC responses and as a control cell line, chemokine release from HPMC cells in response to IL-6 trans-signalling were investigated, as this cell line has been extensively used to investigate such responses in the past.

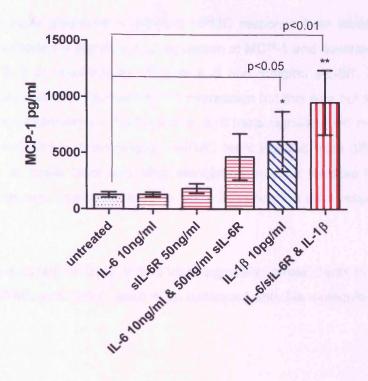
HPMC were isolated from donors and cultured by Dr. C Fielding and Miss C Colmont from the Department of Medical Biochemistry and Immunology at Cardiff University. Donors were not undergoing peritoneal dialysis or an emergency procedure, but further medical details were not available. HPMC were grown to confluency and serum starved for 48 hours. The cells were stimulated with 10ng/ml IL-6 and 50ng/ml sIL-6R, 10pg/ml IL-1 $\beta$  or all stimuli combined for 24 hours (according to previously published procedures). The supernatants were collected and analysed for MCP-1 and IL-8 expression by ELISA. Statistical analysis was performed by Kruskal-Walllis non parametric one way ANOVA and the Dunn's multiple comparisons test. Experiments are n = 3. A parametric test could not be used as the sample size was too small to pass the normality test.

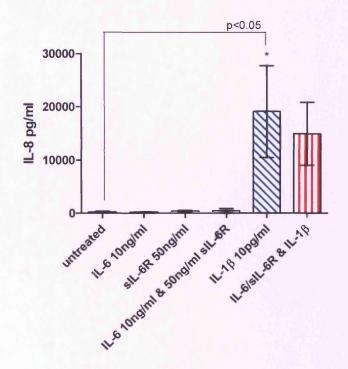
### 6.3.4.1 MCP-1 release from HPMC

Unstimulated HPMC released more basal MCP-1 (1278  $\pm$  235 pg/ml) than SAEC. In addition, IL-6/sIL-6R had elevated MCP-1 to 4663  $\pm$  2058 although it was not significantly different from unstimulated. IL-1 $\beta$  significantly stimulated MCP-1 release to 6034  $\pm$  2666 pg/ml. The combination of IL-1 $\beta$  and IL-6/sIL-6R had elevated MCP-1 levels but was not a significant increase (9502  $\pm$  2872 pg/ml) (figure 6.4).

### 6.3.4.2 IL-8 release from HPMC

HMPC released IL-8 at lower basal level (257  $\pm$  130) compared to SAEC. Similarly, to SAEC, IL-6/sIL-6R had not significantly altered IL-8 release (448  $\pm$  409 pg/ml). IL-1β significantly elevated IL-8 (19129  $\pm$  8641 pg/ml) compared to unstimulated. IL-6/sIL-6R reduced IL-1β-stimulated IL-8 to 14926  $\pm$  8641 pg/ml but it was not a significant downregulation. This is in contrast to previously shown data by Hurst *et al* in which they show IL-6/sIL-6R to significantly downregulate IL-1β-stimulated IL-8 (Hurst, Wilkinson et al. 2001).





**Figure 6.4:** MCP-1 and IL-8 release from HPMC. HPMC were cultured in six well plates. At full confluency, the cells were serum starved for 48 hours prior to cytokine treatment for 24 hours. Supernatants were collected and analysed for IL-8 and MCP-1 expression by ELISA. Experiments are n = 3. Statistical analysis was performed by Kruskal-Wallis and Dunn's multiple comparisons test.

### 6.3.4.3 Conclusion

It was unexpected to have observed a different HPMC response from those reported by Hurst *et al.* They had reported a significant upregulation of MCP-1 and downregulation of IL-8 and IL-1β-induced IL-8 in response to 10ng/ml IL-6 and 50ng/ml sIL-6R. In the current study, IL-6 trans-signalling did upregulate MCP-1 expression but this was not significant due to the variation between experiments. Furthermore, IL-6 trans-signalling did not significantly reduce IL-8 or IL-1β-induced IL-8 expression. HPMC were isolated from different donors. Chemokine secretion at basal level and after stimulation may be variable from donor to donor as disease status and treatment regimes could influence the cells responsiveness to stimuli.

As hyper IL-6 is a more potent inducer of IL-6 trans-signalling, experiments investigating IL-6 trans-signalling in HPMC and SAEC were to be continued with this molecule.

### 6.3.5 HPMC and chemokine stimulated release in response to Hyper IL-6 and IL-1β

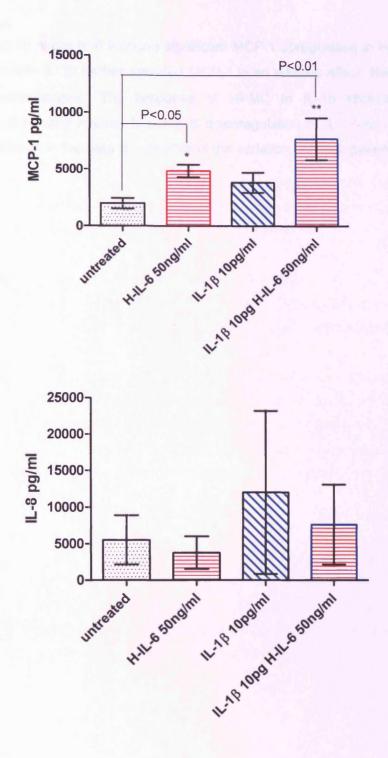
HPMC were cultured in six well plates and serum starved for 48 hours. Confluent HPMC were stimulated with 50ng/ml hyper IL-6 or 10pg/ml IL-1 $\beta$  or the combination of both stimuli for 24 hours. The supernatants were collected and analysed for chemokine release by an ELISA. Statistical analysis was performed by Kruskal-Walllis non-parametric one way ANOVA and the Dunn's multiple comparisons test. Experiments are n = 3. A parametric test could not be used, as the sample size was considered too small to pass the normality test.

### 6.3.5.1 MCP-1 release from HPMC

Unstimulated HPMC released 1993  $\pm$  467 pg/ml of MCP-1 at baseline, which is similar to the previous experiment. Hyper IL-6 significantly upregulate MCP-1 release to 4846  $\pm$  546 pg/ml. IL-1 $\beta$  had elevated MCP-1 but this was not significant compared to unstimulated. Hyper IL-6 in combination with IL-1 $\beta$  significantly upregulated MCP-1 to 7679  $\pm$  1854 pg/ml, compared to unstimulated cells. This was not significantly different from hyper IL-6 or IL-1 $\beta$  alone (figure 6.5).

### 6.3.5.2 IL-8 release from HPMC

HPMC released more IL-8 at baseline (5537  $\pm$  3366 pg/ml) than in the previous experiment. Hyper IL-6 slightly downregulated IL-8 (3772  $\pm$  2227 pg/ml) but this was not significant. Surprisingly, IL-1β did not significantly upregulated IL-8 (12043  $\pm$  11193 pg/ml) as HPMC responses to IL-1β were varied between experiments. Hyper IL-6 in combination with IL-1β downregulated IL-8 to 7638. $\pm$  5491.pg/ml but this was not a significant difference compared to unstimulated or hyper IL-6-stimulated or IL-1β-stimulated IL-8.



**Figure 6.5:** HPMC were cultured in six well plates and serum starved for 48 hours prior to stimulation. Following 24-hour incubation, supernatants were collected and analysed by ELISA for IL-8 and MCP-1. Experiments are n = 3. Kruskal-Wallis and Dunn's multiple comparison test was used for statistical analysis.

## 6.3.5.3 Conclusion

IL-6 trans-signalling by hyper IL-6 induced significant MCP-1 upregulation in HPMC. Hyper IL-6 in combination with IL-1 $\beta$  further elevated MCP-1 in an additive affect. Hurst *et al* also reported similar observations. The response of HPMC to IL-1 $\beta$  stimulation varied, particularly with IL-8 release making hyper IL-6 downregulation of IL-1 $\beta$ -induced IL-8 not significant. This variation in the data is indicative of the variation between patient samples.

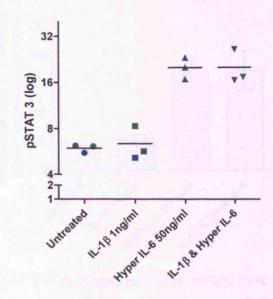
## 6.3.6 Is pSTAT3 upregulated in SAEC in response to hyper IL-6?

SAEC had responded to a higher dose of 200ng/ml of hyper IL-6, by releasing significantly more MCP-1 than unstimulated cells. STAT3 phosphorylation is a key downstream target following gp130 activation. Measuring STAT3 phosphorylation will determine if the hyper IL-6 induced MCP-1 release is mediated through the upregulation of pSTAT3. In the previous chapter, transformed alveolar epithelial cells had slightly upregulated pSTAT3 in response to hyper IL-6 but this was not a significant difference compared to unstimulated. As a comparison, pSTAT3 levels were measured in SAEC and HPMC.

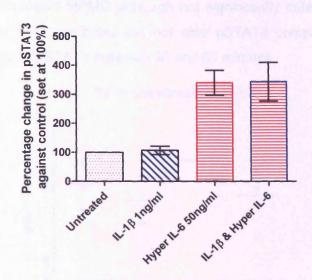
SAEC and HPMC were prepared as outlined in method section 2.7.2 and 2.7.3. HPMC were stimulated with hyper IL-6 or IL-1β or both combined for 30 minutes where as SAEC were stimulated for 1 hour or 30 minutes. Cells were removed by trypsin/EDTA and fixed with 2% paraformaldehyde. Following permeabilisation with methanol, the cells were stained with pSTAT3 and analysed by flow cytometry. The geometric mean was obtained and values plotted individually on a log graph (figure 6.6 and 6.7). In addition, the data is expressed as percentage difference against untreated (figure 6.8 and 6.9). Statistical analysis was performed by Kruskal-Wallis test and the Dunn's multiple comparison tests. Sample size was considered too small for statistical analysis by a parametric ANOVA test.

## 6.3.6.1 Phospho-STAT3 in HPMC

Unstimulated HPMC constitutively expressed pSTAT3 activity (background is  $5.9 \pm 0.2$ ) (figure 6.6). As shown in figure 6.7, pSTAT3 activity was elevated by hyper IL-6 and remained elevated when treated with both hyper IL-6 and IL-1 $\beta$ . Although this was not significantly different from unstimulated, this could be due to a small sample size. Treatment with IL-1 $\beta$  did not affect pSTAT3 activity compared to unstimulated.



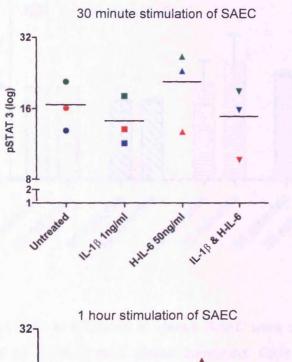
**Figure 6.6:** Activity of pSTAT 3 in response to stimuli. HPMC were grown to confluency in a six well plate and serum starved for 48 hours. HPMC were stimulated for 30 minutes then trypsinised and prepared for intracellular staining. Cells were analysed by flow cytometry and the geometric mean absorbance values were obtained. Individual data points from three separate experiments were plotted on a log scale. Experiments are n = 3. Statistical analysis was performed by Kruskal-Wallis and Dunn's multiple comparison tests.

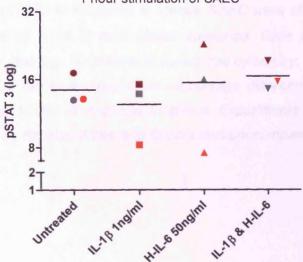


**Figure 6.7:** Activity of pSTAT 3 in response to stimuli. HPMC were grown to confluency in a six well plate and serum starved for 48 hours. HPMC were stimulated for 30 minutes then trypsinised and prepared for intracellular staining. Cells were analysed by flow cytometry and the geometric mean absorbance values were obtained. The data is the same as the data in figure 6.6 but is expressed as percentage difference in pSTAT3 activity against untreated (set at 100%) in response to stimuli. Experiments are n = 3. Statistical analysis was performed by Kruskal-Wallis and Dunn's multiple comparison tests.

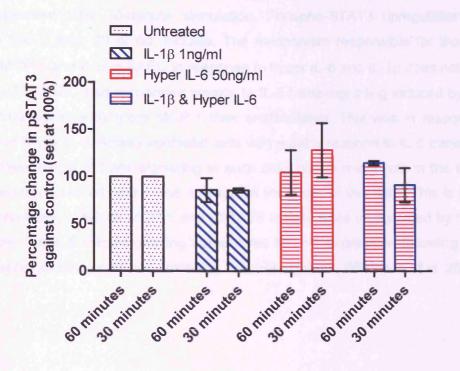
## 6.3.6.2 Phospho-STAT3 in SAEC

Unstimulated SAEC constitutively expressed pSTAT3 activity (figure 6.8). This was at a higher level than unstimulated HPMC although not significantly different. Stimulation with hyper IL-6 or IL-1 $\beta$  or both combined did not alter pSTAT3 compared to unstimulated. There was no difference in pSTAT3 between 30 and 60 minutes.





**Figure 6.8**: Activity of pSTAT3 in response to stimuli. SAEC were stimulated for 30 or 60 minutes with hyper IL-6 or IL-1 $\beta$  or both stimuli combined. Cells were trypsinized and prepared for intracellular staining. Cells were analysed by flow cytometry. The geometric mean absorbance values obtained. Individual data points from three separate experiments were plotted on a log graph. Experiments are n = 3. Statistical analysis was performed by Kruskal-Wallis and Dunn's multiple comparison tests.



**Figure 6.9:** Activity of pSTAT3 in response to stimuli. SAEC were stimulated for 30 or 60 minutes with hyper IL-6 or IL-1 $\beta$  or both stimuli combined. Cells were trypsinized and prepared for intracellular staining. Cells were analysed flow cytometry. The data is the same as the data in figure 6.8 but is expressed as percentage difference in pSTAT3 activity against untreated (set at 100%) in response to stimuli. Experiments are n = 3. Statistical analysis was performed by Kruskal-Wallis and Dunn's multiple comparison tests.

#### 6.3.6.3 Conclusion

In HPMC, hyper IL-6 had induced IL-6 trans-signalling and upregulated pSTAT3 activity. This was observed after 30-minute stimulation. Phospho-STAT3 upregulation was not observed in SAEC after 30 or 60 minutes. The mechanism responsible for the observed increase of MCP-1 and IL-8 in SAEC in response to hyper IL-6 and IL-1β does not appear to involve pSTAT3. SAEC had responded weakly to IL-6 trans-signalling induced by hyper IL-6, by releasing significantly more MCP-1 than unstimulated. This was in response to the higher dose of hyper IL-6. Airway epithelial cells very weakly respond to IL-6 trans-signalling and it is unlikely that IL-6 trans-signalling in such cells play a major role in the transitional change in leukocyte recruitment in the airways on the basis of this data. This is in contrast to HPMC responses, in which MCP-1 and pSTAT3 activity were upregulated by hyper IL-6. This indicates that IL-6 trans-signalling upregulates MCP-1 expression following increased STAT3 phosphorylation, as demonstrated by Hurst *et al* (Hurst, Wilkinson et al. 2001).

### 6.4 Discussion

## 6.4.1 MCP-1 release from SAEC in response to IL-6 trans-signalling under normal conditions

This is the first study to investigate IL-6 trans-signalling in SAEC. The natural IL-6/sIL-6R complex did not induce MCP-1 release. However, SAEC responded to hyper IL-6 by releasing more MCP-1 than unstimulated cells, although this was only significant when stimulated with a high dose of 200ng/ml hyper IL-6. MCP-1 release from SAEC in response to IL-1β was variable. MCP-1 levels remained low indicating that under normal conditions, airway epithelial cells release none or very low amounts of MCP-1. This is a similar observation to a study by Ritter et al, who measured MCP-1 secretion in supernatants by a chemokine/cytokine antibody array and reported SAEC to have no basal expression of MCP-1. A 24 hour stimulation with a ligand for TLR 3, known as poly(I:C) (an analog of viral dsRNA), induced MCP-1 whereas ligands for the other TLRs, such as LPS (TLR 4), flagellin (TLR 5) had no impact on MCP-1 release (Ritter, Mennerich et al. 2005). In contrast, an investigation by Pechkovsky et al reported primary alveolar epithelial cells type II (AEC-II cells) constitutively expressed high level of MCP-1 mRNA and released substantial amounts of MCP-1. A549s up to a passage of nine were used in their comparison study and had demonstrated that MCP-1 release was significantly lower than AEC-II basal level. In addition, MCP-1 was not significantly upregulated by IL-1β in A549s unlike AEC-II (Pechkovsky, Goldmann et al. 2005).

Primary bronchial epithelial cells were shown to constitutively express MCP-1 mRNA. In a study by Becker *et al*, uncultured bronchial epithelial cells spontaneously expressed MCP-1 mRNA, whereas it was variably expressed in cultured bronchial epithelial cells isolated from six healthy individuals. RT-PCR revealed two individuals had not expressed the mRNA for MCP-1 but two weakly expressed it while the remaining two strongly expressed it. MCP-1 release was not measured (Becker, Quay et al. 1994).

## 6.4.2 IL-8 release from primary airway epithelial cells in response to cytokine stimulation

Unstimulated SAEC released high levels of IL-8. This was higher than the IL-8 release from A549s or BEAS2Bs but not as high as HPMC. IL-1 $\beta$  was an excellent inducer of IL-8 in SAEC. A similar effect was observed in the transformed airway epithelial cells. IL-6/sIL-6R did not downregulate IL-8 or IL-1 $\beta$ -induced IL-8. Instead, the addition of hyper IL-6 and IL-1 $\beta$  had further elevated IL-8 release from SAEC. The mechanism involved is unknown but it is unlikely to involve pSTAT3. The results from the HPMC experiments were similar to the findings of Hurst *et al*, although a trend was apparent, a significant downregulation of IL-8 by IL-6 trans-signalling was not observed.

Ritter *et al* used a chemokine/cytokine antibody array to measure chemokine release in SAEC supernatants, and reported IL-8 secretion from untreated SAEC which was greatly enhanced by ligands for TLR 1-6 (Ritter, Mennerich et al. 2005). Thomas *et al* showed SAEC, primary bronchial cells (NHBE) and A549s released IL-8 in a dose-dependent manner in response to IL-1β. The baseline IL-8 was not reported. However, it was demonstrated that in A549s, NF-kB nuclear translocation occurred upon IL-1β stimulation, which included the p50, p65, c-rel subunits. NF-IL-6 and AP-1 were also activated. Unfortunately, the mechanism of IL-1β-induced IL-8 was not included for the primary cells (Thomas, Wickremasinghe et al. 2007).

An interesting study by Van Wetering *et al* showed primary bronchial epithelial cells (PBEC) and A549s to have increased IL-8 mRNA and secreted elevated IL-8 in response to defensins. Neutrophils not only release proteinases but also nonenzymatic polypepetides known as defensins. Stimulated neutrophil-released defensins also increased MCP-1 release from A549s but not from PBEC (Van Wetering, Mannesse-Lazeroms et al. 1997). It is plausible that neutrophils undergoing necrosis could be releasing defensins into the airways of preterm infants, stimulating airway epithelial cells IL-8 and contributing to the neutrophil accumulation in the airways of preterm infants.

## 6.4.3 MCP-1 release from primary airway epithelial cells in response to cytokine stimulation

Although IL-6/sIL-6R had little affect on MCP-1 release from SAEC or HPMC, an analogous but more potent molecule, namely hyper IL-6 was required to significantly elevate MCP-1.

In SAEC, hyper IL-6 in combination with IL-1β significantly enhanced MCP-1 in SAEC compared to IL-1β alone. This was not observed in transformed airway epithelial cells. Interestingly, it does not appear to involve pSTAT3, as pSTAT3 remained unaltered. In contrast to this outcome, IL-6 trans-signalling was shown to stimulate MCP-1 and IL-8 through the upregulation of pSTAT3 in cultured primary endothelial cells (Romano, Sironi et al. 1997). However, this does not appear to be the case in airway epithelial cells under normal conditions.

An additive affect of MCP-1 release was observed from HPMC in response to IL-1β and IL-6/sIL-6R. This supports the findings by Hurst *et al* in which the *in vitro* experiments showed basal release of MCP-1 varied greatly between each condition, from 3000pg/ml to below 500pg/ml. They showed IL-6/sIL-6R did not synergistically modify IL-1β-induced MCP-1 and instead, an additive affect was observed.

A synergistic upregulation of MCP-1 protein and mRNA has been observed in epithelial cells by Struyf *et al.* Hep-2 epithelial cells (human epidermal larynx carcinoma) produced very little MCP-1 which was barely affected by IL-1β or IFN-γ alone. Co-stimulation with both produced an 8-fold increase of MCP-1 (Struyf, Van Collie et al. 1998).

Hyper IL-6 and IL-1β-induced MCP-1 release observed in both the transformed and primary airway epithelial cells could be IL-1β-mediated involving NF-kB activation. The p50 subunit of NF-kB is known to interact with nuclear factor-IL-6 (NF-IL-6) which in turn is induced by cell stimulation with IL-1 or IL-6. NF-IL-6 is a nuclear factor that specifically binds to an IL-1 responsive element in the IL-6 gene (Akira, Isshiki et al. 1990). In BEAS2Bs, Edwards *et al* demonstrated that IL-1β-induced IL-8 required specific NF-kB subunits and NF-IL6 for IL-8 gene expression. In addition, the use of an inhibitor revealed that IL-8 induction was not regulated by MAPKs, JNK or ERK (Edwards, Mukaida et al. 2005), indicative that IL-6 intracellular pathways are unlikely to be involved in IL-8 release from airway epithelial cells as IL-6 has been known to activate those particular pathways.

## 6.4.4 Conclusion

Primary airway epithelial cells were shown to produce substantially more IL-8 than MCP-1 at basal or in response to IL-1 $\beta$ . The outcome of this project has shown IL-6 trans-signalling does not have a direct impact on IL-8 release. Instead, in the presence of IL-1 $\beta$ , IL-8 is elevated. In addition, IL-6 trans-signalling had little effect on MCP-1 although the higher potent dose of hyper IL-6 had evoked a response. In combination with IL-1 $\beta$ , MCP-1 was significantly upregulated. This indicates that IL-6 trans-signalling and IL-1 $\beta$  may have a potential role in recruiting neutrophils and mononuclear cells in airway epithelial cells, but that IL-6 trans-signalling alone has little impact on these cells.

### 7. Final Discussion

#### 7.1 Introduction

Chronic lung disease (CLD) of premature infants is an inflammatory disease characterised by the accumulation of neutrophils in the airways, and their failure to undergo apoptosis. Premature infants are frequently placed on mechanical ventilation to aid lung function. The airways can become damaged from the exposure to hyperoxia, barotrauma and infection. All of which may contribute to a sustained inflammatory response in the airways. The alveolar and bronchiol epithelial cells may also contribute to the sustained inflammation by secreting inflammatory mediators (cytokines and chemokines).

The resolution of an inflammatory response has been demonstrated to be regulated by IL-6 trans-signalling. An *in vivo* murine model of peritoneal inflammation demonstrated that the IL-6/sIL-6R/gp130 complex reduced the influx of neutrophils by down-regulating IL-8 expression and promoting neutrophil apoptosis. This was followed by an influx of mononuclear cells by the upregulation of MCP-1 (Hurst, Wilkinson et al. 2001). It is not known if IL-6 trans-signalling is capable of promoting resolution of inflammation in airway epithelial cells. In addition the role of IL-6 trans-signalling in neonatal neutrophils is unknown. If IL-6 trans-signalling is responsible for the resolution of inflammation in the airways then this could be dysfunctional in premature infants with CLD. This could contribute to some characteristic features of the disease such as neutrophil accumulation and the reduction of neutrophil apoptosis.

The aim of this project was to understand the potential role of IL-6 trans-signalling in CLD of prematurity by using cell lines in an *in vitro* model. The role of IL-6 trans-signalling in adult and neonatal neutrophils, and in airway epithelial cells was investigated *in vitro*.

### 7.2 The role of IL-6 trans-signalling in neutrophils

As previously mentioned, IL-6 trans-signalling requires IL-6 to be bound to sIL-6R and membrane bound gp130 to elicit a cellular response. Soluble IL-6R can be freely available for trans-signalling by shedding of the membrane bound IL-6 receptor or differential splicing. The receptor expression of IL-6R and gp130 on neutrophils was compared between adults and neonates. The expression levels were also monitored in response to bacterial stimuli.

Adult and umbilical cord blood neutrophils were shown to express low levels of IL-6R and gp130. As controls, CD16 and CD11b were also assessed and were found to be highly expressed on both adult and neonatal neutrophils. Although it is difficult to directly compare the intensity of staining from different antibodies. The expression levels of IL-6R, CD16 and CD11b were not significantly different between adult and neonates. FMLP stimulation caused significant downregulation of IL-6R and upregulation of CD11b on both adult and neonatal neutrophils. The most interesting observation was the significant increase of basal gp130 expression on neonatal neutrophils compared to adult neutrophils. This was demonstrated within a mixed leukocyte population. Using a purification method, such as histopaque or percoll, to isolate neutrophils from leukocytes resulted in a loss of IL-6R, CD11b and CD16 from adult neutrophils. Using an isolated leukocyte population reduced the manipulation and ensured the retention of receptors. Leukocytes were either isolated by ACK lysis or by dextran sedimentation of whole blood. Receptor expression and response to stimuli were similar for adult neutrophils using either method. One significant difference between the methods was the upregulation of CD16 in response to fMLP on neutrophils within a leukocyte population isolated by dextran. In addition, neonatal neutrophils appeared to be more sensitive to dextran as expression of CD16 and gp130 was more variable compared to isolating the leukocytes by ACK lysis. The upregulation of gp130 on neonatal neutrophils was observed for both leukocyte isolation methods, although it was only significant on neutrophils from ACK-treated leukocytes. This difference in gp130 expression suggests a potential difference in the way neonates respond to IL-6 trans-signalling. However, it is not known if gp130 is a functional receptor on neonatal neutrophils.

A mixed leukocyte population was used to investigate IL-6 trans-signalling on neutrophil apoptosis. The findings from this project has shown IL-6/sIL-6R does not regulate neutrophil apoptosis *in vitro*. IL-6 through its membrane IL-6R did not affect neutrophil apoptosis. This is in contrast to the studies by Hurst et al and Biffl et al (Biffl, Moore et al. 1996; Hurst, Wilkinson et al. 2001). As this was conducted in a mixed leukocyte population, the leukocytes may have influenced the response of the neutrophil to IL-6 trans-signalling. A suitable method for purifying neutrophils should be sought to evaluate the affect of IL-6 trans-signalling on purified neutrophils. Furthermore, it was shown that apoptotic neutrophils may lose gp130 expression, which may render the cells incapable of responding to IL-6 trans-signalling. These experiments were performed using leukocytes from healthy adult donors and performed in the absence of an infection. Conducting these experiments in the presence of an infection or obtaining neutrophils from neonates known to have an infection, may reveal a more prominent role for IL-6 trans-signalling. Neutrophils and their response to IL-6 trans-signalling may be functionally different in the presence of

an infection compared to healthy conditions. The levels of gp130 expression may also be different during an inflammatory response compared to healthy states. The role of IL-6 trans-signalling was not investigated in cord blood neutrophils, due to a limited availability of cord blood at that time. This would have been an interesting comparison and deserves further study.

## 7.3 The role of IL-6 trans-signalling in airway epithelial cells

The role of IL-6 trans-signalling was investigated in transformed airway epithelial cells (A549 and BEAS2B cells) and primary airway epithelial cells (SAEC) *in vitro*. The cells were shown to express gp130 but not IL-6R indicating that they should respond to IL-6 trans-signalling. MCP-1 release in response to IL-6 trans-signalling was variable and often weak or undetected. The cells responded well to IL-1 $\beta$  stimulation by significantly releasing MCP-1 and IL-8. In all airway epithelial cell lines, there was no evidence of IL-6 trans-signalling down-regulating IL-8 or IL-1 $\beta$ -induced IL-8. In A549 and SAEC cells, the combination of IL-1 $\beta$  and IL-6 trans-signalling significantly elevated IL-8 release compared to IL-1 $\beta$  alone. The same was observed for MCP-1 release in SAEC, but not for A549 cells. This elevation in chemokine release is unlikely to involve pSTAT3 as it was unaffected by IL-6 trans-signalling or IL-1 $\beta$  or both stimuli combined. In HPMC, it was shown that IL-6 trans-signalling significantly upregulated MCP-1 and an upregulation of pSTAT3 were also observed.

Further investigations demonstrated that hyperoxia had no effect on chemokine release from A549s. Although cell viability was significantly reduced compared to normal air, chemokine release in response to cytokine stimulation was unaffected. Treating cells with IL-6/sIL-6R or IL-1β does not appear to promote cell survival during hyperoxia contrary to reports in the literature (Crapo, Barry et al. 1980; Kazzaz, Xu et al. 1996; Ward, Waxman et al. 2000; Romashko, Horowitz et al. 2003; Waxman, Mahboubi et al. 2003; Chetty, Cao et al. 2008; Kolliputi and Waxman 2009; Waxman and Kolliputi 2009).

The role of IL-6 trans-signalling during an infection in the airways remains unknown. The *mycoplasma* infection in A549s demonstrated a potentially interesting aspect of IL-6 transsignalling during an infection. Although not a significant effect, IL-6 trans-signalling increased IL-8 release, compared to unstimulated. Both MCP-1 and IL-8 were significantly released in response to IL-6 trans-signalling and IL-1β. Using LPS, heat-killed *E.coli* or

*U.parvum* did not significantly induce IL-8 or MCP-1 from BEAS2Bs stimulated with IL-6/sIL-6R. However, as chemokine release from A549 and BEAS2B cells was weak compared to *mycoplasma*-stimulated chemokine release, using transformed cells to assess the impact of an infection was not thought to be ideal.

### 7.4 Final Conclusions

The expression of gp130 was greatly upregulated on neutrophils from umbilical cord blood compared to adult neutrophils. This may indicate a potential difference in the way term infants respond to IL-6 trans-signalling. Further investigations also revealed IL-6 transsignalling does not regulate neutrophil apoptosis *in vitro*.

Transformed (A549 and BEAS2B cells) and primary (SAEC) airway epithelial cells express gp130 but not IL-6R. MCP-1 production in response to IL-6 trans-signalling was variable but generally weak. Furthermore, IL-8 release was not downregulated by IL-6 trans-signalling. Instead, an increase in MCP-1 and IL-8 release was observed in response to a combined stimulation of IL-6 trans-signalling and IL-1β in SAEC cells. Furthermore, this did not involve pSTAT3 upregulation. IL-6 trans-signalling may induce the influx of neutrophils and mononuclear cells but only in the presence of IL-1β.

The findings from this project have shown IL-6 trans-signalling is unlikely to promote the resolution of inflammation in the airways. Moreover, the potential dysregulation of IL-6 transsignalling may not be responsible for the retention of ageing neutrophils in the airways of premature infants with CLD.

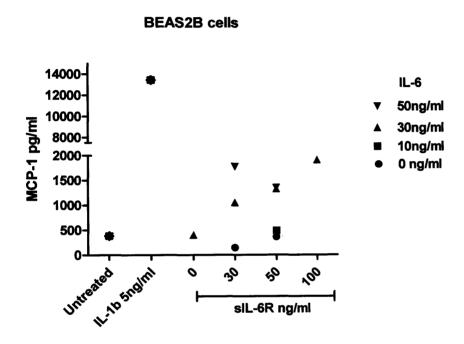
### 7.5 Future work

The role of IL-1 $\beta$  in combination with IL-6 trans-signalling may have an important role in the airways by upregulating MCP-1 and IL-8 release. Furthermore neonatal neutrophils may respond differently to IL-6 trans-signalling than adult neutrophils. This could be investigated further by conducting the following:

- 1. Investigate the indirect role of IL-6 trans-signalling and/or IL-1β on neutrophil apoptosis further by conducting a co-culture of airway epithelial cells and purified neutrophils or isolated leukocytes. The co-culture of cells would be stimulated with an infection to determine the role of IL-6 trans-signalling during an inflammatory response.
- 2. Investigate the role of IL-6 trans-signalling on neonatal neutrophils within an isolated leukocyte population, as conducted for the adult experiments. In addition, include a stimulus such as LPS to IL-6/sIL-6R stimulated leukocytes to determine a role of IL-6 transsignalling during an inflammatory response.
- 3. Determine the impact of hyperoxia or a live infection on chemokine release from SAEC stimulated with IL-1β and IL-6 trans-signalling combined.
- 4. Investigate signalling molecules upregulated by the combined stimulation with IL-1 $\beta$  and IL-6 trans-signalling, such as NFkB, during the synergistic increase of MCP-1 and IL-8. This will help determine the molecules involved in the possible 'cross-talk' between pathways utilised by IL-6 trans-signalling and IL-1 $\beta$ .
- 5. Toll-like receptors utilise similar signalling pathways as IL-1 $\beta$  and are important for the recognition of pathogens (Keating, Maloney et al. 2007). SAEC would be assessed for TLR expression and stimulated with agonists for TLR 2 (Pam 3 Cys) and 4 (LPS) to determine chemokine release. This would be in combination with IL-1 $\beta$  and IL-6 trans-signalling stimulation to determine chemokine release.
- 6. Investigate alternative signal transducing subunits that the IL-6 family of cytokines use to initiate cell signalling. Although gp130 is a common signal transducing subunit, there are members of the IL-6 family, such as IL-31 which do not engage gp130 (Chattopadhyay, Tracy et al. 2007). Investigate if these IL-6 family members via non engagement of gp130 regulate chemokine release from SAEC during an inflammatory response.

## **Appendix**

A preliminary experiment to determine which concentrations of IL-6 and sIL-6R combined induces the most MCP-1 release in BEAS2B cells. This data accompanies the data in chapter 5, section 5.3.5.3.



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