PG/R/06/10

SUMMARY OF THESIS: POSTGRADUATE RESEARCH DEGREES

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SECTION A: TO BE COMPLETED BY THE CANDIDATE AND SUBMITTED WITH THE THESIS

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Title of Degree:	**PhD**
Full Title of Thesis	Mapping the inflammatory process in neonatal lung disease.

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Summary of Thesis:

Chronic lung disease (CLD) of prematurity is a significant complication of preterm birth in which the neutrophil appears to be the key cell of inflammation. Lack of resolution of neutrophilic inflammation, due to delayed or dysregulated neutrophil apoptosis, is thought to be an important component of the pathogenesis of CLD.

In this thesis, I sought to examine the inflammatory process in the lungs of ventilated newborn infants. I have established a reliable method for the use of flow cytometry, a technique not previously reported in the study of neonatal bronchoalveolar lavage (BAL) samples, and have described both the cellular and supernatant components of BAL from term and preterm infants, paying particular attention to the role of infection. I found higher peak neutrophil counts in infants developing CLD, as well as more immature, monocyte-like, macrophages and higher rates of detection of microbial DNA when compared to term infants and infants whose respiratory distress syndrome resolved.

I focused more specifically on the role of neutrophil apoptosis in CLD and sought to understand the mechanism for the delay in neutrophil apoptosis in newborn infants and how this may impact on lung disease. BAL supernatants from term infants showed more pro-apoptotic activity against adult neutrophils than BAL from preterm infants. I found a delay in apoptosis in lung neutrophils in preterm infants and confirmed this delay in blood neutrophils of term infants compared to adults. I have shown that this delay might be due to differential expression of anti-apoptotic proteins Bcl-xl and Mcl-1 between cord and adult neutrophils, which has not previously been described.

Mapping the inflammatory process in neonatal lung disease

A Thesis Submitted for the Postgraduate Degree of Doctor of Philosophy at Cardiff University

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For Luke and James

Contents

List of abbreviations

AIF	Apoptosis inducing factor
AM	Alveolar macrophage
APC	Allophycocyanin
ARDS	Acute (adult) respiratory distress syndrome
AVBB	Annexin-V binding buffer
BAL	Bronchoalveolar lavage
BPD	Bronchopulmonary dysplasia
BPI	Bacterial permeability increasing protein
BSA	Bovine serum albumin
C5a	Complement component 5a
CBA	Cytokine bead array
CD	Cluster of differentiation
CLD	Chronic lung disease of prematurity
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disease
DED	Death effector domain
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribose nucleic acid
DTT	Ditheiothreitol
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain containing protein
FG	French gauge
FSC	Forward scatter
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HBSS	Hank's buffered salt solution
HIE	Hypoxic ischaemic encephalopathy
HFOV	High frequency oscillatory ventilation
HLA-DR	Human leucocyte antigen-DR
HMCO	Human mitochondrial cyclo-oxygenase
HRPO	Horse radish peroxidase
IAP	Inhibitor of apoptosis proteins

ICAM	Intracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin-
IQR	Interquartile range
LBP	LPS binding protein
LBW	Low birth weight
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MCP-1	Monocyte chemotactic protein
MFI	Mean fluorescence intensity
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MNC	Mononuclear cell
MPO	Myeloperoxidase
NF-κB	Nuclear factor kappa B
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Patent ductus arteriosus
PE	Phycoerythrin
PMN	Polymorphonuclear leucocyte (neutrophil)
PPP	Platelet-poor plasma
pPROM	Preterm pre-labour rupture of membranes
PS	Phosphatidylserine
RDS	Respiratory distress syndrome
RIP-1	Receptor interacting protein
RNA	Ribose nucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
RPA	Ribonuclease protection assay
RPMI	Roswell Park Memorial Institute medium
SGA	Small for gestational age
SOD	Super-oxide dismutase

SP-	Surfactant protein-
SSC	Side scatter
TAF	Tracheal aspirate fluid
TBE	Tris borate EDTA
TGF-β	Transforming growth factor beta
TIRAP	Toll IL-1 receptor domain containing protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRADD	TNF Receptor-Associated Death Domain
TRAF	TNF Receptor-Associated Factor
TRAIL	TNF-associated apoptosis inducing ligand
TSP	Thrombospondin
UK	United Kingdom
USA	United States of America
VEGF	Vascular endothelial growth factor
VLBW	Very low birthweight

Chapter 1

Introduction

Chapter 1
Introduction

1.1 Overview

Chronic lung disease (CLD) of prematurity is a significant complication of preterm birth. The pathogenesis of CLD is linked to a number of risk factors, all of which are able to initiate or help to sustain an inflammatory process in the preterm lung and which will be further discussed in this chapter. Historically, the neutrophil appears to be the key cell of inflammation in this disease and a lack of resolution of neutrophilic inflammation, due to delayed or dysregulated neutrophil apoptosis, is thought to be an important component of the pathogenesis of CLD. The role of monocytes and macrophages in the process and resolution of inflammation is well described but their specific role in CLD remains to be elucidated. These concepts and ideas will be discussed and further explored in this chapter.

1.2 Prematurity

Preterm delivery is defined by the World Health Organisation as the delivery of an infant between 20 and 37 weeks' gestation (Wen et al., 2004). A normal pregnancy is 40 weeks in duration. Preterm deliveries account for between 5 and 12% of all births (USA 11%; Europe 5-7% (Goldenberg, 2002); Canada 6.5% (Joseph et al., 2001)). In industrialised countries, preterm birth is responsible for 70% of neonatal mortality and 75% of neonatal morbidity (Challis et al., 2001) and contributes to long term neurodevelopmental problems, visual impairment (Repka, 2002) and lung dysfunction. Early preterm births i.e. those before 32 weeks' gestation, have the highest rates of morbidity and mortality (Lumley, 1993).

Secular trends in preterm births show a steady increase since the early 1980s (Wen et al., 2004). This may reflect increasing numbers of multiple births due to assisted reproduction techniques as well as the advent of more accurate dating of pregnancies by routine ultrasound scanning. In addition, changes in the way births are registered may contribute to the increasing trend, as infants which may previously have been

registered as stillborn or miscarried are now being reflected as preterm births (Wen et al., 2004).

Outcomes for infants born preterm are improving dramatically (Joseph et al., 2002, Horbar et al., 2002) however there are also larger numbers of surviving infants who have a significant disability (Wood et al., 2000).

1.3 Normal lung development

The normal development of the human lung can be divided into 5 phases (Kotecha, 2000b, Kotecha, 2000a, Langston et al., 1984, Langston and Thurlbeck, 1982, Coalson, 2003) (Figure 1.1):

- **embryonic/embryonal phase** (0 to 7 weeks' gestation). The lung begins development as an outgrowth from the ventral aspect of the primitive foregut in the embryo and undergoes repetitive dichotomous branching to form the tracheobronchial tree. The developing airways are accompanied by branches of the pulmonary artery which is derived from the 6th aortic arch.
- **pseudoglandular phase** (7 to 16/17 weeks' gestation). Airway and blood vessel branching continues to the level of the terminal bronchiole. Epithelial cells and cartilage differentiate. Pre-acinar vascular development is completed.
- **canalicular phase** (16/17 to 26/28 weeks' gestation). The respiratory bronchiole, alveolar duct and primitive alveoli are formed. Type II epithelial cells begin to differentiate. The distal pulmonary circulation begins to develop to the level of capillaries at around 20 weeks.
- **saccular phase** (26/28 to 32/36 weeks' gestation). Airway walls thin and gas-exchange area is enlarged. Secondary crests, which are the progenitors of alveolar development, subdivide the cylindrical saccules of the alveolar duct.
- **alveolar phase** (32/36 weeks' gestation to 2 years postnatally). Alveoli increase in number to around 300-600 million (Thurlbeck, 1982) and there is an increase in acinar complexity by increased extension and thinning of secondary crests.

Figure 1.1 Stages of normal lung development (Kotecha, 2000a)

1.4 Respiratory distress syndrome

Respiratory distress syndrome (RDS) is the most immediately life threatening condition to affect most infants born significantly preterm (i.e. before 32 weeks' gestation). RDS is a major cause of morbidity and mortality in preterm infants. It is primarily caused by a deficiency of pulmonary surfactant, which is usually produced by pulmonary type II epithelial cells after about 30-32 weeks' gestation (Fraser et al., 2004).

Infants with RDS present soon after birth with respiratory distress, tachypnoea, intercostal, subcostal and sternal recession, grunting, cyanosis and reduced breath sounds. If untreated, the infant will progress to fatigue, apnoea, hypoxia and respiratory failure which may require assisted ventilation (Fraser et al., 2004). In RDS the lungs are stiff and non-compliant, needing high ventilatory pressures and thus increasing the risk of complications e.g. pneumothorax, pneumomediastinum and pulmonary interstitial emphysema (Fraser et al., 2004).

Over the past 20-30 years administration of antenatal glucocorticoids to pregnant women at risk of preterm delivery has reduced mortality in preterm infants by 40% (Roberts and Dalziel, 2006). Surfactant replacement treatment with bovine (Survanta®, beractant, Abbott, Berkshire, UK) or porcine (Curosurf ®, poractant alfa, Chiesi Pharmaceuticals, Cheadle, UK) surfactants has also achieved a 30-65%

reduction in pneumothorax, thus significantly improving clinical outcomes in RDS (Fraser et al., 2004).

1.4.1 RDS at tissue level

RDS is characterised by impaired gas exchange, decreased lung compliance, pulmonary oedema due to loss of integrity of the alveolar-capillary barrier, impairment of normal surfactant function and an increased tendency of alveoli to collapse (Gitto et al., 2001).

Histologically, normal newborn lungs have few or no inflammatory cells in either term or preterm infants (Robertson, 1964). Bronchoalveolar lavage (BAL) fluid of babies born without risk factors for infection contains $<10^5$ cells, some debris and very few neutrophils (also called polymorphonuclear leucocytes (PMN)) (Grigg et al., 1993). RDS is characterised by alveolar neutrophil infiltration, which disappears as the condition resolves (Grigg et al., 1991). Neutrophils appear soon after initiation of ventilation in animal models and the appearance of neutrophils correlates with lung oedema and the appearance of early indicators of lung injury (Carlton et al., 1997). There is a parallel decrease in circulating neutrophils (Jobe and Bancalari, 2001) with systemic activation of the cells (Brus et al., 1996, Nupponen et al., 2002a).

1.5 Chronic Lung Disease of Prematurity

The most important long term complication of RDS is CLD. The incidence of CLD has been rising over the past decade despite antenatal steroid administration and surfactant treatment (Fraser et al., 2004).

The current definition of CLD is applied to infants who continue to require oxygen supplementation beyond 28 days of age or, more recently and more epidemiologically useful, beyond 36 weeks' corrected gestational age and who have characteristic chest x-ray changes (Kotecha, 1999).

Despite improvements in neonatal care, mechanical ventilatory techniques and the use of exogenous surfactant, CLD remains the most common complication in babies

under 1kg birth weight (Jobe, 1999), occurring in 51% of infants with a birth weight of 501-750 g (Fanaroff et al., 1995, Costeloe et al., 2000) and 35% of infants weighing 751-1000 g (Fanaroff et al., 1995, Stevenson et al., 1998). The reported incidence of CLD varies widely, especially due to the different definitions of the condition currently in use (Bancalari et al., 2003). However, most sources agree that it is uncommon in infants born at more than 32 weeks' gestation (Bancalari et al., 2003) and infrequent at birth weights of more than 1200 g. Gentler ventilation techniques, antenatal steroids and surfactant treatment have reduced severe lung injury especially in older gestations but there are still some, particularly low birth weight (LBW), infants who initially show minimal evidence of RDS but progress to CLD – many of these infants have had possible exposure to chorioamnionitis (Rojas et al., 1995).

Some infants with CLD have severe lung disease requiring assisted ventilation for many months and supplemental oxygen for months or years. In addition, CLD is associated with poor developmental outcome in survivors (Welty, 2005) – it is an independent risk factor for cerebral palsy (Teberg et al., 1991). This is probably due to a generalised inflammatory state in the perinatal period which leads to both lung and central nervous system damage (Welty, 2005).

1.5.1 "New BPD"

Northway *et al* (Northway et al., 1967, Jobe and Bancalari, 2001) originally described bronchopulmonary dysplasia (BPD) as lung injury in preterm infants resulting from oxygen and mechanical ventilation. The terms BPD and CLD have become used interchangeably. The form of CLD described by Northway *et al* (Northway et al., 1967) was characterised by interstitial and alveolar oedema in the early stages and progressed to persistent airway inflammation with significant alveolar septal fibrosis and small airway disease. Chest radiographs showed severe over inflation with a mixture of cystic emphysema and areas of fibrosis with volume loss (Jobe and Ikegami, 1998, Bancalari et al., 2003, Husain et al., 1998). This clinical and radiographic presentation is now seldom seen, except in a few term infants who survive severe meconium aspiration syndrome or congenital diaphragmatic hernia and experience prolonged high pressure ventilation (Jobe and Ikegami, 1998).

The clinical and pathological presentation of CLD described by Northway in 1967 (Northway et al., 1967) has now become infrequent due to the use of antenatal steroids, surfactant and modern ventilation techniques (Husain et al., 1998, Bancalari et al., 2003). Now we see a histologically different, clinically milder form of CLD or so-called "new BPD". Infants with "new BPD" have CLD with less fibrosis which is more diffuse (Husain et al., 1998), more uniform lung inflation, fewer and larger alveoli, increased elastic tissue and decreased pulmonary microvascular development (Jobe and Bancalari, 2001). Chest radiographs initially show uniformly hazy and dense lung fields which progress over time to a lacy parenchymal pattern with moderate hyperinflation but few large cysts (Toce et al., 1984).

Jobe and Ikegami (Jobe and Ikegami, 1998) hypothesise that the "new BPD" is no longer the primary injury and repair mechanism of "old" BPD but rather a sequence of maldevelopment of the lung which is caused by interference with/interruption of the normal developmental signalling for maturation and alveolarisation of the preterm lung.

Alveolar and capillary growth are disrupted in the immature lung causing developmental arrest and abnormal lung repair (Coalson, 2003, Coalson et al., 1995). The common thread in decreased alveolarisation is a persistent increase in airway leucocyte count and over expression of cytokines, particularly tumour necrosis factor α (TNF-α), transforming growth factor β (TGF β), interleukin (IL)-11 and IL-6 in airway secretions (Jobe, 1999, Ogden et al., 1984). These pro-inflammatory mediators interfere with as yet unknown signalling pathways which are important in lung maturation and alveolarisation, with the highest risk being in the most immature infants (Jobe and Ikegami, 1998, Jobe, 1999).

The development of the secondary crest (28-36 weeks) is a critical event in the process of septation and thus of normal acinar development (Coalson et al., 1999, Husain et al., 1998). Coalson *et al* showed that oxygen alone can arrest septation in the saccular stage of lung development in a baboon model of CLD (Coalson et al., 1999).

The effects of oxygen and/or pro-inflammatory cytokines result in decreased alveolarisation and abnormal capillary morphology along with variable fibroproliferation in the interstitium and abnormal elastic fibre deposition giving the overall histopathological features of what we see as CLD (Coalson, 2003).

1.5.2 Risk factors for the development of CLD

The traditional view holds that CLD is caused simply by mechanical ventilation and oxidant injury (Jobe and Bancalari, 2001). This view underestimates the complex pathogenesis of CLD which probably involves the interaction of numerous factors, including mainly immature lung (preterm birth), oxygen injury (premature initiation of pulmonary gas exchange resulting in relative hyperoxia), volutrauma (mechanical ventilation) and an inflammatory response (Coalson, 2003). Additional risk factors include babies who are small for gestational age (SGA), the severity of RDS, duration of ventilation, duration of oxygen administration, patent ductus arteriosus (PDA), chorioamnionitis and postnatal bacterial sepsis (Arnon et al., 1993, Fraser et al., 2004). Fluid overload, most frequently iatrogenic, has also been implicated in the causation of CLD (Shah, 2003, Truog et al., 2007). These factors act additively or synergistically to result in lung injury (Jobe and Bancalari, 2001).

a) Hyperoxia

Endogenous antioxidant activity is relatively deficient at birth and preterm infants have particularly low levels of antioxidants (Rodriguez-Pierce et al., 1994). The intra-uterine environment is relatively hypoxic thus all infants are exposed to relative hyperoxia at birth. In addition to this, most preterm infants are exposed to increased inspired oxygen concentrations during their treatment. The generation of reactive oxygen species can initiate severe inflammatory changes and lung damage especially if antioxidant activity is inadequate (Davis et al., 1991).

Davis *et al* (Davis et al., 1991) showed that hyperoxia consistently evokes a more significant inflammatory response and subsequent acute lung injury than barotrauma alone, even in adult lung models. Hyperoxia increases pro-inflammatory cytokine production (tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-

6) and interleukin-8 (IL-8)) from alveolar macrophages, fibroblasts, type II pneumocytes and endothelial cells (Metinko et al., 1992). Hyperoxia also aggravates the destructive effects of elastase (Bruce et al., 1987, Bruce et al., 1985, Bruce et al., 1992).

b) Mechanical ventilation

Any mechanical ventilation may be injurious to preterm lungs (Jobe and Bancalari, 2001). Mechanical ventilation causes barotrauma and volutrauma due to high peak inspiratory pressures (>35 cm water) (Taghizadeh and Reynolds, 1976, Tremblay et al., 1997). The exact mechanism for the damage caused by ventilation is unclear, however it is thought that positive pressure ventilation disrupts the lung epithelium by over distension of the airways (Oei et al., 2003) leading to leucocyte (particularly neutrophil) (Oei et al., 2003) migration to the lungs, increased alveolar-capillary membrane permeability and alveolar and interstitial oedema. Over distension can stimulate the inflammatory cascade by release of TNF-α, IL-1, IL-6 and IL-8 (Vlahakis et al., 1999, Yoder et al., 2000).

Low end expiratory pressures (Taghizadeh and Reynolds, 1976, Tremblay et al., 1997) may also cause lung injury by causing cytokine release and promoting accumulation and activation of peripheral leucocytes in the lungs (Muscedere et al., 1994).

c) Infection

Antenatal infection is thought to be a major cause of preterm labour. Microbial invasion of the amniotic fluid increases leucocyte recruitment and cytokine production (Keelan et al., 2003) which promote further neutrophil activation and cervical ripening and dilation. This leads to exposure and weakening of fetal membranes and prostaglandin release, which stimulates uterine contractions (Goldenberg et al., 2000).

Up to 80% of women delivering at <28 weeks' gestation have evidence of intrauterine infection, compared to 10-15% of those delivering at term (Goldenberg et al., 2000).

Up to 48% of spontaneous preterm deliveries may be due to infection (Gomez et al., 1997) however it is often subclinical (Klein and Gibbs, 2004). Microbial invasion of the amniotic cavity could be identified in 16% of patients in preterm labour with intact membranes (Jacobsson et al., 2003) and 21% with preterm prelabour rupture of membranes (pPROM) (Satar et al., 2008). This contrasts with the findings of Miralles *et al* (Miralles et al., 2005) who applied polymerase chain reaction (PCR) techniques to detect microbial 16S ribosomal RNA (rRNA) genes to identify the presence of microbial colonisation in intrauterine samples from women delivering at <33 weeks and in gastric fluid or bronchoalveolar lavage fluid from their newborns. They reported microbial genes in at least one tissue or fluid sample (gastric aspirate, amniotic fluid, BAL, fetal membranes or placenta) in 70% of deliveries after pPROM and 80% after preterm labour with intact membranes.

The organisms frequently responsible for antenatal intra-uterine infection are from the cervicovaginal flora – *Mycoplasma* spp., *Gardnerella vaginalis, Ureaplasma spp.* (present in the lower genital tract of 40-80% of pregnant women (van Waarde et al., 1997) and the most common organism grown in the amniotic fluid in chorioamnionitis), *Chlamydia trachomatis* and other anaerobes (Wasiela et al., 2001). Additionally, Group B *streptococci* are an important cause of intra-uterine infection as well as a leading cause of early and late neonatal infection (Gibbs et al., 2004). Animal models in which *Ureaplasma urealyticum* is injected into the amniotic fluid of pregnant baboons have demonstrated the ability of the organism to provoke an inflammatory response in the lungs of the preterm animal once delivered (Yoder et al., 2003) and they may go on to develop CLD.

There is a group of very low birth weight (VLBW) (<1500 g) infants who develop CLD without developing RDS at birth. Some of these infants may be ventilated (with low peak inspiratory pressures and low inspired oxygen concentrations) for apnoea or poor respiratory effort and others may have very mild RDS which responds well to surfactant treatment (Rojas et al., 1995). This group of infants develop a requirement for supplemental oxygen and ventilatory support over the first 2 weeks of life. A number of these babies will have been exposed to chorioamnionitis (Speer, 2001). Colonisation or infection with *U. urealyticum* appears to protect preterm infants from the developing RDS but predisposes them to CLD, independent of gestation

(Hannaford et al., 1999).

Chorioamnionitis was present antenatally in 92% of infants who developed CLD compared to 62% of infants who did not (Yoon et al., 1997). Histological chorioamnionitis was noted in 33% of infants weighing <2000 g with RDS compared to 82% without RDS (Watterberg et al., 1996). In contrast, 63% of babies with CLD had exposure to chorioamnionitis compared to 21% of babies without CLD (Watterberg et al., 1996). Together these data suggest a subgroup of preterm babies who are exposed to chorioamnionitis are destined to develop CLD and that antenatal infection appears to have a specific role in triggering the inflammatory response in fetal lung (Watterberg et al., 1996). Antenatal infection may prime fetal lungs such that minimally injurious postnatal events, like gentle mechanical ventilation, provoke an excessive inflammatory response in neonatal airways and lung tissue (Speer, 2004).

Increased IL-1β has been noted on day 1 of life in tracheal lavage fluid of babies who developed CLD, suggesting pulmonary inflammation prior to delivery (Watterberg et al., 1996). Antenatal exposure to pro-inflammatory cytokines (IL-6, TNF-α and IL-8) has been identified as a risk factor for developing CLD (Yoon et al., 1997). An increase in neutrophils in tracheal aspirates at birth, implies the pathogenesis of CLD commences antenatally (Kim et al., 2004). The presence of pPROM for more than 24 hours is independently associated with raised BAL cell counts (Giles et al., 2000). The importance of pro-inflammatory cytokines and pro-fibrotic growth factors, such as transforming growth factor-β (TGF-β), which are increased in infants who develop CLD, lies in the fact that these factors are also important in promoting normal lung growth (Thibeault et al., 2000).

Antenatal infection and inflammation have also been strongly implicated in the pathogenesis of other important neonatal complications, particularly cerebral palsy (Girard et al., 2009).

The major association, other than birth weight and gestational age, with CLD is postnatal sepsis (Rojas et al., 1995) which is an independent risk factor for the development of CLD. Bacterial infections (Liljedahl et al., 2004, Cordero et al., 1997)

as well as viruses like cytomegalovirus (CMV) and adenovirus (Sawyer et al., 1987, Couroucli et al., 2000) have all been suggested to increase CLD.

d) *Ureaplasma*

The role of *Ureaplasma* in neonatal morbidity and mortality has long been controversial. *Ureaplasmas* are eubacteria which belong to the class Mollicutes. They do not have cell walls and are thought to be the smallest free-living, self-replicating cells. They are limited by the lack of cell wall to a parasitic existence in eukaryotic cells. *Ureaplasmas* were previously designated "*T-mycoplasma*" but later the genus *Ureaplasma* was designated in view of their use of urea as a metabolic substrate. *Ureaplasma urealyticum* was the only species known to infect humans and this species was recently subdivided into 2 separate species, *U. urealyticum* and *U. parvum*, on the basis of their 16S rRNA gene sequences.

Ureaplasma spp. has been shown to be implicated in preterm labour, spontaneous abortion and stillbirth (Embree and Embil, 1980, Schelonka and Waites, 2007, Kataoka et al., 2006). Up to 80% of women have been reported to be colonised with genital mycoplasmas (Quinn et al., 1983) and as many as 22% of women with pPROM or preterm labour have evidence of *U. urealyticum* in their amniotic fluid (Kirchner et al., 2007).

Transmission of *Ureaplasma* from a colonised or infected mother depends on a number of variables, particularly

- gestation

  Preterm infants appear to be more likely to become colonised than their term counterparts (Alfa et al., 1995) with the rate of vertical transmission ranging between 18% and 55% for term infants and 29% and 55% for preterm infants (Sanchez, 1993).
- birthweight

  A higher risk of vertical transmission of *Ureaplasma* in babies of lower birth weight has been reported. For example in babies weighing <1000 g at birth transmission may be up to 89% (Sanchez and Regan, 1990) but a transmission rate of only 15% has been reported among infants of >1500 g birthweight

(Kafetzis et al., 2004). Alfa *et al* (Alfa et al., 1995) showed that VLBW (<1500 g) infants were at significantly higher risk of acquiring *Ureaplasma* spp. in their respiratory tract than larger preterm infants. It is difficult to control for the effects of preterm labour and delivery among these infants as *Ureaplasma* may be a causative factor in the preterm birth which resulted in the infant being classified as low birthwieght.

- route of delivery

  Aaltonen *et al* (Aaltonen et al., 2006) reported a series of 49 infants born at <30 weeks' gestation, in which 45% of 33 spontaneous deliveries were colonised with *Ureaplasma* but none of the electively delivered infants appeared to be colonised. Goldenberg *et al* (Goldenberg et al., 2008) also reported a higher prevalence of *U. urealyticum* or *Mycoplasma hominis* in umbilical cord blood among 351 mother/baby pairs at 23–32 weeks where infants had delivered spontaneously rather than electively.

- pPROM

  Infants with *Ureaplasma* are more likely to have been born following pPROM than those not colonised (Pandey et al., 2007).

Ureaplasma is thought to infect or colonise up to 37% of newborns (Abele-Horn et al., 1998) and has been implicated in neonatal morbidity and mortality including congenital pneumonia, low birthweight, intrauterine growth restriction (Embree et al., 1980), central nervous system infections (Waites et al., 1990, Neal et al., 1994, Hentschel et al., 1993, Chung et al., 2007, Rao et al., 2002) and a systemic inflammatory response (Ohlsson et al., 1993, Ollikainen et al., 1998).

Cultrera *et al* (Cultrera et al., 2006) studied the relationship between RDS and *Ureaplasma* in 50 babies of <37 weeks' gestation. Fifteen out of 24 babies with RDS had *U. urealyticum* or *U. parvum* detected and only 4 of 26 babies without RDS were colonised with either organism, thus suggesting that *Ureaplasma* plays a role in the development of RDS. In contrast, Hannaford *et al* (Hannaford et al., 1999) showed significantly decreased incidence of RDS in infants of <28 weeks' gestation who were colonised with *Ureaplasma* but many of the colonised infants progressed to develop CLD which is in keeping with data showing an association between chorioamnionitis and development of CLD from Watterberg and colleagues (Watterberg et al., 1996).

The association between the presence of *Ureaplasma* and the development of CLD remains controversial. Pulmonary *Ureaplasma* colonisation is strongly linked to preterm delivery and the question remains if this pulmonary colonisation is an independent risk factor for CLD. The consistent observation in many publications is the difficulty in interpreting evidence from available studies due to small sample sizes, vastly different inclusion criteria, different methods of sampling and testing, and different diagnostic criteria for various outcomes including CLD. A 1995 meta-analysis included 1479 babies from 17 studies (Wang et al., 1995) and reported a significant association between CLD diagnosed at 28 days of life and *Ureaplasma* colonisation with an overall relative risk of 1.72 (95% confidence intervals 1.5–1.96). Data on CLD at 36 weeks corrected gestation were not available.

Since that review, several further studies have been completed, including one by Kotecha *et al* (Kotecha et al., 2004) who sought *Ureaplasma* in BAL fluid from 17 preterm neonates without clinical or laboratory evidence of infection in either the mother or infant and reported that of 6 infants who were positive for *U. urealyticum*, 5 developed CLD whereas only 4 of the 11 babies without *Ureaplasma* developed CLD. In a cohort of 126 preterm deliveries, Kafetzis *et al* (Kafetzis et al., 2004) found a significant increase in CLD as well as mortality among *Ureaplasma* colonised infants and vanWaarde *et al* (van Waarde et al., 1997) found that *Ureaplasma* was significantly associated with both CLD and lower gestational age but logistic regression analysis failed to show a correlation between *Ureaplasma* colonisation and CLD. In 2005, a further meta-analysis (Schelonka et al., 2005) including 23 studies and 2216 babies showed an odds ratio of 2.83 (95% confidence intervals 2.29–3.51) for the relationship between the presence of *Ureaplasma* and CLD diagnosed at 28 days of life. There were 751 babies for whom data were available regarding CLD at 36 weeks' gestation, again this showed a significant association.

More recent studies continue to fuel the controversy: Pandey *et al* (Pandey et al., 2007) reported no role for *Ureaplasma* in the development of CLD in a group of 100 babies of <34 weeks' gestation. Goldenberg *et al* (Goldenberg et al., 2008) studied 351 mother/baby pairs at 23–32 weeks, where the umbilical cord blood showed evidence of *Ureaplasma* or *M. hominis* and showed a probable association of *U.*

urealyticum with the development of CLD. In other studies, *U. urealyticum* colonised infants have shown a non-significant trend towards higher neonatal morbidity (longer ventilation, longer hospital stay, younger gestational age, higher incidence of CLD and more late onset sepsis) (Kirchner et al., 2007). The role of *Ureaplasma* in CLD is further complicated by the identification of different patterns of *Ureaplasma* colonisation in the preterm neonate (persistently positive, early transient and late acquisition) which may also impact on the likelihood of a colonised neonate developing CLD, with only the "persistently positive" group showing a higher rate of CLD (Castro-Alcaraz et al., 2002).

If *Ureaplasma* has a causative role in the pathogenesis of CLD, it would be reasonable to expect the incidence of CLD to decrease by eradicating its colonisation with antibiotic treatment. Once again the literature contains a number of small sample size studies which vary in the time of commencement of treatment, type and duration of antibiotic therapy and many lack documentation of eradication of the organism at the end of the course of treatment. However, only two randomised controlled trials have been included in the Cochrane review by Mabanta *et al* (Mabanta et al., 2003) examining studies investigating the treatment of *Ureaplasma* to decrease the rate of CLD. In the first study, Lyon *et al* (Lyon et al., 1998) treated infants prior to knowing their colonisation status and showed no change in the number of infants who developed CLD. In the second study, Jonsson *et al* (Jonsson et al., 1998) treated only those infants with positive cultures from endotracheal or nasopharyngeal samples and were able to show a reduction in colonisation but not CLD. Together these two studies included only 37 colonised patients and there was no significant reduction in CLD with treatment in either study — disparate study designs prevented the results from being combined in the meta-analysis.

1.6 Inflammation

There is now a wealth of evidence that lung inflammation, caused initially by the innate immune response, is a central key participant in the pathophysiology of CLD (Welty, 2005), with all the known risk factors for CLD being able to give rise to inflammation. CLD appears to begin as an acute lung injury which then initiates a series of inflammatory responses. This in turn evolves into the typical clinical and pathological features of CLD (Davis et al., 1991) but how the risk factors impact at the cellular and molecular level is not well described.

1.6.1 The process of inflammation

Inflammation is important to (Janeway et al., 2004):

- deliver effector molecules and cells to the site of infection or wounding and augment the killing of micro-organisms,
- provide a physical barrier to the spread of infection by causing microvascular coagulation,
- promote the repair of injured tissue.

Inflammation is characterised by:

- an inductive or initiation phase (Han and Ulevitch, 2005) characterised by neutrophil infiltration followed by
- a sustained response and then
- resolution (Han and Ulevitch, 2005) or failure of resolution, leading to chronicity of inflammation.

1.6.1.1 Initiation

Any harmful tissue event (infection, trauma, anoxia) is perceived mainly by tissue resident macrophages and monocytes which secrete cytokines (IL-1 and TNF) which act to stimulate other cells to produce a second wave of cytokines (IL-1, IL-2, IL-6 and IL- 8) which serve to amplify the inflammatory response as well as to recruit inflammatory cells (neutrophils, monocytes, macrophages) and systems (complement, coagulation cascade).

a) The neutrophil

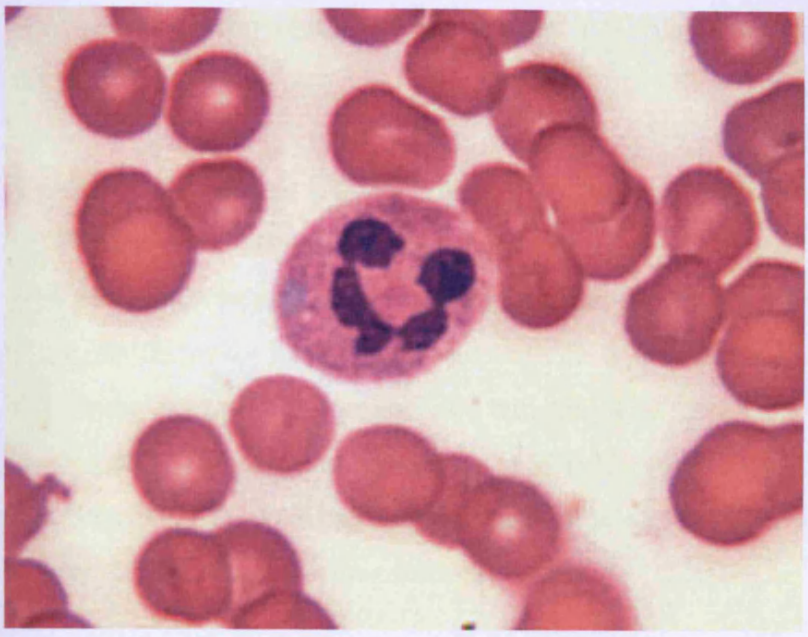

Figure 3.42 Graphs showing ratio of neutrophils to macrophages for individual babies with 3 or more BAL samples.

Neutrophils are the most numerous of the granulocytes and are the key effector cell of the innate immune system (Sabroe et al., 2005). They are produced in the bone marrow from myeloid stem cells and have a relatively short lifespan in the circulation of 8-20 hours (Akgul and Edwards, 2003). Neutrophils are abundant in the blood but are not present in normal healthy tissue (Janeway et al., 2004). Healthy adult BAL fluid has <10% neutrophils (Committee, 1990). They are the first cells to arrive at a site of inflammation (Savill et al., 1989b, Haslett, 1999) and their survival time increases significantly in tissues where they are exposed to pro-inflammatory signals (Akgul and Edwards, 2003).

Merritt *et al* (Merritt et al., 1983) first described the cellular changes in tracheal aspirate fluid (TAF) in the development of CLD. They examined the TAF of 26 ventilated infants and identified neutrophils as the predominant cell type in RDS. They found significantly higher cell counts in TAF from preterm infants with RDS, and particularly in the infants who went on to develop CLD, than in term infants ventilated for non-respiratory reasons. By day three of life infants who would develop CLD had neutrophil counts a hundred times that of the term controls and ten times

that of preterm infants whose RDS resolved. It was also found that the cell count started to fall after three days in infants whose RDS resolved but elevated neutrophil counts persisted for weeks in those getting CLD (Arnon et al., 1993, Kotecha et al., 1995).

A study by Arnon *et al* (Arnon et al., 1993) showed that infants who progressed to CLD had significantly more neutrophils in BAL on day 5 and 7 than infants whose RDS resolved. They identified that the persistence of high neutrophil counts at day 7 was a risk factor for the development of CLD. More recently Kotecha *et al* confirmed that airway neutrophilia is associated with the development of CLD by studying BAL fluid from 32 babies for up to 21 days (134 BAL samples) and 9 term controls (Kotecha et al., 1995). They found higher mean cell counts in CLD babies at day 10, mainly due to an increase in neutrophil count.

b) Initiation of inflammation by neutrophils

Neutrophils gain access to the interstitium of the lung by migrating from the blood. This migration is facilitated by interaction between adhesion molecules on the leucocyte surface and endothelial cells. L-selectin on leucocytes and P- and E-selectins on endothelial cells mediate neutrophil rolling along the endothelium. Firm adhesion and transmigration are mediated by β 2 integrins and intercellular adhesion molecules (ICAM).

ICAM-1 is a glycoprotein that facilitates cell-to-cell contact. It is expressed in response to IL-1 stimulation. It binds to CD11b/CD18 on neutrophils and helps to regulate neutrophil diapedesis. Increased soluble ICAM-1 has been found in TAF of infants with early CLD (Kojima et al., 1993) and a role for ICAM-1 has been suggested in oxygen-induced lung injury.

CD11b/CD18 and ICAM are increased in neutrophils from BAL fluid of babies who develop CLD (Kotecha et al., 1998, Kotecha et al., 1995). Soluble L-selectin increases and remains persistently high in CLD babies – this may reflect leucocyte traffic in the lung i.e. continued neutrophil migration in babies developing CLD (Kotecha et al., 1998).

Tissue transmigration may serve to activate neutrophils and also impart anti-apoptotic signals, although cells retain their susceptibility to Fas (Sexton and Walsh, 2005). Once activated the neutrophils are able to initiate a cascade of defence mechanisms.

c) Toll-like Receptors

Toll-like receptors (TLR) are a family of pattern recognition receptors (Dabbagh and Lewis, 2003) of which at least 10 have been described (Sabroe et al., 2005, Stevens, 2005). TLR initiate host defence in response to unique molecular patterns presented by invading organisms (Strieter et al., 2003, Dabbagh and Lewis, 2003) and are also able to respond to certain host molecules (hyaluronan, heat shock proteins, fibronectin, fibrinogen, surfactant protein-A (SP-A), necrotic cell products and reactive oxygen species (ROS)) (Dabbagh and Lewis, 2003, Chaudhuri et al., 2005, Qureshi et al., 2006). TLR are activated via TIRAP (also called TIR) (Toll-IL-1-receptor domain containing adapter protein) and MyD88 which in turn lead to NF-κB activation and the expression of cytokines (TNF-α, IL-1β, IL-6, IL-8) which amplify the inflammatory response (Stevens, 2005, Forster-Waldl et al., 2005, Dabbagh and Lewis, 2003, Aliprantis et al., 1999). TLR are also important in the formation of the adaptive immune response via dendritic cells (Dabbagh and Lewis, 2003).

Neutrophils express all the TLR except TLR 3 (Sabroe et al., 2005). TLR 2 and 4 regulate important neutrophil functions including (Sabroe et al., 2005):

- recruitment (CD11b/CD18 upregulation, L-selectin shedding, adhesion)
- migration and chemotaxis
- activation (increased reactive oxygen species (ROS) production, increased degranulation, increased phagocytosis, cytokine generation, NF-κB activation)
- survival (inhibition of apoptosis via NF-κB and other molecules).

Neutrophils have TLR 2 and 4 at lower levels than monocytes (Sabroe et al., 2005). TLR 2 but not TLR 4 expression is increased on the neutrophil surface in the presence of pro-inflammatory cytokines (Kurt-Jones et al., 2002). Bacterial lipoproteins can mediate apoptosis via TLR 2 in monocytes and epithelial cells (Aliprantis et al., 1999)

but there is conflicting evidence in neutrophils (Power et al., 2004, Sabroe et al., 2003, Lotz et al., 2004, Aleman et al., 2004).

TLR 4 is the principal lipopolysaccharide (LPS) receptor and is known to reduce apoptosis (programmed cell death) in lung epithelial cells in response to hyperoxia (Qureshi et al., 2006). CD14, MD-2 and LBP (LPS binding protein) are involved in the presentation of LPS to TLR 4 (Chaudhuri et al., 2005). There is less TLR 4 on preterm monocytes than term or adult monocytes thus there may be decreased cytokine production from monocytes after LPS exposure in preterm infants (Forster-Waldl et al., 2005). Genetically impaired TLR 4 function has been described in association with chronic inflammatory conditions, like inflammatory bowel disease in adults (Sabroe et al., 2005).

The TLR are an important part of the innate immune response, however TLR function and expression in neonatal neutrophils is not well described. The role of TLR in the pathogenesis of CLD is unknown.

1.6.1.2 Sustained Response

Once in the tissues, neutrophils recognise and engulf micro-organisms by phagocytosis. Once inside the neutrophil, organisms are killed and degraded by the production and release of ROS and granule proteins which are delivered to phagosomes and the extra-cellular environment.

The "respiratory burst" in neutrophils is usually triggered by phagocytosis and generates ROS. Superoxide is generated via NADPH oxidase and converted by superoxide dismutase (SOD) to H_2O_2. H_2O_2 is in turn converted to hydroxyl radicals and hypochlorous acid (Janeway et al., 2004). These reactive oxygen species are directly cytotoxic and are effective in neutralising invading micro-organisms but are injurious to tissues if released outside of the neutrophil.

Neutrophils store anti-microbial, cytotoxic and proteolytic digestive proteins in their cytoplasmic granules (Cheah et al., 2005a). They contain four different granule types, namely azurophil, specific, gelatinase and secretory granules (Moraes et al., 2006),

with each granule containing a number of different proteins. The granule contents are released upon phagocytosis or when neutrophils disintegrate. Similarly to ROS, uncontrolled release of granule contents may also cause injury to surrounding tissues.

a) Chemokines and cytokines

Cytokines are a category of small molecules that are used extensively in cellular communication. The term cytokine encompasses a large and diverse family of polypeptide regulators that are produced by many different cell types and usually circulate in picomolar concentrations that can increase up to 1,000-fold during trauma or infection.

Pro-inflammatory cytokines may be elevated in newborns due to fetal exposure to maternal inflammatory mediators, postnatal infection or due to mediator release by ventilator-induced injury (Gitto et al., 2001). Activated neutrophils are among the many different cell types that are able to synthesise various inflammatory mediators including chemokines and cytokines to recruit and regulate responses of other effector cells including macrophages, T-cells and other neutrophils (Theilgaard-Monch et al., 2004).

The cytokine response in neonatal lungs may be immature or simply different to that seen in adults (Gitto et al., 2001). A number of studies have examined which cytokines are responsible for the cellular influx seen in RDS and it has been shown that airway secretions of infants developing CLD, in particular, have numerous chemotactic factors for neutrophil and macrophage recruitment (including C5a (complement component 5a), LTB4 (leukotriene B4), TNF-α, IL-8, PAF (platelet activating factor), ICAM-1, fibronectin and elastin degradation products (Lecart et al., 2000, Kotecha et al., 1995, Kotecha, 1996)).

Various cytokines and chemokines have been studied in CLD in both human infants and in animal models of the disease. While animal models are useful in that experimental conditions can be very tightly controlled and regulated, results cannot always be directly extrapolated to human neonatal populations. In previous years, the analysis of inflammatory mediators in BAL fluid has been limited by the types of

assay available, however newer multiplexed assays are becoming more common and are likely to be used extensively in the analysis of multiple mediators in small volume samples such as BAL fluid in the future.

Groneck *et al* showed that although multiple pro-inflammatory and chemotactic factors are present in the airspaces of ventilated preterm infants, they are present in higher concentration in those who develop CLD (Groneck and Speer, 1995). They had already established (Groneck et al., 1994) by examining the TAF from 59 ventilated infants that the supernatants from BAL samples of infants who developed CLD were significantly more chemotactic to adult peripheral blood neutrophils than those of infants who did not develop CLD. As with the cell count, the chemotactic activity in the supernatants increased in both groups from day one until day five before falling again in infants whose RDS resolved while staying high in infants who developed CLD.

i) Chemokines

IL-8 is probably one of the most important chemoattractants for neutrophils and the most extensively investigated in preterm infants (Groneck et al., 2001, Truog et al., 2007, Munshi et al., 1997, D'Angio et al., 2002, Baier et al., 2002). It is produced by alveolar macrophages, neutrophils, fibroblasts, type II epithelial cells and endothelial cells and particularly by stimulated monocytes under the influence of TNF-α or LPS (Groneck et al., 1994). The IL-8 receptor (CD128) is expressed in many different cell types. IL-8 is uniquely able to specifically activate neutrophils where it causes the release of enzymes from granules, enhances the metabolism of reactive oxygen species and increases chemotaxis and the expression of adhesion molecules.

IL-8 concentrations were shown to be up to 200 times higher in TAF than plasma and further increased in infants who develop CLD when compared to infants whose RDS resolves (Groneck et al., 1994, Kotecha et al., 1995, Jonsson et al., 1997). Observations of the timing of the peak concentration of IL-8 are not consistent. IL-8 was significantly elevated on first day of life (immediately preceding the marked peak in neutrophil count) in babies who progressed to CLD in one study (Munshi et al., 1997) but in another study (Kotecha et al., 1995) IL-8 was elevated around day 10

coinciding with the neutrophil peak. These differences were attributed to differences in sampling techniques between the two studies and possible clinical differences between the patient groups such as the presence of infection.

MCP-1 (monocyte chemotactic protein-1) belongs to the family of chemotactic cytokines known as chemokines. MCP-1 is expressed by monocytes, vascular endothelial cells, smooth muscle cells, and human type II pneumocytes under the influence of LPS and IL-1. MCP-1 is chemotactic for monocytes. It regulates the expression of certain cell surface antigens (like CD11b) and the expression of other cytokines (IL-1 and IL-6)

MCP-1 was measured in serial TAF samples from 56 preterm newborns by Baier et al (Baier et al., 2002, Baier et al., 2001) who noted that MCP-1 rose over the first week of life and the highest levels were found in infants who developed CLD. MCP-1 has been found to be elevated in airway secretions where *Ureaplasma urealyticum* has been detected (Baier et al., 2001).

The two **MIP** (macrophage inflammatory protein) proteins are the major factors produced by macrophages following their stimulation with bacterial endotoxins. Both proteins are involved in activation of granulocytes. Both forms of MIP-1 stimulate the production of reactive oxygen species in neutrophils and the release of lysosomal enzymes. They also induce the synthesis of other pro-inflammatory cytokines such as IL-1, IL-6 and TNF. MIP-1α stimulates TNF secretion by macrophages, whereas MIP-1β antagonizes this effect.

The two forms of MIP-1 enhance the activities of GM-CSF and promote the growth of mature haematopoietic progenitor cells. MIP-1α (but not MIP-1β) also acts as an inhibitor of the proliferation of immature haematopoietic stem cells and has therefore been called "stem cell inhibitor". MIP-1α and MIP-1β have been found to be elevated in preterm infants, particularly those developing CLD (Baier et al., 2004) and those exposed to hyperoxic conditions (D'Angio et al., 1998).

ii) Cytokines

As well as chemotactic cytokines, pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α are elevated in the lung lavage fluid of infants developing CLD and have been well studied (Jonsson et al., 1997, Kotecha, 1996, Patterson et al., 1998).

IL1-β is the predominant form of IL-1 in humans. It is produced by activated macrophages and by peripheral blood neutrophils, however monocytes are the main source of secreted IL-1, particularly in response to other cytokines (TNF-α and interferons) and invading pathogens. Many other cell types, including endothelial cells, fibroblasts, smooth muscle cells and lymphocytes also produce IL-1. It has a wide range of biological activities, both locally and systemically. IL-1 helps to initiate and modify the inflammatory cascade by promoting the chemotaxis, adhesion and activation of neutrophils and increasing secretion of inflammatory proteins such as proteases. IL-1 synergises with GM-CSF in promoting macrophage colony growth.

Increased concentrations of IL-1β in tracheal aspirates are associated with the need for mechanical ventilation and supplemental oxygen as seen in CLD (Kotecha, 1996, Cayabyab et al., 2003). IL-1β has the ability to prolong survival of neutrophils by reducing apoptosis and this may be its key role in the development of CLD (Colotta et al., 1992). IL-1β has also been implicated in the disruption of postnatal lung morphology and growth in mice (Bry et al., 2007) and this may be relevant to the altered lung structure seen in infants with "new" CLD.

IL-6 is a pleiotropic cytokine and is one of the major physiological mediators of acute phase reactions. It has a role in upregulation of the inflammatory response but may also have anti-inflammatory effects via inhibition of TNF-α production in macrophages (Aderka et al., 1989). It is produced by many different cell types. The main sources *in vivo* are stimulated monocytes and endothelial cells but macrophages (including alveolar macrophages), lymphocytes, smooth muscle cells and eosinophils can also produce IL-6 after stimulation by IL-1, LPS and TNF among others. IL-6 can also stimulate or inhibit its own synthesis, depending upon the cell type. Intratracheal IL-6 has been shown to block LPS induced lung injury in rabbits (Ulich et al., 1991).

IL-6 was significantly elevated on day 3 and 5 in babies who developed CLD (Munshi et al., 1997). Bagchi *et al* (Bagchi et al., 1994) studied 30 infants of <33 weeks' gestation who were ventilated for RDS along with 10 controls ventilated for non-respiratory reasons (cardiac or gastrointestinal surgery) and found IL-6 activity was 15 fold higher on day 1 in infants who would go on to develop CLD than in controls and 6.6 fold higher in babies with RDS compared to controls at the same time point. IL-6 activity remained elevated for the first 2 weeks of life in babies who developed CLD before returning to similar levels to controls by day 28. Jonsson *et al* suggested that elevated IL-6 levels may be predictive for the development of CLD (Jonsson et al., 1997).

TNF is a member of the TNF ligand superfamily. There are two molecular species of TNF, known as TNF-α (cachectin) and TNF-β (lymphotoxin), which generally display similar biological activities *in vitro*, although TNF-β is often less potent or has partial agonist activity.

TNF-α is derived mainly from macrophages and monocytes following stimulation by LPS, interferons, IL-2, GM-CSF, immune complexes or platelet activating factor. It is a potent chemoattractant for neutrophils and also enhances their adherence, phagocytic and cytotoxic abilities (Strieter et al., 2003). It activates NF-κB and can prolong the survival of neutrophils by reducing apoptosis (Colotta et al., 1992). It stimulates phagocytosis in macrophages and modulates the expression of other cytokines, including IL-1 and IL-6 (Bagchi et al., 1994). TNF-α can be found on monocytes and T-cells after cell activation where it mediates cell destruction by direct cell-to-cell contacts. It is also a potent promoter of angiogenesis.

TNF-α was undetectable in controls and low in infants with RDS but peaked at day 14 in babies who progressed to CLD (Bagchi et al., 1994). TNF-α was elevated in BAL samples taken early in the ventilatory course of preterm infants with the worst pulmonary outcomes in one study (Mahieu et al., 2005).

IL-10 is predominantly an anti-inflammatory cytokine which inhibits production of pro-inflammatory cytokines like TNF-α, IL-1β, IL-6, IL-8, GM-CSF and G-CSF from monocytes and macrophages (Liles et al., 1996, de Waal Malefyt et al., 1991,

Fiorentino et al., 1991) and is known to promote neutrophil apoptosis (Cox, 1996). It also inhibits antigen presentation and macrophage killing. It is produced by stimulated lymphocytes and by monocytes following cell activation by LPS.

IL-10 may be developmentally regulated, as shown by IL-10 being undetectable in 17 preterm ventilated babies but expressed in term infants ventilated for persistent pulmonary hypertension or meconium aspiration syndrome (Whicher and Evans, 1990, Spits and de Waal Malefyt, 1992, Jones et al., 1996). IL-10 was lacking from the lung secretions of preterm infants in one study which may help to explain the continuing inflammatory process in these infants (Jones et al., 1996). In two other studies, IL-10 was detected in the lungs of preterm infants but was lower in infants who developed CLD or showed an early increase and then a fall to lower levels than in infants with RDS (McColm et al., 2000, Beresford and Shaw, 2002).

G-CSF is secreted by monocytes, macrophages and neutrophils after cell activation. The synthesis of G-CSF can be induced by bacterial endotoxins, TNF, IL-1 and GM-CSF. It stimulates the proliferation and differentiation of granulocytes and is able to activate neutrophils. G-CSF synergises with other cytokines, including GM-CSF. The G-CSF receptor (CD114) is expressed on all granulocytes as well as on endothelial cells.

GM-CSF is vital for the proliferation and differentiation of progenitors of granulocytes and macrophages. It enhances microbicidal activity, oxidative metabolism and phagocytotic activity of neutrophils and macrophages. GM-CSF receptors are expressed on the cell surface of myeloid cells and also on non-haematopoietic cells such as endothelial cells.

Neither G-CSF nor GM-CSF has been extensively studied in the preterm infant population but both have been detected in BAL fluid, with increasing levels recorded in infants developing CLD (Papoff et al., 2001).

Vascular Endothelial Growth Factor (**VEGF**) is a growth factor which has a role in the control of angiogenesis and thus plays a crucial role in alveolar development because of the very close relationship between vascular development and the development of the lung. It is produced by a plethora of cell types, including

macrophages and lung epithelial cells. Its receptor is expressed on vascular endothelial cells. VEGF stimulates the proliferation of vascular endothelial cells and significantly influences vascular permeability. VEGF plays an important role in neovascularisation under physiological conditions and its synthesis is induced by hypoxia. It is also a potent chemoattractant for monocytes and is able to induce the synthesis of metalloproteinases which degrade interstitial collagen.

A number of investigators have found reduced VEGF levels in animal models of CLD (Bland et al., 2007, Tambunting et al., 2005). Lassus *et al* found a reduction in VEGF in human infants who developed CLD infants (Lassus et al., 1999) whereas Currie *et al* (Currie et al., 2001) found no difference between term and preterm infants with or without CLD. It has also been proposed that either elevated or decreased VEGF levels can mediate lung injury and emphysematous changes (Voelkel et al., 2006).

TGF-β is a protein which controls proliferation and cellular differentiation in many cell types and also acts as an inducer of apoptosis. TGF-β exists in three isoforms, namely TGF-β1, TGF-β2 and TGF-β3. Immunocytochemistry studies have localised TGF-β to alveolar macrophages obtained by BAL (Kotecha et al., 1996a).

In the past, TGF-β measurement in neonatal lung has produced variable results, possibly because of difficulty in finding an appropriate assay. Elevated levels of TGF-β have been shown in the presence of chorioamnionitis and in developing CLD (Kotecha et al., 1996a, Ichiba et al., 2009, Jonsson et al., 2000). TGF-β levels were also found to correlate with the duration of oxygen administration (Ichiba et al., 2009). However, using two other methods, TGF-β levels were shown to be decreased in a similar population of preterm infants developing CLD (Choi et al., 2008) compared to controls.

b) Proteases

Human neutrophil elastase is the most abundant of the proteases in the lung and the vast majority is produced by neutrophils (Lee and Downey, 2001). Elastase is a serine protease and is stored in the intra-cytoplasmic azurophilic granules of neutrophils, from where it can be released at the time of neutrophil activation. In addition to its

bactericidal role, elastase may also have an important role as a chemotactic agent, being capable of induction of IL-8 (Nakamura et al., 1992) and leukotriene B4 (Hubbard et al., 1991) production.

Elastase is capable of digesting elastin, an important structural component of lung tissue. Elastin is relatively conserved throughout life and once damaged or destroyed is difficult to replace (Bigatel et al., 1999), and repair to elastin networks often results in malformed and dysfunctional elastin filaments (Finlay et al., 1996).

Neutrophil elastase is also capable of digesting almost all components of the extracellular matrix including collagens types I-IV, fibronectin, laminin and proteoglycans. The tissue damage that an excess of uninhibited protease activity can cause has been demonstrated in animal models (Lucey et al., 1985).

In the pre-surfactant era high elastase activity was detected in the majority of lung lavage samples from infants who developed CLD (Merritt et al., 1983, Ogden et al., 1984, Watterberg et al., 1994). More recently, a number of studies have found elastase in only a minority of samples from the lungs of preterm infants and questioned the relationship of elastase to the development of CLD (Speer et al., 1993, Groneck et al., 1994, Sveger et al., 2002, Sluis et al., 1994).

Matrix metalloproteinases (MMP) are proteases which are important in normal lung development (Greenlee et al., 2007) when appropriately controlled by specific protease inhibitors but their unregulated activity, particularly MMP-8 and 9, may be associated with the development of CLD (Sweet et al., 2004, Ekekezie et al., 2004, Cederqvist et al., 2001).

1.6.1.3 Resolution/Chronicity

Chronic inflammation is a continuous interaction between pro-inflammatory mechanisms which act to cause tissue injury and those mechanisms which act to promote resolution of inflammation. It is possible that either uncontrolled pro-inflammatory events, resulting in ongoing initiation of inflammation and/or continued

infiltration and recruitment of neutrophils, or inefficient resolution processes result in chronic inflammation (Haslett, 1999).

Neutrophil contents, like elastase, described above, are not only histotoxic but are also able to amplify the inflammatory response by cleaving matrix proteins (Haslett, 1999, Savill et al., 1989b). The indiscriminate release of these contents as a consequence of neutrophil lysis can damage host tissue and lead to persistence of the inflammatory response (Haslett et al., 1994). Thus the fate of neutrophils is central to the resolution of inflammation (Savill et al., 1989a). Neutrophil persistence characterises the early stages of chronic inflammation (Koenig et al., 2005). Failure to achieve removal of neutrophils and their toxic products from a site of inflammation is associated with chronic/persistent inflammation (Whyte et al., 1993). Apoptosis, or programmed cell death, is critical to the resolution of inflammation as it promotes the removal of effete neutrophils by the reticuloendothelial system.

The persistence of neutrophil infiltration in babies with RDS is strongly associated with the development of CLD (Ogden et al., 1984). Grigg *et al* showed that neutrophils are cleared by apoptosis in RDS (Grigg et al., 1991), leading to the hypothesis that preterm babies are more at risk of lung injury because of reduced, delayed or impaired neutrophil apoptosis (Kotecha et al., 2003, Oei et al., 2003). Inhibition of apoptosis could result in more viable neutrophils persisting in the tissues or more necrotic neutrophils releasing their toxic contents into tissues and thus causing more lung injury (Matute-Bello et al., 1997).

a) Resolution

The usual outcome of inflammation is resolution and repair of damaged tissue rather than persistent or chronic inflammation and continued tissue damage (Serhan and Savill, 2005). It is unknown why some stimuli produce inflammation which completely resolves and others provoke a persistent reaction with tissue destruction and scarring or fibrosis (Haslett, 1999).

The resolution of inflammation requires: (Haslett, 1999)

- removal of the inflammatory stimulus

- removal and/or destruction of pro-inflammatory mediators
- cessation of granulocyte migration
- restoration of normal vascular permeability and removal of extravasated fluid
- stop granulocytes secreting pro-inflammatory and histotoxic substances
- arrival of monocytes and their differentiation to macrophages
- removal of debris and cells (apoptosis)
- final removal of monocytes and macrophages
- repair of injured tissue

In this process there are multiple points at which dysregulation may occur (Han and Ulevitch, 2005).

During the initial phase of resolution of the inflammatory response, prostaglandins are generated which are essential for control of blood flow and blood vessel dilatation for leucocytes to adhere and undergo diapedesis. The generation of arachidonic acid metabolites (e.g. lipoxins) retard entry of new neutrophils to the site of inflammation, reduce vascular permeability, promote non-phlogistic (not causing fever/inflammation) infiltration of monocytes and stimulate macrophages to ingest and clear apoptotic neutrophils (Serhan and Savill, 2005).

In order to resolve inflammation, neutrophil activity must be curtailed and senescent neutrophils disposed of so healing can occur (Oei et al., 2003). In healthy systems neutrophils undergo apoptosis (programmed cell death) and rapid clearance which has the effect of limiting tissue injury and promoting resolution of inflammation (Haslett, 1999). Cells which are not cleared by phagocytes undergo necrosis and disintegrate, releasing their toxic contents, which serve as a further pro-inflammatory stimulus (Savill et al., 2002).

Activated neutrophils initiate an apoptotic programme which facilitates resolution of inflammation and prevents tissue damage which would be caused by necrotic cell lysis (Kobayashi et al., 2003a, Kobayashi et al., 2003b). Following the initial pro-inflammatory response, a further transcriptional response occurs which promotes apoptosis. As part of this, upregulation of genes for pro-apoptotic proteins (TNF-α,

TRAIL and their receptors, caspase 1, Bax and TLR 2 signalling pathway components) and downregulation of receptors for inflammatory mediators occurs.

1.7 Apoptosis

The term apoptosis was coined in 1972 by Kerr *et al* (Kerr et al., 1972) and is derived from ancient Greek, meaning "falling, as of leaves from a tree". It was originally defined to mean a physiological or programmed form of cell death, affecting cells scattered throughout a tissue, which does not induce inflammation and allows for clearance of cells with minimal local injury.

Apoptosis is different to necrosis, which is defined as accidental cell death due to the effect of, for example, toxins, hypoxia or extremes of temperature. Primary necrosis is a passive process and tends to affect large numbers of neighbouring cells. It is characterised by swelling and bursting of the cell with release of cellular contents and is a pro-inflammatory stimulus in most tissues. Secondary necrosis is the disintegration of cells which have originally undergone apoptosis but have not been ingested or cleared by phagocytes possibly because the load of apoptotic cells exceeds the capacity for clearance by phagocytes (Savill et al., 2002).

Apoptosis pathways are broadly similar in phylogenetically diverse organisms which suggests that apoptosis and the phagocytosis of apoptotic cells are important regulatory mechanisms which have been conserved through evolution (Giles et al., 2000). Apoptosis is important in a variety of biological systems for normal cell turnover and maintenance of cellular homeostasis, immune system regulation and the resolution of inflammation, embryonic development and tissue atrophy and remodelling, among others. Apoptosis is normally involved in the structural maturation of the lung, particularly in reducing the number of fibroblasts and type II epithelial cells in thinning of the alveolar septae in animal studies (Schittny et al., 1998).

Apoptosis is a complex process, influenced by the cell's internal genetic code and by its external environment and requires the co-ordinated action of multiple sub-programmes to be effectively carried out (Hengartner, 2000). Inappropriate or

dysregulated apoptosis is implicated in pathologies such as Alzheimer's disease, Huntingdon's disease, ischaemia-reperfusion injury, autoimmune disorders and some cancers (Cohen, 1997).

1.7.1 Neutrophil apoptosis

Neutrophil apoptosis has a major regulatory role in many biological processes, including the inflammatory response (Matute-Bello et al., 1997). Apoptosis is important in the resolution of inflammation as it leads to functional downregulation and clearance of neutrophils. Neutrophils have a constitutive apoptosis programme which is hastened during activation of the cell to ensure clearance from sites of inflammation before they become necrotic and release their toxic contents (Cheah et al., 2005a).

Neutrophil apoptosis is characterised by stereotypical cell morphological changes (Savill et al., 1989b, Cohen, 1997) which are not seen in circulating neutrophils (Wyllie et al., 1980):

- condensation and fragmentation of nuclear chromatin and nucleolar prominence,
- compaction of cytoplasmic organelles, swelling of the endoplasmic reticulum and cytoplasmic vacuolation,
- a decrease in cell volume
- and eventually alterations to the plasma membrane resulting in recognition by phagocytes and phagocytosis.

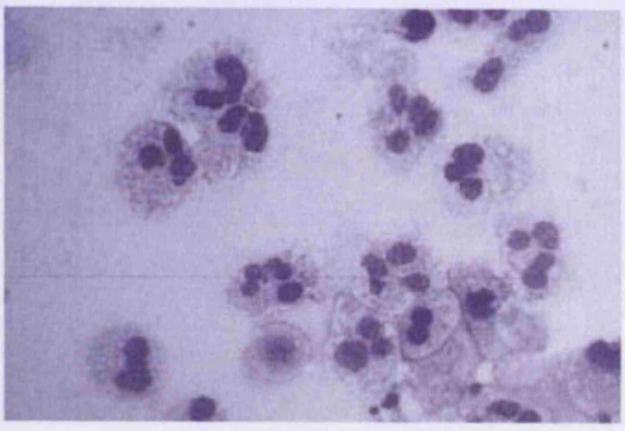

Figure 1.3 Apoptotic neutrophils showing condensation of the nucleus, cytoplasmic vacuolation and decrease in cell volume (Pitrak, 1997).

During apoptosis the cell membrane and organelles remain intact and there is no leakage of cell contents. The neutrophil loses the ability to degranulate and the ability to phagocytose (Haslett, 1999, Savill et al., 1989b). Neutrophil nuclear chromatin becomes fragmented in a characteristic internucleosomal pattern, where each fragment is a multiple of 180bp. This is thought to represent endogenous endonuclease activation. (Savill et al., 1989b)

Neutrophils which have been aged in culture show the characteristic changes of apoptosis but still exclude trypan blue and show little spontaneous release of myeloperoxidase (MPO) for up to 24 hours in culture. Thereafter more cells fail to exclude trypan blue, balloon and disintegrate – undergoing necrosis and releasing their toxic contents (Savill et al., 1989b).

1.7.2 Mechanism/process of apoptosis

Two main apoptosis pathways are thought to occur in neutrophils (Akgul and Edwards, 2003):

- "extrinsic" or "death receptor" pathways (via Fas, TRAIL and TNF receptors) which directly activate the caspase cascade via caspase 8
- "intrinsic apoptosis pathway" which involves mitochondria and the Bcl2 family of genes and activates the caspase cascade via caspase 9.

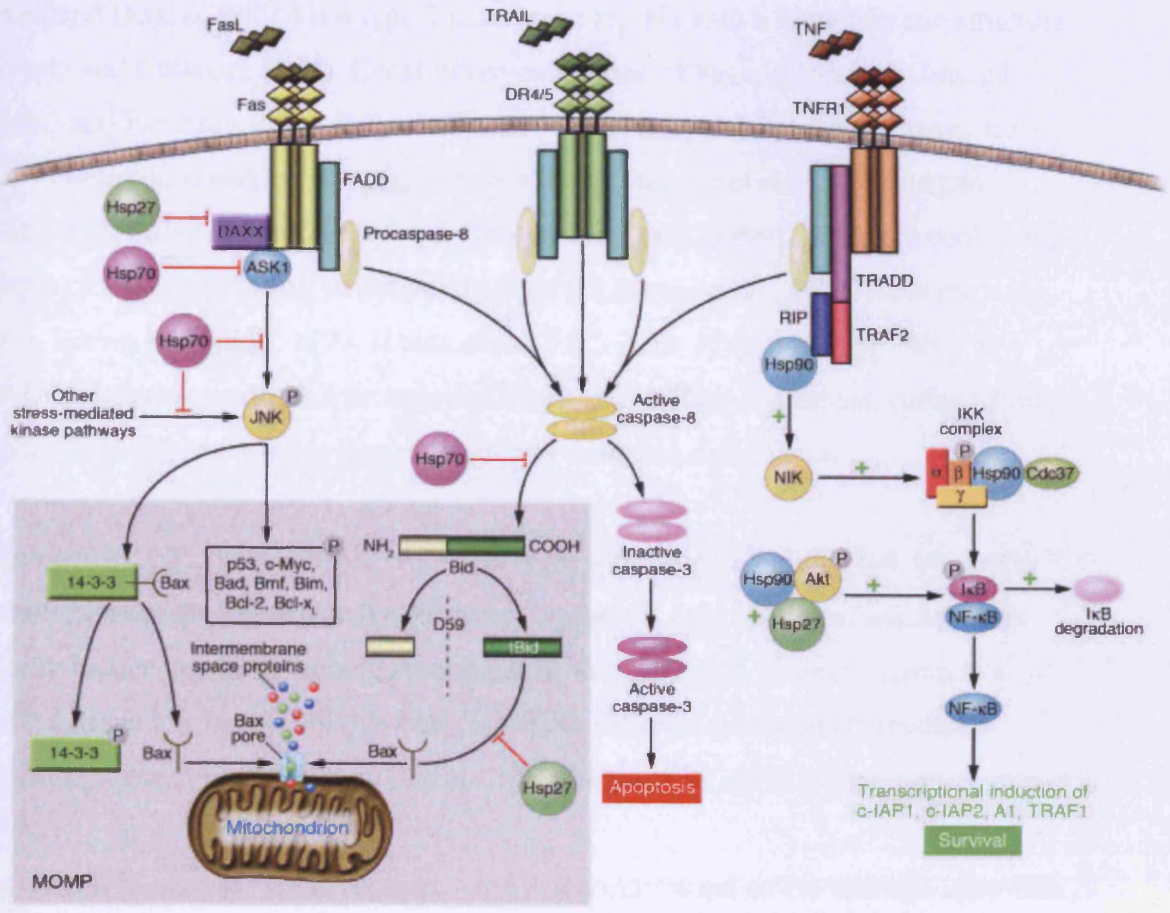

Fig 1.4 Pathways of apoptosis showing both the "death receptor" and "intrinsic" apoptosis pathways (Beere, 2005, Coalson, 2003).

1.7.2.1 Extrinsic or death receptor pathways

a) Fas

The Fas/FasL pathway is important for apoptosis in various cell types (Liles et al., 1996), including neutrophils.

Fas (CD95/Apo-1) is a cell surface molecule (type 1 membrane protein) belonging to the TNF/nerve growth factor receptor family (Hanna et al., 2005, Nagata and Golstein, 1995). There is variable but wide expression of Fas in different tissues (Nagata and Golstein, 1995) including neutrophils (Hanna et al., 2005, Liles et al., 1996) which express more Fas than other leucocytes (Liles et al., 1996).

Fas ligand (FasL/CD95L) is a type 2 membrane protein with a homotrimeric structure (Nagata and Golstein, 1995). Constitutive expression of FasL is relatively limited (Akgul and Edwards, 2003, Hanna et al., 2005). Monocytes/ and macrophages are known to produce and express high levels of FasL (Kiener et al., 1997) and can induce Fas mediated neutrophil death (Brown and Savill, 1999). There are conflicting reports of FasL expression on human neutrophils (Serrao et al., 2001, Renshaw et al., 2000, Brown and Savill, 1999, Hanna et al., 2005, Liles et al., 1996) probably as a result of differing neutrophil processing techniques, culture conditions, cultures being "contaminated" with monocytes and antibody detection differences.

Soluble FasL (sFasL) is derived from cleavage of membrane bound FasL by matrix metalloproteinases. Neutrophils undergoing apoptosis may liberate sFasL and thus weakly induce apoptosis of neighbouring cells in a paracrine manner (Serrao et al., 2001, Liles et al., 1996). Soluble FasL is able to induce a profound chemotactic response in neutrophils (Ottonello et al., 1999, Seino et al., 1998).

FasL binds to the Fas "death receptor" and causes the target cell to undergo apoptosis. In order to trigger apoptosis, Fas needs to be cross-linked (Nagata and Golstein, 1995). Cross-linking of Fas by trimeric FasL results in clustering of intracellular death domains and the recruitment of adaptor proteins (Akgul and Edwards, 2003). The main adaptor protein in this pathway is FADD (Fas-associated death domain-containing protein). Interaction of Fas with FasL (or anti-Fas antibody) activates the FADD/MORT1 complex which contains a death effector domain which allows it to interact with caspase 8 (Akgul and Edwards, 2003). Pro-caspase 8 is cleaved to become an active enzyme and thus initiates activation of other caspases including caspase 3 (Luo et al., 2003). The binding of FasL to Fas also activates p38 MAP kinase and PI3-K (Hanna et al., 2005) and recruits RIP-1 (receptor interacting protein), the activation of which may be sufficient to induce apoptosis (Walczak and Krammer, 2000).

The Fas pathway is independent of extracellular calcium ions and is inhibited by Bcl2 proteins (Nagata and Golstein, 1995). Fas-mediated apoptosis may heavily depend on the involvement of ROS (especially H_2O_2) (Kasahara et al., 1997) but the role of ROS in triggering apoptosis via the Fas pathway is controversial. High levels of ROS can

prevent caspase function and ROS may be involved in the Fas-mediated signalling system (Kasahara et al., 1997). Anti-Fas IgM mimics FasL activity *in vitro*. This antibody activates Fas and increases apoptosis in adult neutrophils (Liles et al., 1996, Hanna et al., 2005).

The Fas/FasL pathway is not the only mechanism for induction of apoptosis in neutrophils. Apoptosis still occurs if the Fas/FasL pathway is blocked (Liles et al., 1996). However, Fas-mediated neutrophil apoptosis can be reduced by G-CSF, GM-CSF, IFNγ (Interferon gamma), TNF-α and dexamethasone (Liles et al., 1996) but Fas ligation, even in the presence of these pro-inflammatory survival signals, causes apoptosis, in other words, inflammatory neutrophils retain their ability to respond to Fas death signals (Renshaw et al., 2000).

Fas and FasL have been studied in neonatal and paediatric blood samples (Ennaciri et al., 2006, Hanna et al., 2005, Sarandakou et al., 2003), but not in lung lavage samples in this age group.

b) TNF-α

TNF-α is a potent pro-inflammatory cytokine produced by macrophages, monocytes and other cell types (lymphocytes, fibroblasts) in response to injury, inflammation or infection (Baud and Karin, 2001).

There are two receptors for TNF-α, namely TNFR1 and TNFR2, which are both type 1 trans-membrane proteins. TNFR1 binding recruits TRADD (TNFR1 associated death domain protein) which in turn recruits FADD and then caspase 8 resulting in apoptosis. TNFR1 binding also recruits RIP-1 (receptor interacting protein) and TRAF2 (TNF receptor associated factor 2) which result in NF-κB and AP-1 (via MAPK, JNK, p38) activation and pro-inflammation (Baud and Karin, 2001). Fas is a better activator of apoptosis than TNF-α because it is a poor activator of NF-κB, which acts to counteract the pro-apoptotic signal (Baud and Karin, 2001). TNFR2 binding directly recruits TRAF2 which recruits TRAF 1 and increases inflammation (Baud and Karin, 2001).

TNF-α is able to accelerate neutrophil apoptosis at early time points via TNFR1 but later inhibits apoptosis (after 12 hours *in vitro*) probably due to NF-κB activation (Baud and Karin, 2001, Murray et al., 1997).

c) Tumour necrosis factor-Related Apoptosis Inducing Ligand

TNF-related apoptosis inducing ligand (TRAIL) is a pro-apoptotic member of the TNF superfamily (Renshaw et al., 2003). It is a 281-amino acid type II transmembrane protein, closely related to FasL and, like FasL has a homotrimeric subunit structure (Pitti et al., 1996, Wiley et al., 1995). TRAIL is expressed on macrophages, monocytes and also T cells and NK cells and induces apoptosis when over-expressed (Walczak and Krammer, 2000).

TRAIL interacts with TRAIL-R1 and TRAIL-R2 which are "death receptors" and with TRAIL-R3 and TRAIL-R4 which may be decoy receptors, although resistance to TRAIL may be mediated at an intracellular level as well (Walczak and Krammer, 2000). Neutrophils have been found to contain mRNA for TRAIL, TRAIL-R2 and TRAIL-R3. TRAIL-R2 and TRAIL-R3 are expressed on the surface of neutrophils but TRAIL, TRAIL-R1 and TRAIL-R4 are not (Renshaw et al., 2003).

Signalling via TRAIL R1 and TRAIL R2 has 2 distinct pathways – one resulting in apoptosis (major outcome) and the other in cell survival. The apoptosis pathway begins with recruitment of FADD. The DED (death effector domain) then recruits pro-caspase 8, which self-cleaves and then activates caspase 3 amongst others. TRAIL receptors can also cause cell survival via NF-κB but NF-κB alone is insufficient to prevent apoptosis - a caspase inhibitor is also required (Kimberley and Screaton, 2004).

It is likely that TRAIL is rapidly cleaved from the cell surface by proteases, in a similar way to FasL cleavage. TRAIL has no effect on neutrophil chemotaxis, unlike FasL (Renshaw et al., 2003). Similar to FasL, TRAIL requires cross-linking of receptors for its activity (Renshaw et al., 2003) and TRAIL uses a caspase-dependent pathway to kill its target cell. Bcl2 and Bcl-xL do not protect cells from TRAIL-induced apoptosis (Walczak and Krammer, 2000).

d) Caspases

A number of proteases play an important role in apoptosis including serine proteases, calpains, proteasomes and IL-1β converting enzyme (ICE)-like proteases (cysteine proteases) called caspases (Cohen, 1997). Most of the morphological changes of apoptosis are the result of caspase activity (Hengartner, 2000).

Caspases are a family of cysteine proteases which are activated specifically in apoptotic cells. Over a dozen caspases have been identified which act on close to 100 caspase substrates characterised to date. All caspases recognise specific polypeptide sequences and cleave their substrates after aspartic acid residues (Hengartner, 2000). Caspases can be inhibited by inhibitors of apoptosis (IAP) proteins (Hengartner, 2000).

Caspases selectively cleave a set of target proteins usually inactivating them (Wyllie, 1980). Additionally, caspases cleave nuclear lamins causing characteristic nuclear shrinkage and budding (Rao et al., 1996) and cleave cytoskeletal proteins (like fodrin and gelsolin) causing loss of cell shape (Kothakota et al., 1997). Not all caspases are required in a single cell for cell death to occur - some may be tissue specific (Cohen, 1997). Most caspases are activated by cleavage and are able to activate each other, especially caspases 3, 6 and 7 which are the more abundant, workhorses of apoptosis (Hengartner, 2000).

Caspase 3 is a key executioner of apoptosis, responsible for the cleavage of many key proteins (Cohen, 1997). It is widely distributed especially in cells of the immune system. Caspase 3 cleaves substrate at Asp-Xaa-Xaa-Asp (DXXD) motif (also seen in caspase 2, 6, 9) (Cohen, 1997). Caspase 3 activation is an early apoptotic event which precedes phosphatidylserine (PS) exposure. Proteolytic activation of caspase 3 occurs when initiator caspases cleave pro-caspase 3 (32kDa) into 2 subunits (p20/p17 and p12) (Cheah et al., 2005a). Cytoskeletal components function as substrates for caspase 3. Caspase 3 may mediate the cleavage of the cytoskeletal element, fodrin, which is linked to PS expression on the outer plasma membrane (Luo et al., 2003). Decreased apoptosis has been observed in caspase 3 deficient mice (Cohen, 1997).

Caspase 6 has a high homology with caspase 3 and may be activated by it. Caspase 6 is the only caspase to cleave lamins, structural proteins of the nuclear envelope (Cohen, 1997).

Caspase 7 is also similar to caspase 3 and may be an important effector of apoptosis (Cohen, 1997) via the Fas pathway but caspase 7 is only minimally expressed in neutrophils (Luo et al., 2003).

Caspase 8 is involved in regulation of Fas or TNF-mediated apoptosis. It is a key initiator of apoptosis and is able to activate all the other caspases (Cohen, 1997). It can also be activated by protein-protein interactions i.e. several caspase 8 molecules can aggregate together at a death effector domain and activate themselves, without the need for cleavage by another molecule (Hengartner, 2000). Caspase 8-dependent cleavage of Bid and subsequent release of cytochrome c integrates the extrinsic and intrinsic pathways of apoptosis (Beere, 2005).

Caspase 9 is similar in structure to caspase 3 and is also activated by caspase 3 (Cohen, 1997). Caspase 9 has a complex activation process and requires both cytochrome c and Apaf-1 for its activation (Luo et al., 2003, Hengartner, 2000).

1.7.2.2 Intrinsic apoptosis pathway

Within the cell, mitochondria sequester pro-apoptotic proteins e.g. cytochrome c – which, in addition to its involvement in oxidative phosphorylation, is important as a co-factor with caspase 9 (Hengartner, 2000). Pro-apoptotic proteins like cytochrome c, apoptosis inducing factor (AIF), endonuclease G and Smac/DIABLO are released from mitochondria under the influence of the opposing pro- and antiapoptotic members of the Bcl2 family of genes (Beere, 2005, Hengartner, 2000).

The Bcl2 family are a group of apoptotic regulators (Hengartner, 2000) which are divided into 3 sub-groups:

- Group 1 (Bcl2, Bcl-xl) which are anti-apoptotic
- Group 2 (Bax, Bak, Bad) which are pro-apoptotic

- Group 3 (Bid, Bim, Bmf) which are pro-apoptotic.

Other Bcl-2 proteins include Bcl-w, Bcl-xs, and Mcl-1.

Bcl-2 family proteins acting on the mitochondria are probably among the key regulators of the apoptotic response (Cook et al., 1999). In one study, peripheral blood neutrophils in adult subjects expressed Bak, Bad, Bcl-w and Bfl-1, but hardly expressed Bcl-2, Bcl-xL, Bik, and Bax (Santos-Beneit and Mollinedo, 2000). In contrast, another study (Ohta et al., 1995) showed expression of Bax, but not other members of the Bcl-2 family in neutrophils, which may reflect the fact that they have the shortest life-span among blood leucocytes.

The expression of Bcl-2 family proteins in the human neonate in comparison to adults has been studied to a very limited extent (Hanna et al., 2005). Some differences have been shown in expression of the proteins in the cerebral cortex of term and preterm guinea pig fetuses (Abedin et al., 2005) and in the cardiac myocytes (Cook et al., 1999) and neuronal tissue of rats (Vekrellis et al., 1997).

a) Bcl-xl

Bcl-xl stands for "Basal cell lymphoma-extra large". It is an anti-apoptotic, transmembrane protein, found particularly in the mitochondrial membranes of cells that are long-lived and postmitotic, such as adult brain cells. Bcl-xl appears to be the dominant regulator of apoptosis. It is known as the survival protein because of its cell death repressor activity.

Two different models of Bcl-xl function have been proposed. In one, Bcl-xl binds to an activator, thereby preventing Bax activation. In the other, Bcl-xl binds directly to activated Bax (Deming and Rathmell, 2006, Harada and Grant, 2003).

b) Bax

Bax is another homologue of the Bcl-2 gene, which acts as a facilitator of apoptotic cell death (Ohta et al., 1995). Although Bcl-xl and Bax are structurally similar, activated Bax forms large oligomers that permeabilise the outer mitochondrial

membrane, releasing cytochrome c and thereby committing cells to apoptosis, whereas Bcl-xl and Bcl-2 inhibit this process (Vekrellis et al., 1997).

c) Mcl-1

Mcl-1 (Myeloid cell leukaemia sequence 1) is an anti-apoptotic member of the Bcl-2 family (Michels et al., 2005) and plays a critical role in promoting the survival of lymphocytes and haematopoietic stem cells (Dzhagalov et al., 2007). Mcl-1 promotes cell survival by interfering in the cascade of events leading to release of cytochrome c from mitochondria. It has a short half life and is a highly regulated protein, induced by a wide range of survival signals, including the cytokine TNF-α (Cross et al., 2008) and also rapidly downregulated during apoptosis. Regulation of Mcl-1 expression occurs at multiple levels, allowing for either the rapid induction or elimination of the protein in response to different cellular events. This suggests that Mcl-1 can play an early role in response to signals directing either cell survival or cell death (Michels et al., 2005).

Accumulating evidence suggests that Mcl-1 plays a critical pro-survival role in the development and maintenance of both normal and malignant tissues as well as being required for embryonic development and the function of the immune system. Targeting of Mcl-1 may be useful as a therapeutic strategy in malignancy, inflammatory conditions and infectious disease where Mcl-1 may play a major role in suppressing apoptosis (Michels et al., 2005).

Studies have implicated Mcl-1 in regulating the survival of both neutrophils and macrophages (Dzhagalov et al., 2007). Mcl-1 conditional knockout mice had a severe defect in neutrophil survival, whereas macrophage survival was normal. The granulocytes in the blood, spleen and bone marrow of Mcl-1 conditional knockout mice exhibited a 2- to 3-fold higher apoptotic rate than in control animals. In contrast, macrophages from Mcl-1-deficient mice showed normal survival. Interestingly, Mcl-1 can also be cleaved by caspases during apoptosis to produce a cell death promoting molecule (Michels et al., 2005).

Overall it is the fine balance of expression of Bcl-2 family proteins which may regulate the life and death of haematolymphoid cells at different stages of cell differentiation and activation. Interactions between Bax, Bcl-xl, Bcl-2 and Mcl-1 play an important role in the control of cell death or survival (Ohta et al., 1995).

1.7.3 Control/modulation of apoptosis

The cellular environment influences apoptosis (Allgaier et al., 1998). The interplay between extrinsic (inflammatory milieu) and intrinsic (endogenous programme of constitutive apoptosis) factors will determine neutrophil longevity (Renshaw et al., 2000). Cell immaturity may also influence the rate of apoptosis, although the mechanism of this is uncertain (Allgaier et al., 1998).

Various inflammatory mediators can modulate neutrophil apoptosis. The presence of pro-inflammatory factors (GM-CSF, G-CSF, TNF-α, IFN gamma, IL-2, IL-6, C5a) and glucocorticoids inhibit neutrophil apoptosis (Haslett, 1999, Leavey et al., 1998, Liles et al., 1996, Allgaier et al., 1998, Colotta et al., 1992, Lee et al., 1993, Matute-Bello et al., 1997). Neutrophil apoptosis may also be delayed by the presence of elevated extra-cellular calcium, LPS (Lee et al., 1993, Liles et al., 1996), hypoxia and corticosteroids (Allgaier et al., 1998, Haslett, 1999). NF-κB is a transcription factor which regulates the expression of many pro-inflammatory proteins including cytokines and adhesion molecules and generates anti-apoptotic signals (Cheah et al., 2005a).

Inhibition of apoptosis serves to preserve and prolong the functional lifespan of the neutrophils (Lee et al., 1993). Neutrophil survival may benefit the host by more neutrophils remaining active at the site of inflammation to neutralise pathogens (Liles et al., 1996) (Leavey et al., 1998) but neutrophils are also capable of producing more tissue damage at the inflammatory site if they do not undergo apoptosis at an appropriate rate (Kasahara et al., 1997, Leavey et al., 1998).

Apoptosis may be enhanced by cycloheximide, TNF-α and nitric oxide donors (Haslett, 1999). Of clinical importance is that a number of drugs in common use may alter neutrophil apoptosis. It is increased by erythromycin (Healy et al., 2002) and

theophyllines (Yasui et al., 2000) and decreased by glucocorticoids (McColl et al., 2007) – all 3 of which are commonly used in clinical neonatal practice.

NF-κB is an ubiquitously expressed transcription factor. It plays a role in expression of genes that regulate apoptosis, as well as controlling cell proliferation, differentiation, immune and inflammatory responses (Shishodia and Aggarwal, 2002). Usually, in non-stimulated cells, NF-κB is found in the cytoplasm, in complex with inhibitory proteins of the I-κB family (Beg and Baldwin, 1993, Finco et al., 1994) which inhibit its ability to bind to DNA. In response to a wide range of stimuli, I-κB is rapidly phosphorylated and degraded, allowing NF-κB translocation to the nucleus, DNA binding and transcription of target genes (Chu et al., 1997, Karin, 1999).

There are numerous reports showing the anti-apoptotic effect of NF-κB (Shishodia and Aggarwal, 2002, Whyte et al., 1997, Ward et al., 1999). NF-κB regulates apoptosis by regulating expression of genes that play a role in blocking apoptosis (e.g. IAP) and it may also have role in the activity of Bcl 2.

1.7.4 Results of apoptosis

The onset of apoptosis is closely associated with functional impairment of neutrophils and their "isolation" from stimulation by inflammatory mediators (Whyte et al., 1993). Whyte *et al* (Whyte et al., 1993) showed significant reductions in a number of neutrophil functions with the onset of apoptosis, including

- reduction in spreading ability,
- reduced neutrophil polarisation (both spontaneous and in response to stimulation *in vitro*),
- reduced phagocytosis of opsonised particles,
- reduced chemotaxis in both stimulated and unstimulated cells,
- reduced MPO enzyme release in response to stimulation and
- reduced superoxide anion release.

Dransfield *et al* (Dransfield et al., 1995) also showed that the ability of neutrophils to secrete granular contents was reduced with the onset of apoptosis and that their adhesive ability is also reduced as part of the damage limitation strategy of apoptosis.

Some functional loss may precede the onset of morphologically recognisable apoptosis (Whyte et al., 1993).

The process of apoptosis induces changes to the expression of various cell surface markers and receptors related to cell function e.g. CD16 (Haslett, 1999). Some of these alterations are important for neutrophil function (Dransfield et al., 1995). CD11b and CD11c are increased on apoptotic neutrophils (but may be functionally inert), but CD15, CD43, CD62L, CD35, CD11a are decreased. CD66 expression is maintained (Dransfield et al., 1995). Other surface changes may be important for recognition of apoptotic neutrophils by macrophages, so that phagocytosis can occur and the apoptotic neutrophils can be "mopped up" before the onset of secondary necrosis.

a) CD16

CD16 (Fcγ receptor III) mediates the binding of immunoglobulin opsonised particles to phagocytes (Dransfield et al., 1994). Neutrophil apoptosis is accompanied by proteolytic cleavage of CD16 from the cell surface (Dransfield et al., 1995). Not all CD16 is removed and the CD16 remaining on CD16 low apoptotic cells is the portion that is relatively resistant to protease cleavage (Dransfield et al., 1994). Levels of CD16 expression can therefore be used to define apoptotic and non-apoptotic cells (Dransfield et al., 1994) as the number of CD16 low cells strongly correlates with the number of apoptotic cells in culture.

b) Phosphatidylserine

Phosphatidylserine (PS) is a lipid normally confined to the inner leaflet of the plasma membrane. Its externalisation is early and widespread in apoptosis, regardless of the apoptosis initiating stimulus (Martin et al., 1995). The externalisation of PS has been proposed to occur during apoptosis as a result of an increase in intracellular calcium which activates the scramblases. Also, aminophospholipid translocase activity is thought to be suppressed by the increased calcium concentration, with the overall result that PS equilibrates between the inner and outer leaflets of the plasma membrane (Vance and Steenbergen, 2005). Changes in PS localisation can be

detected before the morphological changes of apoptosis can be seen by microscopy. The externalisation of PS appears to be one of the signals by which apoptotic cells are recognised and subsequently removed by phagocytes (Vance and Steenbergen, 2005).

Apoptotic neutrophils would normally be rapidly ingested by macrophages before losing membrane integrity. In the absence of macrophages or in conditions where macrophages do not adequately recognise apoptotic cells (cations, low pH, autoantibodies) or where macrophage clearance pathways are overwhelmed by large numbers of apoptotic cells, secondary necrosis will occur leading to liberation of the neutrophil contents and local tissue damage (Haslett, 1999).

1.8. Mononuclear phagocytes

1.8.1 Monocytes

Monocytes appear in the circulation as early as 20 weeks' gestation (Forster-Waldl et al., 2005). They can be rapidly recruited to the lung when required, usually following neutrophil influx. Monocytes are able to phagocytose cell debris and inflammatory products (Rosseau et al., 2000b, Gordon and Read, 2002). They can produce pro-inflammatory cytokines, ROS and proteolytic enzymes and may contribute to acute and chronic lung inflammation (Maus et al., 2002a). Monocytes have an important role in T-cell and macrophage activation via cytokine signalling (Gordon and Read, 2002) and may be important in the alveoli even in the absence of inflammation to expand the alveolar macrophage (AM) pool (Maus et al., 2002a).

The process and interactions of monocyte trafficking across the pulmonary endothelial/alveolar epithelial barrier *in vivo* are poorly characterised, when compared to what is known about neutrophil migration (Maus et al., 2002a). What is known is that the monocyte response is dependent largely on CD11b/CD18 on monocytes and ICAM-1 on pulmonary epithelial and endothelial cells (Maus et al., 2002b). Peripheral blood monocytes which are precursors of alveolar macrophages have an immature monocyte-like immunophenotype and may have augmented release of pro-inflammatory mediators (Rosseau et al., 2000a). In addition, recruited monocytes have increased CD14 expression when compared to peripheral blood monocytes and have greater TNF-α response to LPS (Maus et al., 2002b).

Monocytes from newborn infants have reduced capacity to phagocytose apoptotic cells (Kramer et al., 2003) which may be important in the persistence of neutrophils in CLD. There may be some significant differences between adult and infant monocytes which may play a role in the pathogenesis of CLD, for example H_2O_2 production in response to LPS in preterm and term sheep monocytes is reduced and delayed and monocytes from preterm infants have decreased HLA-DR expression (Hallwirth et al., 2004). CD14 expression on monocytes has been shown to be the same as adults (Forster-Waldl et al., 2005) but, paradoxically, also been shown to be gestation-dependent (Gengenbacher et al., 1998).

1.8.2 Alveolar macrophages

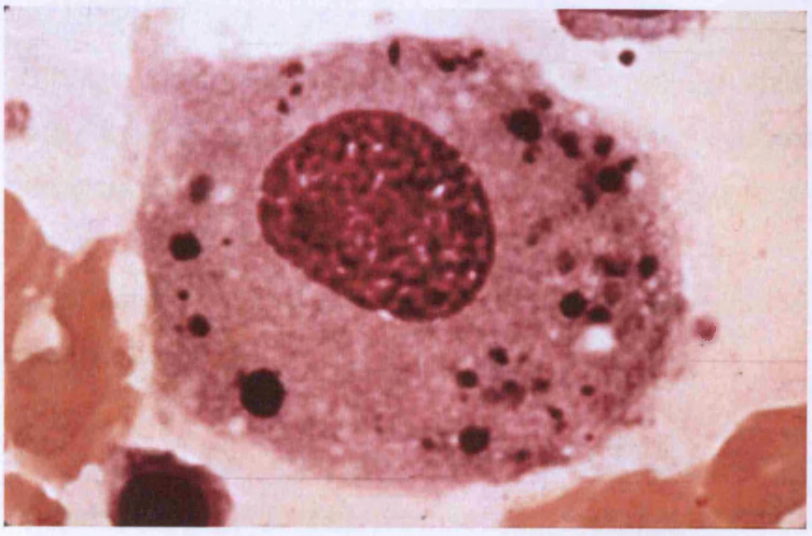

Fig 1.5 An alveolar macrophage by light microscopy. Image from Ohio State University Department of Pathology (www.pathology.med.ohio-state.edu)

Alveolar macrophages (AM) originate in the bone marrow as monocytes from haemopoetic precursor cells (Gordon and Read, 2002) and are the major resident population of immunocompetent cells in the lower respiratory tract. AM are resident lung phagocytes but are relatively poor antigen presenting cells. However they are able to release an array of inflammatory mediators (oxygen radicals, proteases, arachidonic acid metabolites and cytokines) and are thus implicated in the pathogenesis of lung injury (Maus et al., 1998). Resident macrophages in the peritoneum live for about 2 weeks in an uninflamed state (Bellingan et al., 1996).

Monocytes from the circulation enter the fetal lung in the third trimester in response to MCP-1, specific for the recruitment of mononuclear leucocytes, and mature to tissue macrophages under the influence of local factors (type II alveolar cells, bronchial epithelium, cytokines, surfactant) (Gordon and Read, 2002, Rosseau et al., 2000a). Most only differentiate into macrophages after term birth (Alenghat and Esterly, 1984, Bellanti et al., 1979, Kramer et al., 2003). The presence of AM in the infant lung correlates mainly with the length of postnatal survival and the presence of pulmonary lesions. Gestational age shows some correlation in post-mortem studies but is not thought to be a major influence on the presence of AM as they can be seen

as early as 20 weeks' gestation if an infection such as congenital pneumonia is present (Alenghat and Esterly, 1984).

Alveolar macrophages account for 95% of the cell burden in healthy lung BAL fluid, with the remaining cells being lymphocytes (Gordon and Read, 2002). Macrophages which are present in the lung in acute inflammation have been found to be monocytic in origin, rather than as a result of proliferation of resident tissue macrophages which are terminally differentiated and not able to proliferate (Rosseau et al., 2000a, Van Furth et al., 1973).

Arnon *et al* (Arnon et al., 1993) showed that infants who progressed to CLD had fewer macrophages in BAL fluid on day 5 and 7 than infants whose RDS resolved. Macrophages peaked at around day 4 in other studies and persisted in the lung in CLD infants (Ogden et al., 1984, Clement et al., 1988).

1.8.3 Macrophage recognition of apoptotic cells

The final step in the process of apoptosis is the phagocytosis of cell "corpses". The uptake of senescent neutrophils by macrophages was recognised by Metchnikoff in 1891 but the process of apoptotic cell removal by professional phagocytes is complex and remains incompletely described at the molecular level. It has 2 central elements, namely

- recognition ("eat me")
- engulfment/phagocytosis (Lauber et al., 2004).

Neutrophil uptake by macrophages occurs via a number of specific recognition mechanisms, with different signals mediating recognition and engulfment (Lauber et al., 2004).

The process of apoptosis leads to recognition of apoptotic cells by macrophages (Savill et al., 1989b). This recognition process is complex (Lauber et al., 2004, Savill et al., 2002) and there are many possible receptors on the phagocyte for many different markers on apoptotic cells (Giles et al., 2000). Macrophage recognition of apoptotic neutrophils is different to recognition for red blood cells or apoptotic

thymocytes (Savill et al., 1989a). Macrophage receptors for opsonins (Fc, C3b, iC3b) or for advanced glycosylated end products of proteins or N-acetylglucosamine-specific lectin may not be used in apoptotic neutrophil recognition (Savill et al., 1989b). Apoptotic cells may be at different stages of apoptosis, so different mediators of phagocytosis in early and late apoptosis may be required (Giles et al., 2000). Recruited monocytes may also have to undergo a maturation process before they are able to recognise and remove apoptotic neutrophils as macrophages (Newman et al., 1982).

PS is a well known "eat me" signal but the underlying mechanism of this signal to the macrophage is incompletely understood. PS is recognised by several different receptors, possibly including a specific PS receptor (Fadok et al., 2000) but may bind to it via a bridging molecule. It has been shown that the externalisation of PS alone is insufficient for phagocytosis by macrophages *in vitro* (Devitt et al., 2003). The mechanism of recognition of apoptotic cells via PS may vary with different macrophage populations (Fadok et al., 1992).

Phagocytosis via the PS receptor pathway produces an increase in TGFβ-1. TGFβ-1 is multi-functional and modulates diverse cellular activities e.g. inhibits growth and differentiation of many cell types and regulates inflammatory response (Huynh et al., 2002).

There are a number of other possible signals which mediate apoptotic neutrophil recognition by macrophages (Savill et al., 1990, Lauber et al., 2004), including (Figure 1.6):

- The thrombospondin (TSP) receptor (CD36, a class B scavenger receptor) is an 88kD macrophage surface molecule. It acts as a receptor for a wide range of cells and molecules, including thrombospondin 1, and is synthesised and secreted by neutrophils and macrophages in culture (Savill et al., 1992). Macrophage synthesised thrombospondin mediates neutrophil/macrophage interaction by forming a molecular bridge between the cells. TSP binds to CD36 and to the vitronectin ($\alpha_v\beta_3$) receptor (Savill et al., 1992). This recognition process appears to be dependent on Ca++ and Mg++ for the interaction to occur. CD36 may also be able to work independently of the

vitronectin receptor because transfecting CD36 into non-professional phagocytes induces the ability to phagocytose apoptotic neutrophils (Ren et al., 1995)

- some resembling oxidised LDL,
- sites to bind C1q and C3b/bi,
- collectin binding sites e.g. for MBL, SP-A, SP-D,
- CD91,
- ABC-1 (ATP binding cassette 1 transporter),
- class A scavenger receptor and
- CD 14 is a 55kDa glycoprotein cell surface receptor and differentiation marker (Ziegler-Heitbrock and Ulevitch, 1993). It functions as a receptor for LPS and is found on mononuclear phagocytes and activated neutrophils (Hasday et al., 1997). CD14 is capable of interactions with phospholipids and could function as alternative PS receptor but does not appear to do so (Devitt et al., 2003).

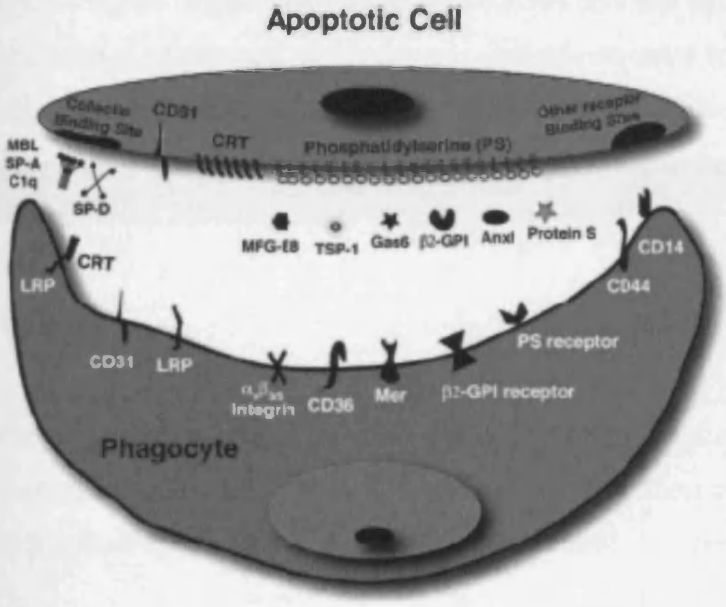

Figure 1.6 Diagram showing the numerous possible signals which mediate interactions between apoptotic cells and phagocytes (Vandivier et al., 2006).

Phagocytosis clears dead neutrophils but could also initiate a signal to macrophages to begin the process of egress from the inflamed site to draining lymphatics and lymph nodes (Bellingan et al., 1996, Serhan and Savill, 2005).

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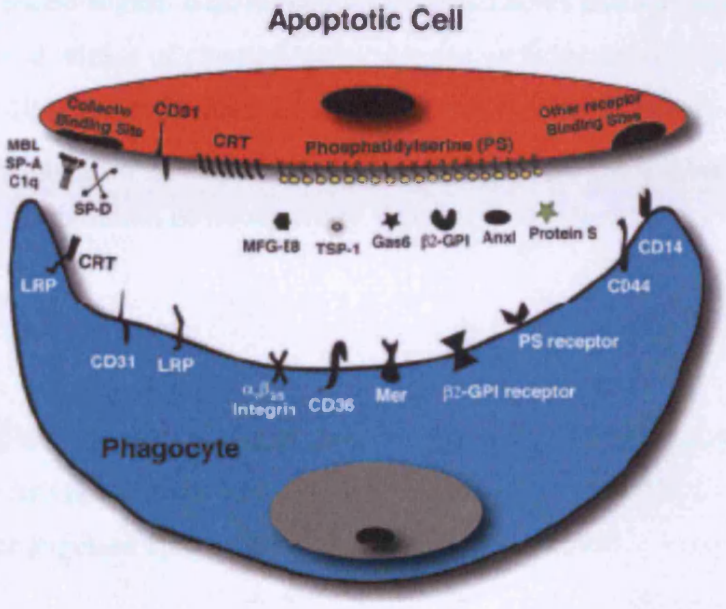

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1.8.4 Rate/regulation

Recognition and ingestion of neutrophils occurs very rapidly. *In vitro*, neutrophils can be phagocytosed by macrophages and digested to the point of being unable to be assessed morphologically within 30 minutes (Savill et al., 1989a).

The rate of macrophage ingestion of apoptotic cells can be altered. Macrophage clearance of apoptotic cells can be enhanced by various early inflammatory cytokines and by corticosteroids and CD44 ligation (Haslett, 1999). Surfactant protein A (SP-A) and, to a lesser extent, surfactant protein D (SP-D) accelerate alveolar macrophage phagocytosis of apoptotic neutrophils (Schagat et al., 2001).

Macrophage recognition of apoptotic cells is inhibited by cationic monosaccharides, amino sugars, heparin and basic amino acids and low pH. Changes in interstitial pH and release of charged molecules are well documented in inflammation – this could delay macrophage uptake of apoptotic neutrophils and potentially lead to neutrophil disintegration during secondary necrosis and thus persistence of inflammation and exacerbation of tissue injury (Haslett, 1999, Savill et al., 1989b).

1.8.5 Outcome

Phagocytosis usually provokes a marked pro-inflammatory response from macrophages, however, macrophages do not have their usual inflammatory response to ingested apoptotic neutrophils (Haslett, 1999).

Phagocytosis of apoptotic neutrophils results in:

- Suppression of release of pro-inflammatory agents like IL-1β, GM-CSF, IL-8, LT C4 and TNF-α (Fadok et al., 1998). ThromboxaneB2 release is suppressed to lower than normal background level (Meagher et al., 1992).
- An increase in release of anti-inflammatory factors like IL-10, TGFβ, prostaglandinE_2 and PAF (Fadok et al., 1998).
- Release of FasL from monocytes and macrophages and thus further Fas-mediated apoptosis of neutrophils (Brown and Savill, 1999).

Macrophages retain their ability to liberate pro-inflammatory cytokines after apoptotic neutrophils are phagocytosed and digested, thus the "anti-inflammatory" effect of ingesting apoptotic neutrophils is dependent on the macrophage recognising that the neutrophil it is about to ingest is apoptotic, not as a result of the apoptotic particle itself. Ingestion of opsonised apoptotic neutrophils is able to cause a pro-inflammatory reaction (Meagher et al., 1992).

1.9 Neutrophil apoptosis in adult respiratory disease

Adult/Acute Respiratory Distress Syndrome (ARDS) is considered to be similar to neonatal RDS in some respects, particularly as neutrophil apoptosis is thought to play a significant role in its pathogenesis and patient outcome.

Matute-Bello *et al* (Matute-Bello et al., 1997) investigated neutrophil apoptosis in 34 ARDS patients as well as 13 patients at risk for ARDS and a group of healthy adult controls. Neutrophil apoptosis was suppressed in ARDS and this effect was maximal in early disease but there was no significant difference in patients who survived compared to those who died from the disease. They also showed that BAL fluid was anti-apoptotic to human neutrophils and that apoptosis suppression is mostly mediated by anti-apoptotic cytokines, of which G-CSF and GM-CSF were most important in prolonging neutrophil survival.

Rosseau *et al* (Rosseau et al., 2000a) studied 49 ARDS patients by repeated BAL sampling. The first BAL sample on each patient showed an increase in total cell count with massive neutrophil influx and an overall expansion of the alveolar macrophage population due to peripheral blood monocytes being recruited to the alveolar compartment. Sequential samples showed increasing macrophage counts and neutrophil counts decreased only slightly. They found two sub-groups of patients - one in which there was a transition to a mature macrophage phenotype and another which had prolonged predominance of immature monocyte-like macrophages. There was a clinical correlation with improved oxygenation index, lung function and survival in the mature macrophages group.

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease of adult lungs. Exacerbations of COPD are associated with viral or bacterial infection and airway inflammation, accumulation of neutrophils in the bronchial tree, tissue damage and bronchial obstruction. Increased neutrophil accumulation in COPD has been related to prolonged neutrophil survival/decreased apoptosis, similar to events in ARDS or bacterial pneumonia (Droemann et al., 2000, Matute-Bello et al., 1997).

Pletz *et al* (Pletz et al., 2004) studied 36 COPD patients and 10 healthy non-smoking volunteers and showed reduced spontaneous apoptosis in peripheral blood neutrophils during exacerbations of COPD and an increased rate during recovery.

1.10 Neonatal vs. adult neutrophils

There is a well recognised developmental immaturity of the neonatal immune system as evidenced by several studies on umbilical cord blood samples (Bortolussi et al., 1993, Koenig et al., 1996).

Neutrophils from term newborn infants have shown defects in:

- adherence (of which CD11b/CD18 is an important mediator),
- transendothelial migration (Anderson et al., 1990),
- chemotaxis (marked),
- phagocytosis and oxidative metabolism and
- non-oxygen dependent bactericidal activity (Levy et al., 1999)

All of which may contribute to the increased susceptibility of newborns to infection.

CD11b is part of the integrin family and mediates cell adhesion to the endothelium (Koenig et al., 2005). It is highly expressed on neutrophils and less is found on monocytes/macrophages. CD11b in combination with CD18 forms CR3, a receptor for C3bi. Both quantitative and qualitative defects in CD11b have been described in neonatal neutrophils. CD11b has been found in equivalent amounts on adult neutrophils and those of vaginally delivered neonates but is lower in babies born by Caesarean section (Nupponen et al., 2002a, Molloy et al., 2004, Abughali et al., 1994). However, there are also contradictory reports that non-apoptotic neutrophils from cord blood express more CD11b than adult neutrophils but that the total cell

content of CD11b/CD18 is lower in neonates (Koenig et al., 2005) (Abughali et al., 1994). Both term and preterm infants have significantly less upregulation of CD11b in response to stimulation (Anderson et al., 1987, Bruce et al., 1987, Nupponen et al., 2002a). Upregulated CD11b may be protective against Fas-mediated apoptosis (Watson et al., 1997, Coxon et al., 1996).

Neonatal neutrophils have deficiencies in specific granule number and also possibly abnormal morphology of specific granules (Ambruso et al., 1984). Neonatal neutrophils have diminished lactoferrin (from specific granules) but release of lactoferrin on stimulation is comparable to adults (Ambruso et al., 1984, Anderson et al., 1987). Azurophilic granules in neonates contain less BPI (bactericidal/permeability increasing protein), which has a high affinity for LPS, than adults and preterm neonates and have a lower capacity to release BPI than term infants or adults (Levy et al., 1999, Nupponen et al., 2002b) - this may be a reason for the increased susceptibility of newborns to Gram negative infections. However, large variations between amounts of BPI present in neonatal neutrophils have been noted (Levy et al., 1999). Neonatal neutrophils release very similar amounts of MPO (Nupponen et al., 2002a, Levy et al., 1999) from azurophilic granules when compared to adults. MPO and BPI both occur in the azurophil granules, so low BPI is probably not due to a problem with its release from the granule.

The oxygen-dependent killing ability of neonatal neutrophils shows some conflicting data and ROS killing capacity may be increased or decreased in neonates. Nupponen *et al* showed that preterm infants and infants with proven sepsis have reduced ROS (Nupponen et al., 2001) but there is enhanced release of ROS in non-apoptotic cells of cord blood compared to adult after 24 hours in culture (Koenig et al., 2005). This may be an important factor in tissue injury.

Cord blood neutrophils may have less capacity to undergo apoptosis than adult neutrophils (Allgaier et al., 1998, Luo et al., 2003). Several recent studies have shown reduced or delayed apoptosis, both spontaneous and induced, in neonatal compared to adult neutrophils (Oei et al., 2003, Molloy et al., 2004, Hanna et al., 2005, Allgaier et al., 1998). The reasons for this delay in apoptosis are unclear but there are a number of possible causes:

- Lower levels of pro-apoptotic proteins in neonates (Bak, Bax, Bad) (Hanna et al., 2005).
- Reduced pro-caspase 3 and caspase 3 amount and activity in neonates (Hanna et al., 2005, Luo et al., 2003), but similar levels of caspases 1 and 8.
- Caspase 9 may also be reduced in neonates (Molloy et al., 2005).
- FasL appears to be reduced in neonates (Hanna et al., 2005) although there are similar amounts of Fas on the surface of neonatal and adult neutrophils (Allgaier et al., 1998). Anti-Fas antibody did not induce apoptosis significantly in neutrophils of infants born by Caesarean section whereas adult neutrophils and neutrophils from vaginally delivered neonates had a significant increase in apoptosis with Anti-Fas antibody (Hanna et al., 2005, Molloy et al., 2004, Allgaier et al., 1998).
- Molloy *et al* (Molloy et al., 2004) showed that spontaneous neonatal neutrophil apoptosis is delayed in infants born by Caesarean section and further delayed if an infant is vaginally delivered. Labour may thus cause "priming" of neutrophils and prolong their survival (Weinberger et al., 2007). Neonatal neutrophils may have altered responses to extracellular stimuli. For example, G-CSF (and not GM-CSF) further delays apoptosis in neonatal neutrophils. Both substances delay it in adults. In neonates at high risk for infection, neither substance delays apoptosis (Molloy et al., 2005). GM-CSF increases CD11b expression and ROS production but G-CSF has no effect (Molloy et al., 2005) possibly because there are reduced G-CSF receptors on neonatal neutrophils (Gessler et al., 1999).

However not all studies have shown a delay in neonatal neutrophil apoptosis. Uguz *et al* (Uguz et al., 2002) found increased Fas expression on neonatal neutrophils (following Caesarean delivery) compared to adult neutrophils and more rapid apoptosis in neonatal neutrophils.

Apoptotic neutrophils have diminished functional capacity. The function of surviving neutrophils is more important in disease processes. Koenig *et al* (Koenig et al., 2005) showed that neonatal neutrophils with prolonged survival exhibited enhanced inflammatory and cytotoxic responsiveness with more CD11b expressed and enhanced release of ROS compared to surviving adult neutrophils.

1.11 Neutrophil apoptosis in chronic lung disease of prematurity

Grigg *et al* (Grigg et al., 1991) produced the first report of neutrophil apoptosis and removal by macrophages in neonatal lungs. They studied 8 babies - five <34 weeks' gestation who were ventilated for RDS and three >34 weeks' gestation without RDS. Intact neutrophils were identified within macrophages in BAL fluid by detecting MPO on cytospins and by electron microscopy. The neutrophils showed features of apoptosis by light microscopy. They questioned the pathophysiological relevance of this process in neonatal lung inflammation as their findings did not correlate with clinical outcome.

In a study of 52 babies, 23 of which were preterm and ventilated for RDS (Oei et al., 2003), twice weekly tracheal aspirates were performed. Cell counts from the aspirates were lowest in preterm infants who did not progress to CLD and highest in term infants. All the samples were neutrophil predominant but the lowest proportion of neutrophils was in preterm infants who did not progress to CLD. Term babies had the highest proportion of apoptotic neutrophils in the first week, significantly more than all the preterm infants or the CLD group alone. There were significantly fewer apoptotic neutrophils in the first 4 days in the CLD group than in the RDS-only group or in term infants. They concluded that neutrophil apoptosis increases with gestational maturity and thus preterm babies may be more at risk of lung injury because of reduced neutrophil apoptosis. They also studied IL-10 in all the aspirate samples but found no correlation between IL-10 and apoptosis in any group.

Kotecha *et al* (Kotecha et al., 2003) studied 134 BAL samples from 32 infants who were ventilated for RDS. They found that the development of CLD is associated with persistent neutrophilia in BAL samples with the difference between RDS and CLD apparent at day 10. Macrophage counts were significantly higher in the RDS group on day 4. The percentage of apoptotic neutrophils in the BAL samples increased from 1% on day 1 to 17% on day 7 in RDS but fell from 4.1% on day 1 to 0.7% at 7 days in the CLD group. The differences were statistically significant on day 7. In other words, increased survival may be the mechanism of neutrophil persistence in CLD. They also studied BAL fluid apoptotic activity against adult neutrophils and found RDS BAL fluid was significantly pro-apoptotic on days 1 and 2, which was not seen in BAL of

babies who progressed to CLD. They also looked at GM-CSF, TNF-α and IL-10 but found no correlation between the levels and the amount of neutrophil apoptosis on day 1.

In apoptosis, cytochrome c released from mitochondria triggers cleavage of pro-caspase 9 which leads to activation of caspase 3 (Hanna et al., 2005). Caspase 3 levels in tracheal aspirate fluid (TAF) were studied in 27 ventilated preterm infants with RDS, 14 of whom developed CLD (Cheah et al., 2005a). No statistically significant relationship was found between caspase 3 activation and the development of CLD. The role of caspase 3 in the differences between neonatal and adult neutrophil apoptosis is still unclear.

1.12 Summary

The progression of RDS to CLD, rather than its resolution, is a cause of significant morbidity and mortality among infants born at preterm gestations. CLD is thought to be a process of lung maldevelopment, predisposed to by ventilation, hyperoxia and pulmonary and systemic infection in the preterm infant. These risk factors all play a role in the generation of an innate inflammatory response in the lung, in which neutrophils are the predominant cell type. This neutrophilic inflammation needs to resolve by a process of neutrophil apoptosis and ingestion of the effete neutrophils by macrophages in order for RDS to resolve. It has been postulated that a failure of this resolution process is an important factor in the development of CLD.

It has previously been shown that apoptosis in neonatal neutrophils may be significantly delayed when compared to adults but the reason and mechanism for this remain unclear.

1.13 Aims and hypotheses

This project aims to describe the cellular component of BAL fluid from preterm infants who have RDS which resolves and compare this with preterm infants who develop CLD and with term infants who have no pulmonary pathology, using flow cytometry, a novel technique for this type of sample in this population of patients.

1. I aim to characterise the BAL cells in terms of their cell type (neutrophil or macrophage) and then further characterise the different cell types according to their cell surface markers. The differences in BAL cells between infants in whom microbial infection or colonisation is detected will be described. I hope to be able to identify a factor or characteristic of the BAL cells which may predict the later development of CLD.
2. I will study macrophage surface markers as I hypothesise that a difference in these markers may highlight an altered ability to interact with and ultimately phagocytose apoptotic neutrophils, contributing to the pathogenesis of CLD.
3. I will determine the proportion of apoptotic cells in each BAL sample as well as an indication of the apoptotic activity of the BAL supernatant and compare this between groups, seeking a relationship between, in particular, neutrophil apoptosis and the pathogenesis of CLD,
4. I will attempt to understand the relationship between the different BAL cellular components and some of the inflammatory substances present in the BAL supernatant and the development of CLD as well as some of the relationships between the BAL supernatant, neutrophil apoptosis and the presence of microbial infection or colonisation.
5. In light of the association between infection and the development of CLD and the controversy in neonatal circles regarding the role of *Ureaplasma* in this disease, I will attempt to relate BAL cell and supernatant findings to the presence of micro-organisms in BAL samples.
6. It is probable that neutrophil apoptosis is delayed in newborn infants and I aim to confirm this and compare it between infants and adults, using umbilical cord blood, before going on to investigate possible mechanisms for any dysregulation of apoptosis in the newborn. This dysregulation of apoptosis may occur as a result of:

- neutrophil factors, which will be investigated in BAL samples and in cord blood,
- dysfunctional macrophage recognition or phagocytosis of apoptotic neutrophils, and macrophage surface markers will be investigated in BAL,
- the inflammatory environment in which the neutrophils find themselves, leading to analysis of some of the constituents of BAL supernatants, particularly in relation to apoptosis.

Chapter 2

Materials and Methods

Chapter 2
Materials and Methods

2.1 Chronic Lung Disease of Prematurity study

2.1.1 Patient Group

Patients were recruited in the regional neonatal intensive care unit at the University Hospital of Wales, Cardiff between February 2006 and June 2008.

Two groups of infants were recruited:

- Preterm infants (< 32 weeks' gestation) who required mechanical ventilation were recruited within the first 12 hours of life. This group was later subdivided into those infants who went on to develop CLD (oxygen dependence at 36 weeks' corrected gestation) and those infants whose RDS resolved.
- Term control infants (> 37 weeks' gestation) who required mechanical ventilation for non-respiratory reasons (for example infants ventilated peri-operatively for gastroschisis).

I excluded (did not recruit) any infant ventilated for hypoxic ischaemic encephalopathy, those with known chromosomal abnormalities and infants who were so unwell that they were extremely likely to die or be unlikely to tolerate the lavage procedure.

Parents whose infants met the study criteria were approached either prior to, or shortly after, delivery and invited to participate in the study. The study was explained to them and they were given an information leaflet about the study (appendix 1) before fully informed, written consent was obtained.

The study was approved by the Cardiff and Vale NHS Trust Research and Development committee and the South East Wales Research and Ethics Committee (Reference number 05/WSE04/85, 7th September 2005).

2.1.2 Bronchoalveolar lavages

Infants requiring mechanical ventilation regularly have endotracheal suction performed as part of their routine care. Bronchoalveolar lavage (BAL) is a well established clinical and research technique that is safe and well tolerated (Grigg et al., 1992, Shields and Riedler, 2000) in mechanically ventilated neonates (Vyas et al., 2002), provides reproducible results and no long term adverse effects (Kotecha, 1999, Vyas et al., 2002).

For the purpose of this study, BAL was performed daily for the first week of life and then twice weekly until the infant was 28 days old or until the infant was extubated, whichever occurred first. Preterm infants in the neonatal unit in Cardiff routinely receive exogenous surfactant therapy at birth and again at 12 hours of age. In order to minimise any wash out of surfactant, the first BAL was performed at 12 hours of age, immediately prior to the administration of the second surfactant dose. Timing of subsequent lavages was co-ordinated daily with the nursing staff on the neonatal unit in order to replace the routine endotracheal suction and avoid extra suctioning procedures being performed on the infant. If an infant was judged too unwell by the attending clinician to tolerate a BAL, the procedure was withheld.

Bronchoalveolar lavages were performed using the guidelines set out by the European Respiratory Society task force on BAL in children (de Blic et al., 2000). The procedure was performed with the infant lying supine and the head turned to the left, thus increasing the likelihood of the suction catheter being introduced into the right lower lobe. If the infant was conventionally ventilated, the ventilator was briefly disconnected and a size 6 French Gauge (FG) catheter was gently introduced down the endotracheal tube until resistance was felt. If the infant was receiving high frequency oscillatory ventilation (HFOV) the procedure was carried out using the in-line suction port to avoid the need to disconnect the ventilator. Once resistance to further advancement of the catheter was felt, 1 ml/kg of 0.9% saline (up to a maximum of 2 ml) was instilled via the catheter. The catheter was then connected to 8-12 kPa of suction pressure and the lavage fluid was suctioned back and collected in a suction trap as the catheter was withdrawn. The ventilator was then reconnected and the infant's heart rate and saturations, which may have dipped during suction, were

allowed to return to normal before the procedure was repeated a second time. The BAL fluid was pooled from the two lavages, placed on ice and immediately transported to the laboratory for analysis.

2.1.3 Processing of bronchoalveolar lavage samples

The sample was transported on ice from the patient's bedside to the laboratory within 10 minutes of collection. An aliquot of 25 µl was taken from the BAL sample at this stage for culture for *Ureaplasma* (see 2.1.6 below). The sample was placed in a pre-cooled centrifuge (Jencons, Leighton Buzzard, UK) and centrifuged at 4°C at 1 000 x*g* for 10 minutes, resulting in the sample being separated into a cell pellet and supernatant. The supernatant was carefully removed and stored as 25 µl aliquots at -80°C for later analysis.

The cell pellet was re-suspended in 1 ml of phosphate buffered saline (PBS) with 5 mM EDTA. In order to break up the thick mucus present in most of the lavage samples, 100 µl of 50 µg/ml dithiothreitol (DTT), a reducing agent, was added to the resuspended cell pellet and incubated for 15 minutes at room temperature. A cell count was performed on the re-suspended cells using a haemocytometer. A portion of the sample was mixed with an equal volume of trypan blue (Invitrogen, Paisley, UK) prior to counting, in order to facilitate counting and gain information about cell viability.

2.1.3.1 Use of DTT as a mucolytic

a) Effect of DTT of neutrophil surface antigens at different concentrations

A sample of 15 ml of peripheral venous blood was drawn from an adult volunteer and placed in a tube containing 1.5 ml of 3.8% sodium citrate (Martindale Pharmaceuticals, Romford, UK). The blood was then mixed with 3 ml of 6% dextran 70 (Baxter Healthcare Ltd, Thetford, UK) and left to stand at room temperature for 40 minutes. This allowed the red blood cells to sediment to the bottom of the tube, leaving a clear interface between an upper layer of serum containing leucocytes and a lower layer containing red blood cells. The leucocyte portion was carefully aspirated

from the red blood cell layer and placed in a sterile universal container. The leucocytes were then centrifuged at 400 x*g* for 5 minutes, washed twice in 20 ml of flow cytometry (FACS) buffer and then resuspended in FACS buffer to a given volume. The resulting suspension was divided into two equal aliquots. To one aliquot, DTT was added at a concentration of 50 μg/ml and to the other, an equal volume of PBS (diluent for DTT) was added. Both tubes were left to stand at room temperature for 15 minutes.

Each sample was then centrifuged at 400 x*g* for 5 minutes and washed twice in 20 ml of FACS buffer to remove any trace of DTT. Thereafter each sample was counted on a haemocytometer and resuspended to a density of 10^6cells/ml using FACS buffer.

Cells were then placed into a round-bottomed 96 well plate (Nunc brand, Fisher Scientific, Loughborough, UK) (10^5cells/well) and prepared for flow cytometry as described in 2.1.4.1 (below). Wells were allocated for each isotype control antibody and for each surface marker that I intended to use. Each antibody was tested in duplicate and the experiment was performed on 3 occasions.

A further, similar set of experiments were performed, using concentrations of DTT of 0, 5, 25, 50, 100 and 200 μg/ml.

2.1.3.2 Cytospins

At least 2 cytospins per BAL sample were made from the re-suspended cells, using a Cytospin 3 (Shandon, Runcorn, UK). A volume of 50 μl of cells resuspended in FACS buffer at a density of 0.5 x 10^6 cells/ml was added to the cytospins chamber and spun at 300 rpm for 4 minutes. The cytospins were air dried for at least 24 hours and then fixed in methanol and frozen at -20^0C. The cytospins were defrosted and stained with Diff-Quik (Medion Diagnostics, Limerick, Ireland) before a differential cell count was obtained by counting at least 300 cells per sample under direct vision with a high powered light microscope. Counting for the neonatal arm of the study was performed by Miss Sharon Gill, laboratory technician in the Department of

Respiratory Medicine at the University of Sheffield, who was blinded to the clinical condition of the children.

2.1.4 Analysis of bronchoalveolar lavage cell pellet

2.1.4.1 Preparation of samples for cell phenotype analysis by flow cytometry

Following the haemocytometer count, the remaining cells were washed in 20 ml flow cytometry buffer (PBS containing 0.1% sodium azide, 1% bovine serum albumin, 5% fetal calf serum and 5 mM EDTA) to remove the DTT and then resuspended to a density of ~10^6 cells/ml in flow cytometry buffer

Using a round bottomed 96 well plate, 100 µl of the cell suspension was added to each well, including wells for all antibody staining as well as controls. The number of cells obtained from the lavages varied greatly and the combinations of antibodies which could therefore be used was limited by the number of cells in some samples. Three separate templates (appendix 2) were designed to be used, depending on the number of cells obtained from the BAL sample in order to maximise the information obtained but still obtain a standard minimum data set for each lavage.

All antibodies were monoclonal mouse anti-human antibodies, produced commercially for use in flow cytometry and used in appropriate titres (Table 2.1).

Antigen	Antibody clone (supplier)	Isotype	Conjugate	Cells identified
CD11b	ICRF44 (eBioscience, San Diego, CA)	IgG1	Biotin	Monocytes, macrophages, granulocytes, activated lymphocytes
HLA-DR	G46-6 (BD Pharmingen, Oxford, UK)	IgG2a	Allophycocyanin (APC)	Macrophages, monocytes, B cells
CD16	3G8 (BioLegend, San Diego, CA)	IgG1	Phycoerythrin (PE)	Neutrophils, activated monocytes (also Natural Killer (NK) cells and dendritic cells)
CD36	TR9 (BioLegend)	IgG1	PE	Monocytes, Macrophages (also platelets and erythrocytes)
CD15	HI98 (BioLegend)	IgM	APC	Neutrophils
CD14	61D3 (Southern Biotech, Birmingham, AL)	IgG1	Biotin	Monocytes, macrophages, dendritic cells
TLR 2	TL2.1 (eBioscience)	IgG2a	Biotin	Any cell expressing TLR 2
TLR 4	HTA125 (eBioscience)	IgG2a	Biotin	Any cell expressing TLR 4
IgG1 isotype control	(eBioscience)	IgG1	Biotin	
IgG1 isotype control	MOPC-21 (BioLegend)	IgG1	PE	
IgG2a isotype control	(eBioscience)	IgG2a	Biotin	
IgG2a isotype control	(eBioscience)	IgG2a	APC	
IgM isotype control	MM-30 (BioLegend)	IgM	APC	

Table 2.1 Table showing the antibodies which were used, their isotype, the flurophor to which they were conjugated and an indication of the cell type which each antibody could be used to identify, together with the selected isotype controls.

The cells in the plate were then incubated for 30 minutes at 4°C in a blocking buffer containing PBS, 0.1% sodium azide, 1% BSA, 1% heat inactivated normal mouse serum and 5 mM EDTA, to block non-specific antibody binding sites on the cell surface. The cells were then centrifuged at 500 x*g* for 4 minutes and the supernatant removed. Biotinylated antibodies were diluted in flow cytometry buffer at pre-determined optimal concentrations and 80 µl of the appropriate antibody solution was added to each well of the plate. Wells not requiring antibody at this stage received the same volume of flow cytometry buffer alone. The plate was incubated at 4°C for 30 minutes. Following the incubation, cells were centrifuged at 500 x*g* for 4 minutes and then washed 3 times using 200 µl of flow cytometry buffer. Secondary fluorescent conjugated reagents or directly conjugated antibodies, again diluted to pre-determined optimal concentrations in 80 µl of flow cytometry buffer, were added to the cells and a further incubation at 4°C for 30 minutes followed. Thereafter, cells were again washed 3 times and resuspended in 200 µl flow cytometry buffer. Samples were then transferred to test tubes ready for immediate FACS analysis.

2.1.4.2 Preparation of samples for apoptosis analysis by flow cytometry

Cells obtained by BAL for apoptosis analysis were stained using phycoerythrin-conjugated Annexin-V (MBL International, Woburn, MA) which binds to exposed phosphatidyl serine on the cell membrane of apoptotic and necrotic cells and To-Pro-3 (Invitrogen, Paisley, UK) which binds to DNA exposed in cells undergoing necrosis.

Cells were prepared in a similar way to those for phenotypic analysis. After the initial blocking step, cells were incubated in flow cytometry buffer for 30 minutes at 4°C and then washed three times in Annexin-V binding buffer (AVBB) (BD Pharmingen, Oxford, UK) to remove any EDTA containing flow cytometry buffer. Annexin-V binding to phosphatidylserine is a calcium dependent process which would be prevented by the presence of EDTA. An appropriate, pre-determined, volume of Annexin-V-PE was diluted in AVBB (5 µl of Annexin-V-PE per 100 µl AVBB) and 80 µl of this solution was added to each appropriate well. As a negative control, Annexin-V-PE was added to AVBB containing 20 mM EDTA which would prevent Annexin-V binding and 80 µl of this solution was added to the appropriate well of the plate. The plate was then incubated at 4°C for 30 minutes before washing three times

in AVBB. The cells were resuspended in either in 100 µl To-Pro-3 solution (diluted 1:30 000 with AVBB) or AVBB, as appropriate, and transfered to FACS tubes, each containing 100 µl Annexin-V binding buffer, for analysis.

2.1.4.3 FACS analysis

All processed BAL samples were analysed on the same Becton Dickinson FACScalibur flow cytometer (Becton Dickinson, Oxford, UK) immediately following staining, regardless of the time of day or night.

All samples had appropriate negative and positive control antibodies included in the staining panel and compensation was adjusted appropriately for each individual sample. A polyhedral gate was drawn in order to exclude cell debris (very low forward and side scatter) and 10 000 gated events were collected for each control and each antibody combination.

In order to ensure consistency between FACS measurements over the duration of the project, Spherotech Ultra Rainbow beads (Spherotech, Lake Forest, IL) were run on the FACS machine from time to time and results compared and any minor variations noted so they could be adjusted for.

FACS data was analysed using Cellquest software (Becton Dickinson, Oxford, UK).

2.1.4 Polymerase chain reaction (PCR) for 16S rRNA

Ribosomes are the cellular organelles that translate messenger RNA into proteins. Eukaryotic ribosomes have a sedimentation of rate of 80 Svedburg (S) units, while prokaryotic ribosomes are highly homologous and have a sedimentation rate of 70S. The 70S ribosome consists of 2 subunits:- a smaller 30S subunit and a larger 50S subunit. The 30S subunit contains a 16S ribosomal RNA (rRNA) transcript associated with 21 bacterial proteins. The 16S rRNA is distinct from the analogous 18S eukaryotic equivalent, but conserved enough amongst prokaryotes that primers can be designed that detect the presence of most bacteria without cross-reacting with eukaryotic host sequences. The presence of 16S rRNA gene is thus indicative of the

presence of bacteria in a sample. Polymerase chain reaction (PCR) may be used to amplify the DNA that codes for 16S rRNA, without concern for RNA degradation, and is used to demonstrate the presence of bacterial DNA within a sample (Lane, 1991).

In order to examine the relationship between bacterial presence in the airways and the development of CLD, 16S rRNA gene PCR was performed on the DNA extracted from any remaining BAL cell pellet after sufficient material had been obtained for flow cytometry analysis. The DNA extraction, PCR and sequencing was performed by Mr Michael Beeton, a PhD student in the department of Child Health in Cardiff.

2.1.5.1 DNA extraction

The DNA extraction was performed using a kit from Qiagen (Crawley, UK) in accordance with the manufacturer's instructions.

The cell pellet was resuspended and the cells dissolved using 0.5 ml of lysis buffer. Equilibration buffer was then added and the cellular debris removed by centrifugation at 15 000 x*g* for 20 minutes at 4°C. The supernatant, containing the nucleic acids, was removed and ice cold isopropanol added, causing the nucleic acids in the sample to precipitate. The mixture was centrifuged at 15 000 x*g* for 30 minutes and this time the nucleic acids formed a pellet. The supernatant was discarded and the nucleic acids were resolublised in 150 µl of lysis buffer and heated for 6 minutes at 60°C. Then 1.35 ml of equilibration buffer was added and any debris removed by centrifugation for 5 minutes at 5 000 x*g*. The supernatant, containing the nucleic acids, was removed and added to the top of an equilibrated Qiagen® tip, which is a small column containing resin beads. In the first run through the column, RNA binds to the column, whilst the DNA passes through and is collected. A wash buffer is added to the column and an elution buffer is used to remove the RNA from the column; this is collected for separate analysis. The collected DNA is added back to the column and, due to a change in the buffer, this now binds to the column. DNA elution buffer is then added to the column and a purer DNA sample is collected. The DNA is then precipitated with isopropanol and centrifuged to give a pellet of DNA. This pellet is washed twice in 70% ethanol before being resuspended in 100 µl of RNase-free water and heated to

60°C for 6 minutes. The extracted DNA was then stored in this state at -20°C until the PCR was performed.

2.1.5.2 16S rRNA gene PCR

The PCR was performed by making a "master mixture" of Taq polymerase (Go Taq, Promega, Southampton, UK), forward and reverse primers consisting of 20 base pairs complementary to 16S rRNA (Forward 5'-3' AGAGTTTGATCCTGGCTCAG, Reverse 5'-3' ACGGCTACCTTGTTACGACTT) (Weisburg et al., 1991) ordered from MWG Biotech, Ebersberr, Germany), deoxynucleoside triphosphates, magnesium (to aid nucleotide binding), buffer and water to a final volume of 20 µl.

In a PCR tube (Starlab, Milton Keynes), 19.5 µl of the master mixture and 0.5 µl of extracted DNA were mixed. Further tubes containing a positive control with known bacterial DNA and a negative control with no DNA were also made up. The PCR tubes were then placed in a thermocycler (Quanta QD96, Quanta Biotech, Byfleet, UK). This heats the mixture to 94°C for one minute to denature the DNA, then cools the sample to 67°C for 40 seconds to allow annealing of the primers to the open strands of DNA and then the sample is heated to 72°C for one minute which is the optimal temperature for the Taq polymerase to extend the primer to a full complementary strand of DNA. Detection of bacterial 16S rRNA genes was optimised by performing 20 such cycles but with each cycle the temperature of the annealing step was reduced by 1°C. A second set of 20 cycles was then performed using 94°C for one minute, 47°C for a further minute and 72°C for 90 seconds. The mixture was kept at 72°C for eight minutes before finally being cooled to 4°C then stored at -20°C until needed.

2.1.5.3 DNA Gel Electrophoresis of PCR products

To visualise amplified DNA and determine the size of DNA product, gel electrophoresis was performed. The gel was made up by adding 1g agarose (Invitrogen, Paisley, UK) to 100 ml of 0.5X Tris borate EDTA (TBE) (Sigma-Aldrich, Irvine, UK) and 1 µl of the intercalating agent, ethidium bromide. The lanes were loaded with samples, positive control, negative control as well as a DNA ladder (Hyperladder 1, Bioline, London, UK) consisting of several strands of DNA of known

length. The gel electrophoresis was run at 130V until the bromophenol blue in the sample loading buffer had moved sufficiently to indicate good separation had occurred (about 30 minutes). The gel was placed under a transilluminator and ethidium bromide which has intercalated into the amplified DNA fluoresces and was captured by a digital camera. Those samples that contain 16S rRNA as well as the positive control have a visible band at 1.2 kbp. Successful extraction of DNA from samples was judged by a second amplification to detect the human mitochondrial cyclo-oxygenase (HMCO) gene using specific primers and cycling conditions. Samples that failed to show either 16S rRNA or HMCO genes were considered indeterminate, while samples that were positive for HMCO but negative for 16S rRNA were deemed to be free of bacterial infection.

2.1.5.4 Sequencing of 16s rRNA genes to identify organisms

Samples that were positive for 16s rRNA genes went on to have the 16s rRNA gene sequenced in order to identify the organism that was present in the lavage (Lane, 1991). If an infant had several samples in which the 16s rRNA gene was detected then the first positive sample and/or the sample closest to the peak BAL cell count was sequenced.

Selected samples had the 16s rRNA gene PCR repeated, as in section 2.1.5.2, and scaled up five fold to yield a sufficient quantity of DNA for sequencing. These were pooled and purified using a Novagen spin-prep PCR clean-up kit ™ (Merck Chemicals, Nottingham, UK), according to the manufacturer's instructions, to remove the nucleotides, primers and enzymes and leave only the amplified 16s rRNA genes. The genes are then fluorescently labelled using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK), according to the manufacturer's instructions and analysed by an ABI Prism 3130xl Genetic Analyzer, (Applied Biosystems). This enabled different organisms to be identified as each has a specific variation in their 16s rRNA gene. Gene sequences were analysed using BLAST (Basic Local Alignment Search Tool) via the National Center for Biotechnology Information, U.S. National Library of Medicine (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.1.6 Detection of *Ureaplasma* in bronchoalveolar lavage samples

In order to further study the controversial association between *Ureaplasma* and CLD, as soon as each BAL sample had been obtained, 25 µl of BAL was placed into 2ml of *Ureaplasma*-specific culture medium (Mycoplasma Experience, Surrey, UK). This culture was incubated at 37°C for 5 days.

Ureaplasma contains the enzyme urease which breaks down urea. This causes a change in the pH of the culture medium, resulting in the orange culture medium turning a clear cherry red indicating a positive culture, usually within 24 hours. Cultures which remained orange were negative. Some cultures became cloudy due to the presence of other microbes which prevented accurate interpretation of any colour change and these samples were subjected to PCR for 16S r RNA and for *Ureaplasma* (see 2.1.5.2 above) in order to obtain an accurate assessment of the microbial colonisation in the sample.

2.1.7 Analysis of bronchoalveolar lavage supernatant

2.1.7.1 Elastase Activity Assay

One of the secondary objectives of this study was to measure enzymatically active elastase that was present in our lavage samples as a marker of "tissue damage potential ". I therefore performed a kinetic activity assay on all the lavage samples. Measurement of the rate of chromogenic conversion of the colourless Suc-Ala-Ala-Pro-Val-pNA substrate to a yellow product is a well established technique for measuring elastase activity and has been used previously in bronchoalveolar lavage samples from both neonatal and cystic fibrosis patients (Birrer et al., 1994, Speer et al., 1993, Sluis et al., 1994).

Stocks of standard human neutrophil elastase (Athens Research and Technology, Athens, GA) were prepared by reconstituting 100 µg of lyophilized elastase powder in a 200 µl solution of 50mM sodium acetate (pH 5.5) and 150 mM sodium chloride.

The 16.9 μM enzyme solution was then divided into 20 μl aliquots and stored at -20°C.

To make up a standard curve, a 20 μl aliquot of elastase was thawed and diluted using activity buffer (0.1 M Tris (Fisher Scientific, Loughborough, UK), 0.5 M Sodium Chloride, pH 7.4, 0.05% Triton X-100) to make up concentrations of 20 nM, 10 nM, 5 nM, 2.5 nM, 1.25 nM, 0.625 nM and 0.3125 nM of neutrophil elastase. These standard dilutions were then added in duplicate to a 96 well plate at a volume of 100 μl per well.

BAL supernatants were thawed and 10 μl from each sample was added to the 96 well plate, again in duplicate, and made up to 100 μl with the activity buffer.

A stock solution of neutrophil elastase-specific chromogenic substrate, Suc-Ala-Ala-Pro-Val-pNA (Bachem, St Helens, UK), was prepared by reconstituting 50 mg of lyophilized powder in 10 ml dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Irvine, UK). The 8.67 mM substrate solution was divided into 1mL aliquots and stored at -20°C.

Prior to use, the substrate was thawed and diluted to a 2 mM concentration using "activity buffer". Each well of the 96 well plate containing sample or standard concentrations of neutrophil elastase received 100 μl of the substrate solution. The kinetic assay was read at 405 nm in a heated (37°C) plate reader (Dynex Magellan Industries, Chantilly, VA) which shakes the plate and takes readings at 1 minute intervals for an hour. Integral Dynex Revelation software calculates concentration of elastase by comparing the rate of substrate conversion in the samples against the standard curve of purified elastase.

Any samples with elastase activity in excess of the standard curve were diluted further and reanalysed. The standard curve profile was examined for its consistency and concordance between duplicate values was also assessed.

2.1.7.2 Cytometric Bead Array (CBA)

The environment in which the BAL cells are found may also have a profound effect on the behaviour of the cells, as well as reflecting products produced by them.

CBA is a technique which uses the ability of flow cytometry to discriminate between particles on the basis of size and color. CBA uses a series of beads with distinct fluorescence intensities which have been coated with specific capture antibodies to simultaneously detect multiple soluble analytes. Each bead in the CBA kit acts as a capture surface for a specific protein, in this case cytokines. A single set of diluted standards is used to generate a standard curve for each analyte so that concentrations of the cytokine can be determined in each sample. I used this technique to measure concentrations of 12 potentially relevant cytokines in BAL samples.

The CBA work was performed in the Central Biotechnology Services at University of Sheffield on my behalf. BAL samples were defrosted and 25 µl of BAL was diluted 1:10 for MCP-1, IL-8, IL-6, MIP-1α, MIP-1β and G-CSF or used undiluted for IL-1, TNF-α, FasL, IL-10, GM-CSF and VEGF, based on a series of optimisation experiments. The samples were then incubated with the antibody-coated beads and the subjected to flow cytometry analysis on a FACSArray machine.

2.1.7.3 Bioassay

In order to assess the pro- or anti-apoptotic activity of BAL supernatants, their ability to induce apoptosis in fresh adult neutrophils was studied, similar to Kotecha *et al* (Kotecha et al., 2003). These experiments were performed by Sharon Gill, laboratory technician at the Department of Respiratory Medicine, Royal Hallamshire Hospital, Sheffield.

Peripheral blood neutrophils from healthy adult volunteers were isolated as described in 2.2.2 below and then resuspended in HBSS at a density of 5 x 10^6/ml. A 50 µl aliquot of the cell suspension was mixed with 25 µl of RPMI containing 10% FCS in each well of a 96 well plate. BAL supernatants were thawed and 25µl of BAL was

added to the neutrophil /RPMI mixture. Each BAL sample was assayed in duplicate at 2 time points and the rate of spontneous apoptosis in the adult neutrophils was assessed by adding 25 μl of 0.9% saline (as this is the fluid in which BAL was originally collected from the infants), instead of BAL supernatant, as a control. The plate was incubated at 37°C in 5% CO_2 for either 5 or 20 hours.

The percentage of apoptotic neutrophils at the outset and at 5 and 20 hours of incubation was assessed by cytospins, made as described in 2.1.3 above and stained with Diff-Quik.

2.2 Peripheral Cord Blood Neutrophil study

2.2.1 Patient Groups

As well as examining neutrophil apoptosis in cells obtained by bronchoalveolar lavage from infants' lungs, I also aimed to gain information on the differences in rates of neutrophil apoptosis between adults and newborn infants.

Blood sampling from healthy term neonates poses a number of ethical concerns and there are also clinical constraints on the volume of blood obtainable. For this reason umbilical cord blood was used as it is normally discarded following delivery of the infant and volumes of up to 40 ml can be obtained relatively easily. Similarly blood sampling from preterm neonates poses even more complex ethical issues. Preterm infants are infrequently born by elective Caesarean section due to the more complex and higher risk nature of this procedure for the mother at preterm gestations. A majority of preterm infants are born following spontaneous onset of preterm labour which may be due to sub-clinical infection in a significant proportion of cases (Gomez et al., 1997, Klein and Gibbs, 2004). The combination of possible infection along with the activating effect of labour on neutrophils (Molloy et al., 2004, Weinberger et al., 2007) made a majority of preterm infants ineligible for our study. For this reason, only term infants born by elective Caesarean section were included in the study.

Healthy pregnant women who were undergoing elective Caesarean section (most frequently because of previous Caesarean delivery) at 37 weeks' gestation or more were asked in advance of delivery whether cord blood could be taken once their infants were born. An information sheet (appendix 1) was given to the women and written, informed consent was obtained.

At the Caesarean section, following delivery of the infant, the placenta is delivered and inspected for completeness by the attending midwife, before it is discarded. Within 5 minutes of the delivery of the placenta, blood was carefully obtained from the umbilical cord vessels using a 21G (green) needle and syringe and immediately placed into a 50 ml conical tube and mixed 1:6 with 3.8% sodium citrate (Martindale

Pharmaceuticals, Romford, UK) to prevent coagulation. The volume of blood obtained varied with the size and length of the portion of the umbilical cord available for sampling and ranged from less than 5 ml to over 60 ml from different cords.

For comparison, a similar volume of blood from healthy adult volunteers was obtained by venepuncture and prepared identically to cord blood samples.

The study was approved by both the Cardiff and Vale NHS Trust's Research and Development committee and the South East Wales Research and Ethics Committee (Reference number: 05/WSE04/85, 7th September 2005).

Eight pairs of cord and adult samples were used for apoptosis experiments and four further pairs for the RT-PCR (section 2.2.5.3) and RPA (section 2.2.5.2) work.

2.2.2 Isolation of peripheral blood neutrophils

Both adult and cord blood samples were mixed by gentle inversion following collection and then centrifuged at 450 x*g* for 20 minutes at room temperature. The plasma (upper layer) was then carefully removed from the cellular component in each sample and placed into a clean conical tube. This plasma was centrifuged at 1300 x*g* for 20 minutes at room temperature to remove any remaining red blood cells and platelets. This platelet poor plasma (PPP) was saved in a clean 15 ml conical tube and used to make up the plasma percoll gradient later.

Percoll (Sigma-Aldrich, Dorset, UK) consists of polyvinylpyrrolidone (PVP)-coated colloidal silica particles which are 15-30 nm in diameter (23% w/w in water). The PVP coating renders the Percoll non-toxic to cells. Percoll is well suited for density gradient experiments due to its low viscosity and low osmolarity.

In a 15 ml conical tube, 1.02 ml of 90% Percoll and 0.98 ml of PPP were mixed (51% gradient layer). In a separate container a 42% gradient layer was made consisting of 0.84 ml of 90% Percoll and 1.16 ml of PPP. This mixture was very carefully layered over the 51% layer in the 15 ml tube to avoid mixing of the layers. One or two such

gradients were made for cord blood and another set for adult blood, depending on the original volume of blood obtained.

Following the initial centrifugation step and removal of the plasma layer, the lower layer of each sample was gently mixed 1:5 with warmed 6% dextran 70 (Baxter Healthcare Ltd, Thetford, UK) and then made up to the original volume of the blood sample with warmed 0.9% saline. These mixtures were left to settle at 37°C in a waterbath for 30 minutes with the lids loosely on until a clear line of demarcation could be observed between the lower layer containing red blood cells and the upper layer containing the white blood cells. The upper white blood cell-rich layers were carefully removed from the tube and centrifuged at 200 x*g* for 6 minutes at room temperature. The supernatants were discarded and the cell pellets gently resuspended in 2 ml of PPP each. These mixtures were then very carefully layered on top of the appropriate adult or cord plasma percoll gradient, once again to avoid mixing of the layers.

The gradients were centrifuged at 350 x*g* for 13 minutes at room temperature without braking. This allows the formation of 3 layers of cells in each tube. The upper layer consists of mononuclear cells, the middle layer contains polymorphonuclear leucocytes (mainly neutrophils) and the cell pellet in the bottom of the tube contains any remaining red blood cells. The layers were carefully aspirated from the gradient and placed into clean tubes.

The neutrophils were then washed once in 20 ml of warmed Hanks buffered saline solution (HBSS) without calcium and magnesium and twice more in HBSS with calcium and magnesium before being counted on a haemocytometer and resuspended at a density of ~10^6cells/ml in flow cytometry buffer for FACS staining and analysis or DMEM containing 5% FCS for culture.

Cytospins of the separated neutrophils were made as described in 2.1.3.1 (above) and at least 300 cells per slide were counted to obtain a differential cell count and thus confirm the purity (percentage neutrophils) of the separated cells.

2.2.3 Culture

Working in a sterile hood, 100 µl (10^5 cells) aliquots of the isolated neutrophils were placed into each well of a 96-well flat bottomed plate, allowing at least 1 well per test for each subsequent planned timepoint as well as additional wells so that cytospins could also be made at each time point.

Half the neutrophils were cultured in DMEM medium alone and a further 100 µl of media was added to these wells. The other half of the cells were cultured in medium containing 50 ng/ml of bacterial lipopolysaccharide (LPS) from *E.coli* O157:B8 (Sigma-Aldrich, Irvine, UK). For this, a solution of medium containing 100 ng/ml of LPS was made up and 100 µl of this solution was added to each appropriate well, already containing 100 µl of cells in medium.

The plates were covered with plastic lids and placed in an incubator (Hera cell 240, Heraeus) at 37°C containing 5% CO_2 for the required period (6 or 20 hours).

To remove cells from the plate at the end of the incubation period, cells were gently pipetted up and down twice before placing them in a round bottomed plate (or FACS tubes for caspase 3) staining. The plate was centrifuged at 500 x*g* for 4 minutes to pellet the cells and the supernatants were carefully removed and frozen at -80°C for later analysis.

Cytospins of apoptotic neutrophils at each time point were made as described in 2.1.3.1 above.

2.2.4.1 Preparation of cord and adult neutrophils for flow cytometry analysis

Using a round bottomed 96 well plate, 100 µl of the cell suspension was added to each well, including wells for all antibody staining as well as controls for both adult and cord neutrophils, according to a standard template (appendix 2) which I designed to assess the purity of neutrophil preparations and any relative difference in the expression of cell surface markers at the start of the culture period, using the antibodies listed in Table 2.1 (above).

The cells in the plate were incubated for 30 minutes at 4°C in a blocking buffer containing PBS, 0.1% sodium azide, 1% BSA, 1% heat inactivated normal mouse serum and 5 mM EDTA, to block non-specific antibody binding sites on the cell surface. The cells were then centrifuged at 500 x*g* for 4 minutes and the supernatant removed. Biotinylated antibodies were diluted in flow cytometry buffer at pre-determined optimal concentrations and 80 µl of the appropriate antibody solution was added to each well of the plate. Wells not requiring antibody at this stage received the same volume of flow cytometry buffer alone. The plate was incubated at 4°C for 30 minutes. Following the incubation, cells were centrifuged at 500 x*g* for 4 minutes and then washed 3 times using 200 µl of flow cytometry buffer. Secondary fluorescent conjugated reagents or directly conjugated antibodies, again diluted to pre-determined optimal concentrations in 80 µl of flow cytometry bufer, were added to the cells and a further incubation at 4°C for 30 minutes followed. Thereafter, cells were again washed 3 times and resuspended in 200 µl flow cytometry buffer. Samples were then transferred to test tubes ready for immediate FACS analysis.

FACS analysis was performed as described for BAL samples in 2.1.4.3 (above).

2.2.4.2 Apoptosis

Isolated neutrophils from both cord and adult blood samples were assessed for the proportion of apoptotic cells at the start of the culture period (time 0), again at 6 hours from commencement of culture (time 6) and again at the end of the culture period (time 20) using annexin-V-PE and To-Pro 3 using an identical staining and FACS analysis protocol to that used for BAL samples (see 2.1.4.2 above).

2.2.4.3.1 Caspase 3

The amount of activated caspase 3 in cord and adult neutrophils was assessed at each of the three time points using Apo Logix ™ - SR Sulforhodamine Caspase Detection Kit (Peninsula Laboratories Inc, California, USA) according to the manufacturer's instructions.

Caspases specifically recognise a 4 amino acid sequence on their substrate, which in the case of Caspase 3 is the DEVD sequence. Sulforhodamine (SR)-labelled - DEVD – fluoromethyl ketone (FMK) (SR-DEVD-FMK) is a potent inhibitor of caspase activity which enters the cell and binds irreversibly to activated caspase 3, rendering these cells visible by flow cytometry. It has lower binding affinity for caspases 8, 7, 10 and 6.

A stock solution of SR-DEVD-FMK was made up by reconstituting the supplied vial of the peptide with 50 µl of DMSO to give a 150x stock solution which was stored in aliquots at -20°C. Immediately prior to use the 150x solution was thawed and diluted 1 part SR-DEVD-FMK to 4 parts PBS to give the 30x working dilution.

Neutrophils were prepared as described in 2.2.4.1 above and then resuspended at a density of 10^6 cells/ml in tissue culture medium and divided into 300 µl aliquots in FACS tubes, allowing a control and a test sample for each condition. Each tube received 10 µl of a 30x working dilution of SR-DEVD-FMK and were gently mixed by flicking the tube. The control sample also received 2 µl of Suc-Ala-Ala (Bachem, St Helens, UK), an inhibitor of caspase 3 (molar equivalent of 10 µl of SR-DEVD-FMK). The tubes were incubated in the dark at 37°C and 5% CO_2 for 1 hour.

Following incubation, 2 ml of 1X working dilution of the supplied wash buffer was added to each tube before centrifuging at 500 x*g* for 4 minutes. The supernatant was discarded and the cells washed twice more, each time with 2 ml of wash buffer per tube, before resuspending the cells in 200 µl of flow cytometry buffer for analysis.

2.2.4.3.2 FACS analysis for caspase 3

The pink SR-DEVD-FMK reagent fluoresces in the red spectrum and 10 000 ungated events were collected for both control and test samples. Mean fluorescence intensity (MFI) was measured as well as the percentage of cells which were positive for activated caspase 3 and compared between control and test samples for both adult and cord neutrophils.

2.2.4.4 Bax

I chose to investigate the pro-apoptotic protein Bax in cord and adult neutrophils. Bax is an intracellular protein and thus neutrophils needed to be fixed and then rendered permeable to the Bax antibody (monoclonal phycoerythrin-conjugated mouse anti-human Bax (Clone 2D2), Santa Cruz Biotechnology). The Fix &Perm kit (An Der Grub Bio Research GmbH, Austria) was used for this.

Using a 96-well round bottomed plate, 100 µl of neutrophil suspension (either fresh or from culture) was added to each required well. The cells were pelleted by centrifuging at 500 x*g* for 4 minutes. Each cell pellet was gently resuspended in 100 µl of Reagent A (a fixative containing formaldehyde) and the plate was incubated at room temperature, in the dark, for 20 minutes. The cells were then washed twice with 200 µl of flow cytometry buffer before adding 100 µl of the supplied Reagent B (the permeabilising solution) containing an optimal concentration of Bax antibody or appropriate isotype control antibody (IgG1-PE (clone MOPC-21, Biolegend). The plate was again incubated for 20 minutes at room temperature in the dark before a further 2 washes. The cells were resuspended in 200 µl of flow cytometry buffer and analysed immediately.

FACS analysis was performed on the same BD FACScalibur machine as the BAL analysis, using appropriate settings which were saved and used for every cord/adult blood experiment. FACS data was again analysed using CellQuest software.

2.2.5 Analysis of neutrophil mRNA Expression

2.2.5.1 Extraction of neutrophil RNA

Neutrophils were isolated by the plasma-Percoll gradient method (section 2.2.2) described above and a cell count was performed using a haemocytometer. Neutrophils were spun at 450 x*g* for 6 minutes at room temperature in HBSS with Ca^{2+} and Mg^{2+} and the cells were resuspended at 10^7/ml in HBSS with Ca^{2+} and Mg^{2+} then transferred as 1 ml aliquots to 1.5 ml RNase-free eppendorfs and microcentrifuged at 2 000 rpm for 2 minutes. Each pellet was resuspended in 250 µl cold PBS and

following resuspension, 750 µl Tri-Reagent was added. The tubes were inverted to mix the contents and then incubated at room temperature for 5 minutes for cell lysis to occur. Each tube then had 200 µl chloroform added. Again the tubes were inverted and incubated at room temperature for a further 15 minutes. Cells were then spun in a microcentrifuge at 11,500 rpm for 15 minutes at 4°C. Following this, three layers are visible in each tube: at the bottom is a red layer of protein, in the middle a white band of DNA and on top a colourless, aqueous layer containing RNA. The top layer was carefully aspirated from each tube, without contamination of the sample with the DNA layer and transferred to new 1.5 ml RNase-free eppendorfs. The RNA solution was further suspended in 500 µl isopropanol per tube and incubated at room temperature for 10 minutes, followed by centrifugation at 11,500 rpm for 8 minutes at 4°C. All the supernatant was carefully removed and discarded and 1ml 75% ethanol added (not resuspended) to the pellet to wash the cells. The tubes were centrifuged at 9200 rpm for 5 minutes at 4°C and, once the supernatants had been removed, the pellets were left to air dry. One dry, the pellets were resuspended in 10 µl RNase-free water and stored at -80°C until analysis.

2.2.5.2 Ribonuclease (RNase) Protection Assay (RPA)

The RPA is a highly sensitive and specific method for the detection and quantitation of mRNA species. The assay uses DNA-dependent RNA polymerases for the synthesis of high-specific-activity RNA probes from DNA templates each of distinct length and each representing a sequence in a distinct mRNA species. The probe set is hybridized in excess to target RNA in solution, after which free probe and other single-stranded RNA are digested with RNases. The remaining "RNase-protected" probes are purified, resolved on denaturing polyacrylamide gels, and quantified by autoradiography. The quantity of each mRNA species in the original RNA sample can then be determined based on the intensity of the appropriately-sized, protected probe fragment.

The BD RiboQuant Multi-probe RNase Protection Assay System (BD Biosystems) was used according to the manufacturer's instructions to analyse the RNA obtained from cord and adult blood nutrophils. The RPAs were performed by Vanessa

Singleton in the Department of Respiratory Medicine at the Royal Hallamshire Hospital, Sheffield, where she has acquired appropriate expertise in this process.

RNA is very sensitive to RNase contamination and degrades very easily, so for this reason, all supplies and reagents used were RNase-free.

a) Synthesis of probe

The [α-32P]UTP, GACU nucleotide pool, DTT, 5x transcription buffer and RPA template set were brought to room temperature. The following reagents were added, in order, to a 1.5 ml Eppendorf tube: 1 µl RNasin, 1 µl GACU nucleotide pool, 2 µl DTT, 4 µl 5x transcription buffer, 1 µl RPA template set, 10 µl [α-^{32}P] UTP, 1 µl T7 RNA polymerase. The T7 RNA polymerase was kept at –20°C until use and returned to –20°C immediately after use. These were mixed and then incubated at 37°C for 1 hour. The reaction was ended by adding 2 µl of DNase, mixed again and incubated at 37°C for 30 minutes. Each Eppendorf tube then received (in order) 26 µl 20 mM EDTA, 25 µl Tris-saturated phenol, 25 µl chloroform:isoamylalcohol (50:1) and 2 µl yeast RNA. These were mixed and then micro-centrifuged for 5 minutes at room temperature. The upper, aqueous layer was transferred to a new 1.5 ml Eppendorf tube and 50 µl of chloroform: isoamylalcohol (50:1) was added. This was mixed and then micro-centrifuged for 2 minutes at room temperature. Again the upper, aqueous phase was transferred to a new 1.5 ml Eppendorf tube and 50 µl 4M ammonium acetate and 250 µl ice-cold 100% ethanol were added. The tubes were mixed by inverting them and incubated at –70°C for 30 minutes, after which they were micro-centrifuged for 5 minutes at 4°C. The liquid layer was carefully removed and 100 µl ice cold 90% ethanol was added before micro-centrifuging for 5 minutes at 4°C. The liquid was removed and the contents of the tube allowed to air dry for 5-10 minutes. Finally 100 µl of hybridisation buffer was added and the pellet solubilised by gently vortexing for 30 seconds. The probe was then stored at –20°C until needed.

b) Hybridisation

The RNA prepared in 2.2.5.1 was removed from –70°C storage and the quantity of RNA was measured using a NanoDrop Spectrophotometer ND-100 (Labtech

International, Lewes, UK). Each RNA sample was diluted to the same concentration and had 8 µl of hybridisation buffer added to the sample. The RNA was solublised by gently vortexing and then quickly spinning in the microcentrifuge. The chosen probe was diluted with hybridisation buffer and 2 µl of diluted probe was added to each RNA sample and mixed by pipetting. A drop of mineral oil was then placed on top of the mixture. The tubes were then placed in a pre-heated heat block at 90°C and immediately turned down to 56°C. They were then left to incubate for 12-16 hours. Samples were removed from the heat block 15 minutes prior to the RNase treatments and placed at room temperature to allow the temperature to equilibrate slowly.

c) RNase treatment

An RNase cocktail was prepared consisting of 2.5 ml RNase buffer and 6 µl RNase A + T1 mix for twenty samples. To the RNA samples that had been removed from the heat block, 100 µl of cocktail was pipetted underneath the mineral oil into the aqueous layer and then incubated at 30°C for 45 minutes. During this time, the Proteinase K cocktail was prepared, consisting of 390 µl Proteinase K buffer (prewarmed to 37°C to solubilize the SDS), 30 µl Proteinase K and 30 µl Yeast tRNA for 20 samples. This was mixed and 18 µl added to new, labelled Eppendorf tubes. The aqueous layer was then removed from underneath the oil and placed in the vials containing the Proteinase K cocktail. This mixture was incubated for 15 minutes at 37°C before adding 65 µl of Tris-saturated phenol and 65 µl of chloroform: isoamylalcohol and centrifuged for 5 minutes at room temperature. The upper aqueous layer was aspirated and transferred to a new tube and 120 µl 4M ammonium acetate and 650 µl ice-cold 100% alcohol were added. This was mixed by inverting and incubated at –70°C for 30 minutes, then centrifuged for 15 minutes at 4°C. The liquid supernatant was removed, 100 µl of ice-cold 90% ethanol added before centrifugation for a further 5 minutes at 4°C. The ethanol was removed and the pellet allowed to air dry before adding 5 µl of loading buffer.

d) Gel Resolution of Protected Probes

The protected probes were then run on a 4.75% acrylamide sequencing gel composed of 35.82 g of urea, 22.35 ml dH_2O, 7.45 ml of 10x TBE, 8.85 ml of 40% acrylamide,

9.31 ml of 2% bis acrylamide, 450 µl ammonium persulfate (10%) and 60 µl TEMED. Using the recommended acrylamide concentration and acrylamide:bis acrylamide ratio is critical for the correct resolution of unprotected and protected probe bands. After polymerisation the gel was run in a vertical gel rig with 0.5x TBE running buffer in the upper and lower reservoirs.

The samples were heated for 2-3 minutes at 90°C before loading and running the gel. Once complete, the gel was removed, dried and exposed to X-ray film. Initial exposures were done overnight but finally required exposure for a week for optimal results.

In interpreting the film, it is important to note that the probe lengths are greater than the "protected" fragment lengths, due to the presence of flanking sequences in the probes which do not hybridise with target mRNA.

2.2.5.3 Reverse transcription – Polymerase Chain Reaction (RT-PCR)

RNA that has been extracted from neutrophils was used for reverse transcription (RT) followed by PCR to analyse mRNA expression in a semi-quantitative manner. All RNA work was performed using RNase free pipette tips, eppendorfs and solutions.

The RT and PCR were performed by Kate Vaughan in the Department of Respiratory Medicine at the Royal Hallamshire Hospital, Sheffield, where she has acquired expertise in this process. The RNA was thawed and DNase-treated to remove any contaminating DNA by using a DNA-*free*™ kit (Ambion, Warrington, UK). Using this kit 2 µg of RNA was treated with 1 µl of the supplied DNase-I buffer and 1 µl of DNase-I for 25 minutes at 37°C, then 2 µl of stop-reagent beads was added and incubated for 2 minutes at room temperature before centrifuging the mixture at 10 000 x*g* for 90 seconds. The RNA was then transferred to a fresh tube. The quantity of RNA was then measured using a NanoDrop Spectrophotometer ND-100 (Labtech International, Lewes, UK).

Following this, the RNA was converted into cDNA as follows:
In thick walled 0.5 ml eppendorfs, each 1 μg of RNA was made up to a volume of 12.4 μl with RNase-free water, then 8 μl of AMV (Avian Myeloblastosis Virus) buffer, 16 μl of 10 mM dNTPs and 1.2 μl each of RNasin, Random primers and AMV-RT (all reagents from Promega, Southampton, UK). These tubes were then placed in an MJ Research Peltier Thermal Cycler-200 which keeps the reaction mixture at 23°C for 5 minutes which allows the oligo d(T) primers to anneal to the poly-A tails of the RNA. The temperature is raised to 42°C for 2 hours during which cDNA is synthesised, catalysed by reverse transcriptase. The mixtures are then heated to 99°C for 2 minutes which denatures the reverse transcriptase enzymes to stop the reaction. The products are then cooled to 4°C and held at this temperature until the resultant cDNA can be used in the quantitative PCR process.

2.2.5.4 Quantitative PCR

Quantitative PCR (Q-PCR) is a technique which is used to amplify and simultaneously quantify a specific sequence of DNA. Simply, cDNA samples are produced by the RT process (2.2.5.3 above). Specifically designed forward and reverse primers are added and annealed to specific cDNA sequences. A specifically designed fluorescent reporter probe is also allowed to anneal to the sequence between the forward and reverse primers. The probe has a 5' high energy reporter dye (6-FAM) and a 3' low energy non-fluorescent quencher (TAMRA) attached. Due to the close proximity of the reporter to the quencher its fluorescence is prevented. When the DNA polymerase (Taqman Gold), which has 5'-exo-nuclease activity, begins to copy the cDNA, it reaches the area where the probe has annealed. Using its 5'-exo-nuclease activity it cleaves the reporter dye from the quencher, enabling fluorescence of the reporter to be detected. This increase in fluorescence corresponds directly to the exponential increase in the number of amplicons generated and this is used to determine the threshold cycle (CT) in each reaction. The cycle at which fluorescence from a sample crosses the threshold is called the cycle threshold (Ct) (the threshold is above background) and since we know that the quantity of DNA doubles every cycle during the exponential phase, we can accurately quantify relative amounts of DNA in the samples by comparing the results to a standard curve produced by real-time PCR of serial dilutions of a known amount of DNA and thus make comparisons between

samples. The measured amount of product from the gene of interest is divided by the amount of product from a housekeeping gene measured in the same sample to normalize for possible variation in the amount and quality of DNA/RNA between different samples.

The procedure was carried out as follows: Primers/probes, samples and the mastermix were kept on ice. A mastermix was made up for each set of primers. For primer/probe sets from Applied Biosystems (HIF-1α: Hs00153153_m1; β-actin: Hs99999903_m1; GAPDH: Hs99999905_m1; Bcl-xl: Hs99999146_m1; Mcl-1: Hs00172036_m1; A1: Hs00187845_m1)(Applied Biosystems, Gloucester, UK) the mastermix consisted of 10μl of buffer which contains the DNA polymerase (RT-QP2X-03, Eurogentec, Southampton, UK), 1 μl primer/probe set and 8 μl of sterile water. For the HIF-2α primer/probe set (HIF-2α: sense = CTCCCACggCCTgTACggACAC; anti-sense =AgTgCTCCCgCTgAATgACTCCACT; probe = CTCggATTgTCACACCTATggCATATC)(Sigma-Genosys, Dorset, UK) the mastermix contained 10 μl of buffer, 0.1 μl forward primer, 0.1 μl reverse primer, 0.02 μl probe and 8.78 μl water.

Standard curves of known amounts of cDNA were made up in water as follows: For Bcl-xl and Mcl-1 normoxic neutrophil cDNA was used in 1:4 serial dilutions to produce the standard curve. A1, GAPDH and β-actin used 1:10 serial dilutions of normoxic cDNA. HIF-1α and HIF-2α used hypoxic cDNA in the standard curve at serial dilutions of 1:10 for HIF-1α and 1:2 for HIF-2α.

Then, 19 μl of each master mix was accurately pipetted into appropriate wells of a polypropylene 384-well plate (Greiner, Gloucester, UK) allowing wells for each sample to be analysed and the standard curve in duplicate. Thereafter 1 μl of the appropriate standard curve concentration or cDNA sample was added to the appropriate wells. The plate was covered and sealed (Biorad, Hemel Hempstead, UK) then centrifuged at 800 x*g* for 2 minutes to remove any trapped air bubbles from the bottom of the wells.

The plate was was then placed in the 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) and allowed to run. Data was analysed using SDS 2.2.1 software (Applied Biosystems).

2.3 Statistical Analysis

Statistical analysis for this thesis was performed using Microsoft Excel, SPSS, Graphpad Prism and Instat as applicable. In general, lavage data were non-parametric in nature and comparisons between data sets were made using Mann-Whitney U tests, one way ANOVA (Kruskal-Wallis) and Chi Squared analysis, whilst correlations between groups were performed using Spearman correlation co-efficients. Data for lavages are expressed as median values with interquartile ranges given, to reduce the impact of any results which were marked outliers.

For cord and adult blood data results were normally distributed and as such data were also expressed as median and interquartile range (IQR) due to small numbers of repeat experiments. Significance was taken as a p value <0.05.

Chapter 3

Bronchoalveolar lavage cell types and influx

Chapter 3

Bronchoalveolar lavage cell types and influx

3.1 Introduction

The primary focus of this thesis is the nature and progress of the inflammatory response in the lungs of preterm infants and how this may influence the development of CLD.

Recent years have seen many significant changes in the treatment of infants born prematurely as well as in the survival and long-term complications in those infants who are now surviving extreme preterm birth (Horbar et al., 2002, Wood et al., 2000). CLD continues to be the major respiratory complication in preterm infants, both in hospital and after discharge (Jobe, 1999). The aberrant lung development which "new" CLD is thought to represent is somewhat different to the simple "injury and repair" mechanism seen in historically reported "classical" CLD (Jobe, 1999). Although the pathogenesis of "new" CLD is multifactorial, the role of neutrophils appears to be pivotal. Their role in maintaining the inflammatory process is key to the disturbance of lung development seen in "new" CLD.

In this thesis I have sought to clarify the role of neutrophils and other cellular components of bronchoalveolar lavage fluid in infants at risk of developing CLD. In this chapter I have chosen to specifically examine whether there is a relationship between the number or proportion of neutrophils or macrophages in the BAL fluid and gestation or the development of CLD. Additionally I have looked in more detail at the surface markers on both neutrophils and macrophages in order to assess whether there are differences in the phenotypes of the cells present in different groups of infants.

It has been suggested that antenatal infection and the intra-uterine environment have an important role to play in the development of CLD and I will test this hypothesis by looking in more detail at cell counts and cell surface markers in BAL on the first day of life; looking particularly at whether any parameter in BAL on day 1 may be

predictive of the development of CLD. The role of postnatal infection in the development of CLD will also be studied further by looking at the effect of the presence of microbial DNA on BAL cell counts and cell phenotypes.

In order to obtain the information required to test the above hypotheses, it was necessary to first develop a method for working with and obtaining the largest amount of relevant data by flow cytometry from the very small amounts of BAL fluid obtained from the smallest preterm infants. The development of this method will be described in this chapter. Previous studies of BAL cells (Grigg et al., 1991, Oei et al., 2003, Kotecha et al., 2003) have relied mainly on data from microscopy and immunocytochemistry performed on cytospins and I will compare data gained by flow cytometry with cytospin findings.

For the purpose of this thesis the definition of CLD as oxygen dependence at 36 weeks' corrected gestation will be used as this implies a more severe form of lung disease than oxygen dependence at 28 days. It would be reasonable to assume that an infant born at 23 weeks might continue to require supplemental oxygen at 28 days of life as this is only a corrected gestation of 27 weeks. The "36-weeks" definition of CLD allows infants to be compared at what should be similar stages of lung development rather than comparing very different babies who have simply been ex-utero for the same length of time.

3.3 Results

3.2.1 Patient Characteristics

Thirty two infants were recruited into the BAL arm of the study between February 2006 and June 2008. Of these, 27 were born at less than 32 weeks' gestation and 5 were term controls ie. babies who were ventilated for non-respiratory reasons. In the preterm group, 6 babies had resolved their RDS by 28 days of life, 20 remained in oxygen at this stage (CLD) and 1 infant died before a diagnosis could be assigned. By 36 weeks' corrected gestation, 11 of the surviving preterm babies were classified as having resolved RDS and the remaining 15 had CLD (Table 3.1).

The infant who died was the second of non-identical twins, born at 26+5 weeks' gestation. The first twin died within hours of birth due to respiratory complications of prematurity combined with a large congenital diaphragmatic hernia and was not recruited into the study. The second (recruited) twin died on day 5 of life as a result of pericardial effusion and cardiac tamponade, a rare complication caused by migration of an intravenous long line (Pettit, 2003). This infant was excluded from analysis as her diagnosis at both 28 days and 36 weeks was unknown.

All 5 of the term control infants were born with gastroschisis and were operated on during the first few hours of life. In most cases, children born with gastroschisis who undergo primary closure of the abdominal defect are ventilated for only a brief period following surgery, however some require longer periods of ventilation, particularly if the gastroschisis closure is performed as a staged procedure due to a relatively small abdominal cavity. Ventilation of term infants for other non-respiratory reasons is unusual, the exception being in infants with hypoxic ischemic encephalopathy (HIE) and resultant poor respiratory drive. The pathogenesis of HIE implies a period of intra-uterine hypoxia which may have pro-inflammatory effects on the lung leaving them far from "normal" and for this reason these infants were excluded from the study. As a result of the entry criteria, the number of term control infants recruited was limited.

A statistically significant difference was found between the gestational age of infants who went on to develop CLD (mean 26+2 weeks; median 26+0 weeks, range 23+4 weeks to 29+3 weeks) and that of infants whose RDS resolved (mean 28 weeks; median 27+1 weeks, range 25+4 weeks to 31+6 weeks) ($p=0.024$). The term infants had a median gestation of 38+1 weeks (range 37+2 to 38+3 weeks). The gestational age profile of the infants recruited is shown in Figure 3.1.

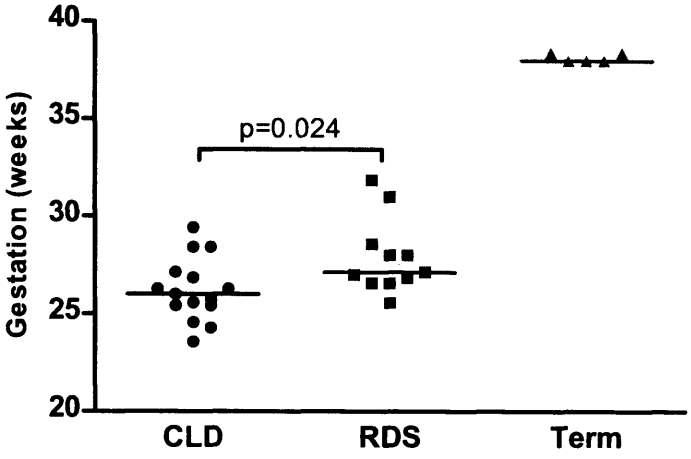


Figure 3.1 demonstrates the gestational ages of the infants recruited, grouped according to diagnosis. Each marker represents one infant and the horizontal lines represent the median value for each group.

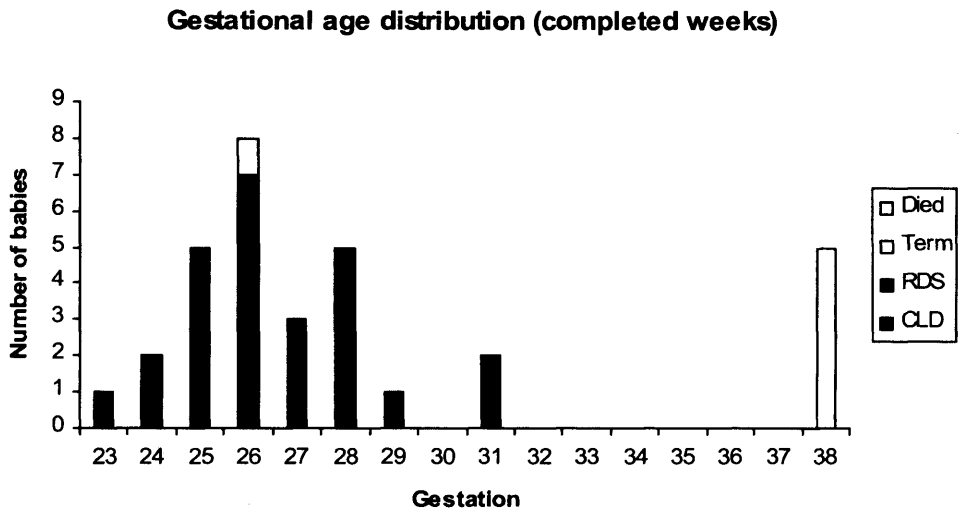


Figure 3.2 demonstrates the gestational age profile of the recruited infants. Each bar represents infants born during the stated week of life. Infants between 32 and 37 weeks' gestation were excluded from this study. The colours indicate the diagnostic categories to which the infants were assigned.

Patients who developed CLD did not have a significantly lower birth weight (mean 875g ; median 850g; range 560-1230g) than infants whose RDS resolved (mean 1120g; median 1000g; range 700-1900g, p=0.102, Mann-Whitney U-test), figure 3.3.

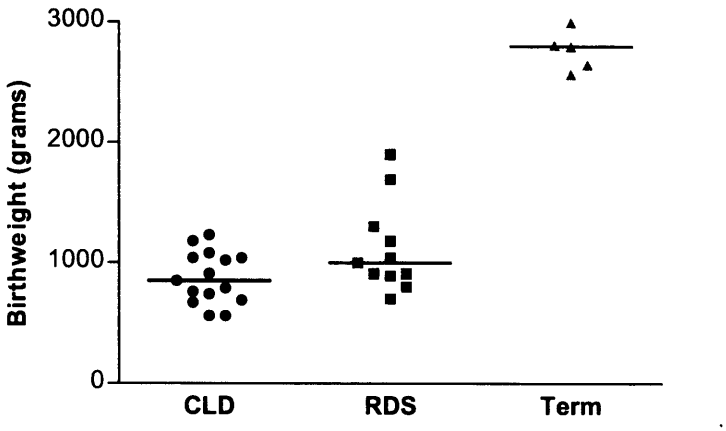


Figure 3.3. Scatterplot comparing birthweight of infants. Horizontal lines represent median values.

There was no difference in the male: female ratio between groups, although all the term controls were male which is consistent with the increased incidence of gastroschisis in males (Forrester and Merz, 1999, Calzolari et al., 1993, Alvarez and Burd, 2007) (Table 3.1). A similar proportion of infants in each group were born by Caesarean section (Table 3.1).

The majority of preterm infants received antenatal steroids (Table 3.1). Not all babies in each group had received a full 2-dose course of antenatal steroids (6/11 in the RDS group and 9/15 in the CLD group) but this was not a statistically significant difference (p=0.46, Chi square test). All 11 babies in the RDS group had received at least one dose of steroids which was significantly more than the CLD group where only 10/15 babies had been at least partially treated (p=0.033). One of the term infants had received a course of antenatal steroids for threatened preterm labour several weeks prior to delivery.

In line with the neonatal unit policy to administer exogenous surfactant to all intubated preterm infants as soon as possible after intubation, 100% of preterm babies received exogenous surfactant therapy (Curosurf™, Trinity-Chiesi Pharmaceuticals Ltd, UK) (Table 3.1).

There was no statistically significant difference between the number of infants with either RDS or CLD who were born following prolonged rupture of the membranes

(RDS 4/11 and CLD 2/15) (p= 0.18, Fisher's exact test) or between the groups when comparing for suspected maternal infection (based on maternal pyrexia, offensive vaginal discharge, elevated maternal CRP or WBC count) (p=0.69, Chi square test) (Table 3.1).

All the infants who developed CLD had a patent ductus arteriosus diagnosed during their admission compared to only 5/11 babies with RDS (p=0.002, Fisher's exact test). In the CLD group 9/15 required medical (indomethacin) or surgical (ligation) intervention compared to only 1 of the 5 in the RDS group (p=0.152, Fisher's exact test) (Table 3.1).

It is not surprising that those infants with apparently worse lung disease, who frequently went on to develop CLD, were ventilated for significantly longer than those infants whose RDS resolved. CLD infants were ventilated for a median of 36 days (range 12-92 days) compared to infants whose RDS resolved who were ventilated for a median of 4 days (range 2-12 days), p=0.001. As a consequence, fewer samples were inevitably obtained from the resolved RDS group than the infants who developed CLD (Table 3.1).

Of the 15 infants who developed CLD, 7 were defined as having severe disease using the NICHD/NHLBI criteria (Jobe and Bancalari, 2001), as they were on IPPV or CPAP and/or requiring >30% oxygen at 36 weeks' corrected gestation.

In total 207 BALs were performed. Of these 152 were from infants who developed CLD, 32 were from infants whose RDS resolved, 4 were from the infant who died and 19 were from term controls. The median fluid volume recovered from the BALs performed was 61% of the volume of fluid introduced (mean 61.2%, range 23.5% - 145.5%).

An element of bias is inherent in the nature of any study which requires infants to be intubated in order for lower airway secretions to be collected. Very few RDS infants remained ventilated beyond days 4 and 5 of life, thus it is only possible to make direct comparison between groups for the first few days of life and it is not possible to comment on what changes may be occurring in infants' lungs following extubation,

although we assume that if these infants remain extubated, that any changes within the lung are unlikely to be causing any significant respiratory compromise, compared to the intubated infants. Clearly for CLD infants, more samples will give the opportunity for greater amounts of data to be accumulated and for more peaks or troughs in various parameters to occur. Whilst these issues need to be considered when comparing groups, the longitudinal nature of our samples in individual infants does allow us to assess the nature of the inflammatory response and its resolution in these infants and to compare the relationships between numerous parameters for individual infants as well as groups of babies. Details of patient demographics are shown in table 3.1.

	CLD group	RDS group	Term controls
Number of Patients	15	11	5
Number of Samples	152	32	19
Median Gestational Age (range)	26^{+0} (23^{+4}-29^{+3} wks)	27^{+1} (25^{+4}-31^{+6} wks)	38^{+1} (37^{+2}-38^{+3} wks)
Median Birth Weight (range)	850g (550-1230g)	1000g (700-1900g)	2766g (2570-3000g)
Male:Female	7:8	8:3	5:0
Vaginal : Caesarean delivery	10:5	5:6	2:3
At least 1 dose antenatal steroids	10/15 (67%)	11/11 (100%)	1/5 (20%)
Full course steroids	6/15 (40%)	6/11 (55%)	1/5 (20%)
Patent Ductus Arteriosus	15/15 (100%)	5/11 (45%)	0/6 (0)%
PDA treated medically or surgically	9/15	1/5	0/0
Surfactant therapy (%)	15/15 (100%)	11/11 (100%)	0/5 (0%)
Rupture of membranes >24 hours	2/15 (13%)	4/11 (36%)	0/6 (0%)
Infection suspected peripartum	7/15 (47%)	6/11 (55%)	0/6 (0%)
Median days of ventilation (range)	36 (12 - 92 d)	4 (2 - 12 d)	3 (2-9 d)

Table 3.1. Patient characteristics. Values are shown as medians or total numbers with percentages in brackets.

3.2.2 Development of BAL cell processing method

3.2.2.1 DTT

In order to analyse cells from BAL samples, it is necessary to "extract" the cells from the thick mucus in which they are enveloped in the airways and thus in the BAL sample. Various methods of obtaining cells for analysis from sputum samples have been tried, including addition of DTT, deoxyribonuclease (DNAse), N-acetyl-L-cysteine, sodium EDTA or trypsin as well as mechanical methods such as mechanical blending or filtration of sputum through gauze or glass wool (Tockman et al., 1995, Pang et al., 1995). Given the very small volume (frequently less then 1 ml) of neonatal BAL samples, I felt that mechanical methods were unlikely to yield sufficient remaining sample for analysis and could potentially damage or activate any cells present. The use of DTT is the most frequently cited method in the literature in a range of concentrations, the most frequently used being around 50 μg/ml (Lensmar et al., 1998, Saraiva-Romanholo et al., 2003).

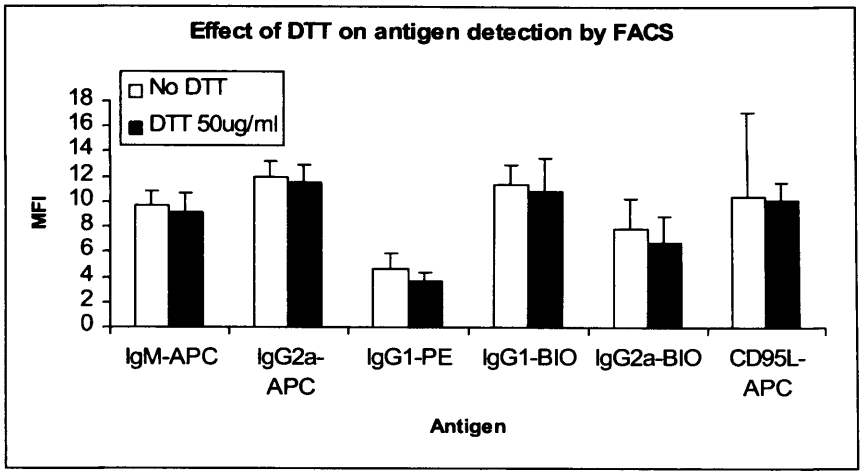
DTT is a reducing agent which breaks down disulphide bonds in sputum, rendering it less viscous and allowing cells within a sputum sample to be liberated for analysis. I was concerned that the reducing effect of DTT may alter the conformation of cell surface markers, thus changing the binding properties of the antibodies I was using for flow cytometry.

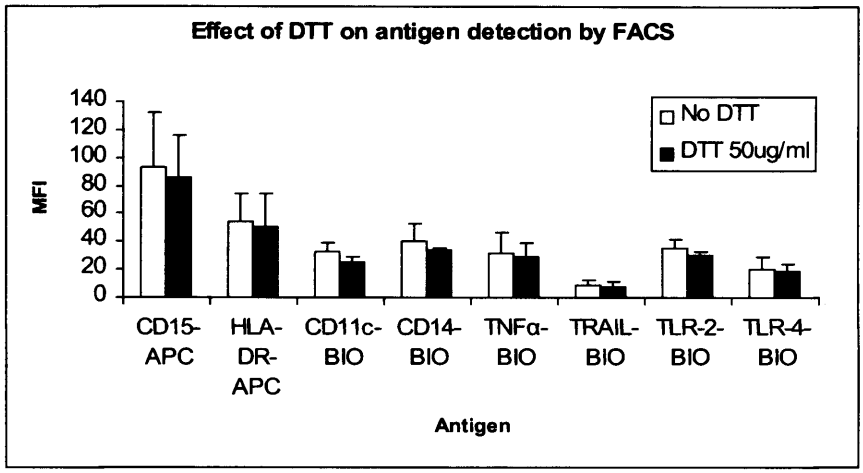
Although DTT has been used frequently in studies on adult sputum, there have been limited descriptions of its effect on cell surface markers detected by flow cytometry (Dominguez-Ortega et al., 2002, Loppow et al., 2000, Qiu and Tan, 1999) and these did not include a number of the antibodies which I was planning to use.

I studied the effect of DTT on the detection of the cell surface markers I had chosen to study in our neonatal BAL samples by exposing adult peripheral blood leucocytes to DTT and comparing cell surface marker expression against cells from the same blood sample not exposed to DTT.

Two sets of experiments were performed - firstly to assess the effect of DTT on cell surface marker detection by flow cytometry and secondly to determine the optimal concentration of DTT.

There was no difference in the mean fluorescence intensity between DTT treated and untreated cells using DTT at a concentration of 50 μg/ml (Figure 3.4).





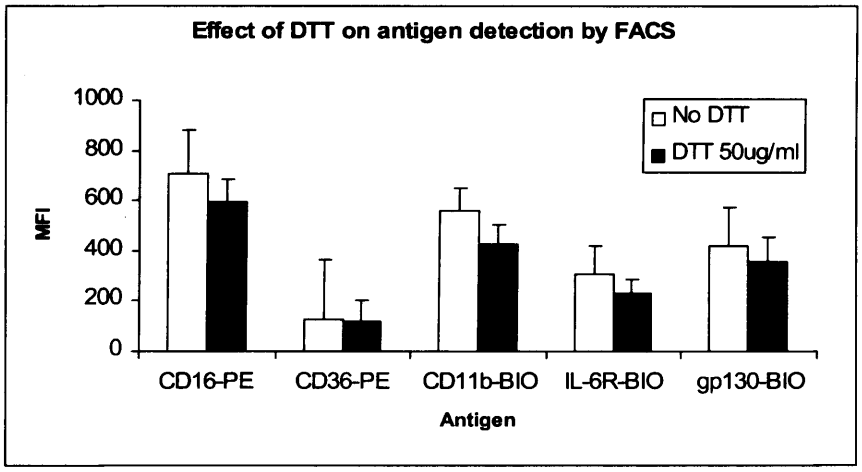


Figure 3.4 Graphs showing mean (and standard deviation) fluorescence intensity for 19 different antibodies by flow cytometry in DTT treated and untreated peripheral blood neutrophils. n=6 DTT treated and 6 untreated.

Adding DTT to clinical BAL samples according to the method described above produced a visible alteration in the viscosity of the mucus component of the BAL sample, making the cell pellet easier to resuspend with much less vigorous pipetting, which could otherwise potentially have activated the neutrophils.

Flow cytometry analysis showed no significant difference in the mean fluorescence intensity between DTT treated and untreated cells for all of the antibodies and isotype controls used, which suggested that DTT did not cause a change in any of the epitopes to which our antibodies would bind. Thus, DTT at 50 μg/ml for 15 minutes at room temperature was selected as the method of dealing with the mucus component of BAL samples in order to obtain an appropriate cell suspension for analysis, particularly for flow cytometry purposes. All BAL samples were treated in this way before analysis.

3.2.2.2 Working with very small numbers of cells

One of the biggest challenges of designing flow cytometry experiments on neonatal lung lavage samples is to be able to use the very small amount of sample available to obtain the largest possible amount of information. From previous studies (Mildner et al., 2005, Curley et al., 2004) and previous experience gained in our laboratory, I was able to estimate that the average return from a BAL procedure was of the order of 50-60% of the fluid instilled, however reported cell counts in the returned BAL varied widely.

Using mixed peripheral blood leucocyte populations, separated using dextran sedimentation, as described in section 2.2.2, I was able to optimise antibody concentrations for each antibody used, working with 10^5 cells in 100 μl of buffer in each well of the 96 well plate in which the cells were being stained. The number of cells was then reduced to as few as $3x10^4$ cells with a corresponding reduction in antibody added and I was still able to obtain 10 000 gated events on the flow cytometer with similar mean cell fluorescence values when cells were analysed. It was then possible, once sufficient cells for cytospin preparation had been removed from the BAL sample, to calculate the number of aliquots of at least 30 000 cells into which the remaining sample could be divided for flow cytometry staining. With cell

counts as low as $3x10^5$/ml or as high as $2x10^7$/ml, 3 templates were designed so that a basic level of information could be obtained from all BAL samples and additional data could be obtained from samples in which there was a relative abundance of cells (appendix 2). Even so, there were 3 BAL samples, of the 207 obtained, in which there were so few cells that only Annexin-V/To-Pro-3 staining for apoptosis was possible.

3.2.3 Flow cytometry of neonatal BAL samples

I was unable to find published flow cytometry plots of neonatal lung lavage, although some data for adult lung lavage has been published in this format (Rosseau et al., 2000a, Guth et al., 2009). By running antibody-stained and unstained BAL cells from 8 infants of different gestations and postnatal ages, appropriate flow cytometer settings were developed which could be used across the range of samples. These 8 BAL samples were obtained from infants enrolled in another study of BAL samples in our department and were surplus to the requirements of the study. I am very grateful to Dr Phil Davies for his generosity in sharing these samples with me.

3.3 Bronchoalveolar lavage cell counts

Cell counts were performed using a haemocytometer and differentials performed by flow cytometry and by microscopy. The microscopy was undertaken by Sharon Gill using cytospins, as described in section 2.1.3.1.

3.3.1 Non-viable cells

Cells counted on the haemocytometer were stained with trypan blue (section 2.1.3 of Materials and Methods) to assess the viability of the BAL cells. The mean percentage of non-viable cells was 14.8% (range 0-60%). There was no significant difference between term and preterm infants (Mann-Whitney U-test, p=0.90), nor between babies who developed CLD and those whose RDS resolved (Mann-Whitney U-test, p=0.75) (Figure 3.5).

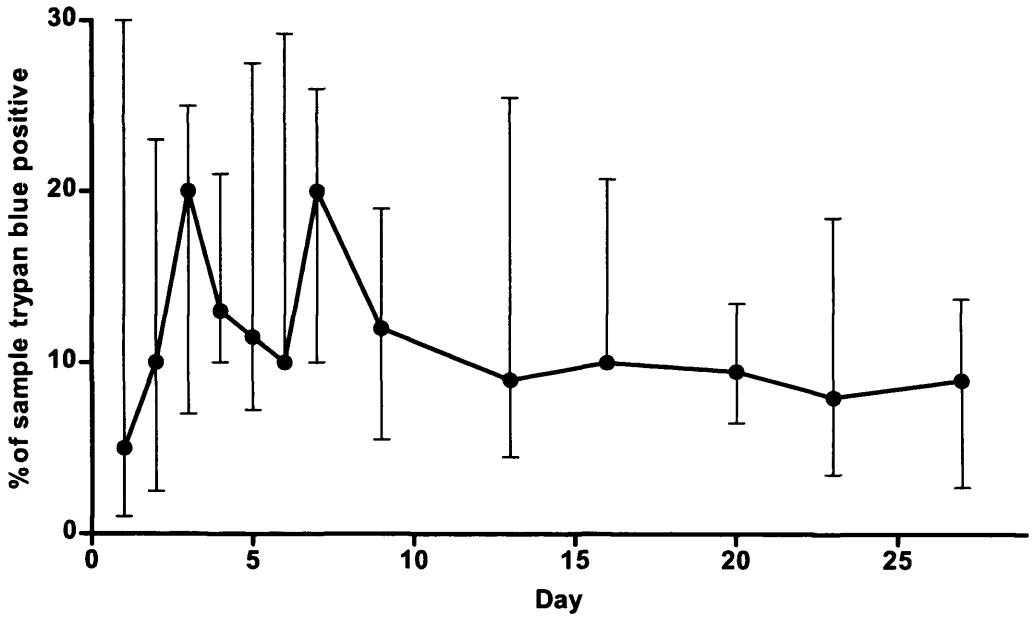


Figure 3.5 Graph showing the median percentage of non-viable (trypan blue positive staining) cells in all the BAL samples on different days of collection. The error bars represent interquartile ranges.

3.3.2 Debris in BALs

From the outset it was noticeable that there was a large amount of low forward scatter/low side scatter material present in BAL samples which did not stain with any of the antibodies which I used to detect neutrophils or monocytes/macrophages by flow cytometry. The material was seen in the lower left-hand corner of the FSC/SSC plots, indicating material of small size and low granularity (Figure 3.6). It was most abundant in the first few days of life and reduced as the babies got older. From cytospins, much of this material was thought to be damaged fragments of cells and cell membranes, platelets and red blood cells and which I termed "debris".

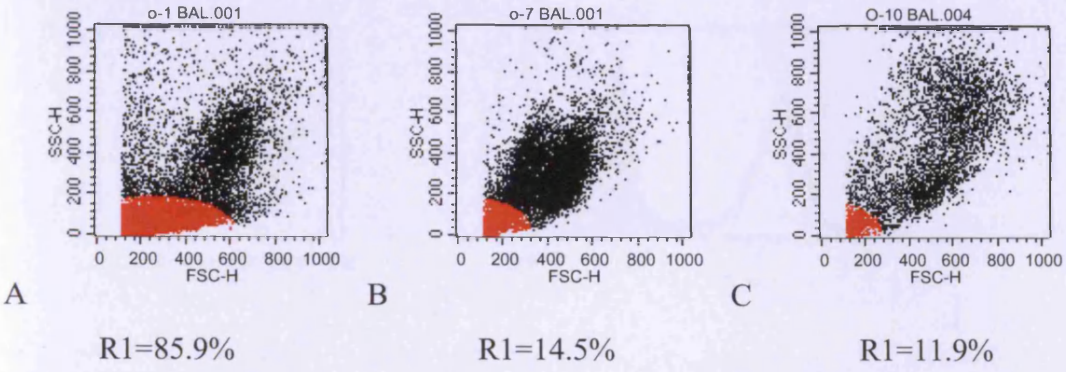
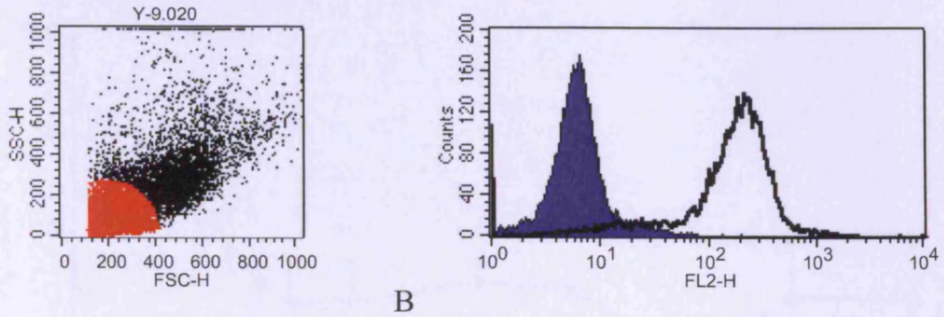


Figure 3.6 Forward scatter (FSC) (horizontal axis) vs side scatter (SSC) (vertical axis) flow cytometry plots showing low forward scatter/low side scatter material identified as debris in BAL samples. Figure 3.6A represents a sample taken on day 1, figure B is a sample from the same baby on day 7 and figure C is from the same baby on day 20. R1 corresponds to the cells marked in red and the percentage of the sample represented by the red area.

In order to further elucidate the nature of this debris, I used a phycoerythrin-conjugated antibody to CD59 (Mouse anti-human CD59-PE, Caltag Laboratories, Buckingham, UK), a complement regulatory protein (protectin) which is found on all human cells. Most of the material I had labelled debris was positive for CD59, indicating that it originated from human cells (Figure 3.7). The proportion of the sample that was CD59 positive however, was variable and was higher in the earliest BAL samples from each baby. Of the 207 samples taken, 5 were noted to be blood stained and these samples had very large amounts of so-called debris, an example of which can be seen in Figure 3.7. Additionally antibodies to CD3 and CD4 were used to identify lymphocytes. There were no lymphocytes detected in any of the 10 BAL samples from different gestations and postnatal ages which were analysed for these markers.

The range of non-cellular (ie. CD59 negative) material varied between 0 and 90% of the total number of events counted by the flow cytometer for each sample. In the majority of samples it made up around 40% of the total event count. Debris which was CD59 negative stained positively for To-Pro-3, indicating the presence of DNA among the debris, confirming the possibility that much of the debris consisted of cell fragments.



A B

Figure 3.7 Forward scatter (horizontal axis) vs side scatter (vertical axis) plot (A) and histogram (B). "Debris" in a BAL sample taken on day 16 from a preterm infant who developed CLD (Baby Y) is marked in red. The histogram shows only the material marked in red in plot shown in (A). The black line represents CD59-PE staining of the "debris" material and the blue histogram is an appropriate IgG1-PE isotype control.

When the amount of debris was averaged over the whole period of ventilation, preterm infants had significantly less debris present ($p<0.0001$) but it can be seen in figures 3.8 and 3.9 that the amount of debris in the BAL samples falls steeply over the first few days of life and relatively little debris remains in BALs of babies who remain ventilated and develop CLD. In the first 5 days of life when term and preterm infants can be adequately compared as the term and RDS groups are still ventilated, there was no significant difference between the diagnostic groups or between term and preterm infants.

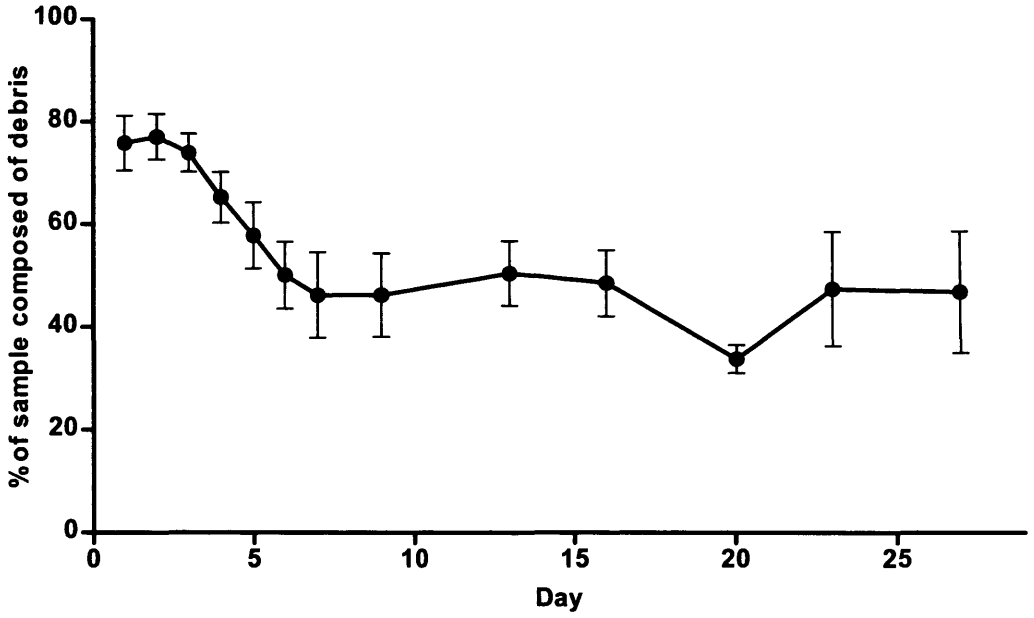
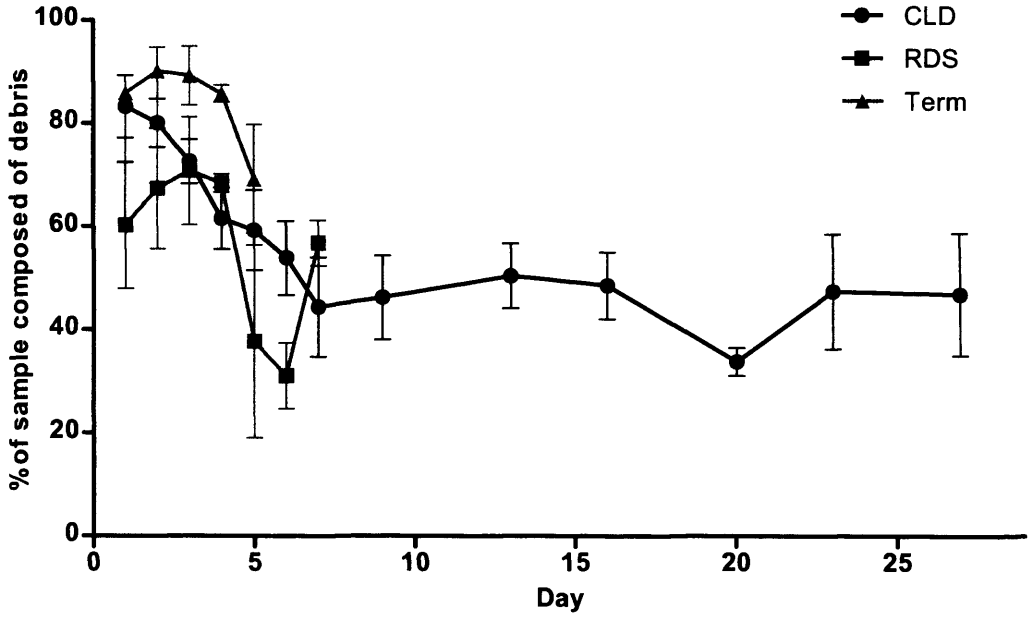
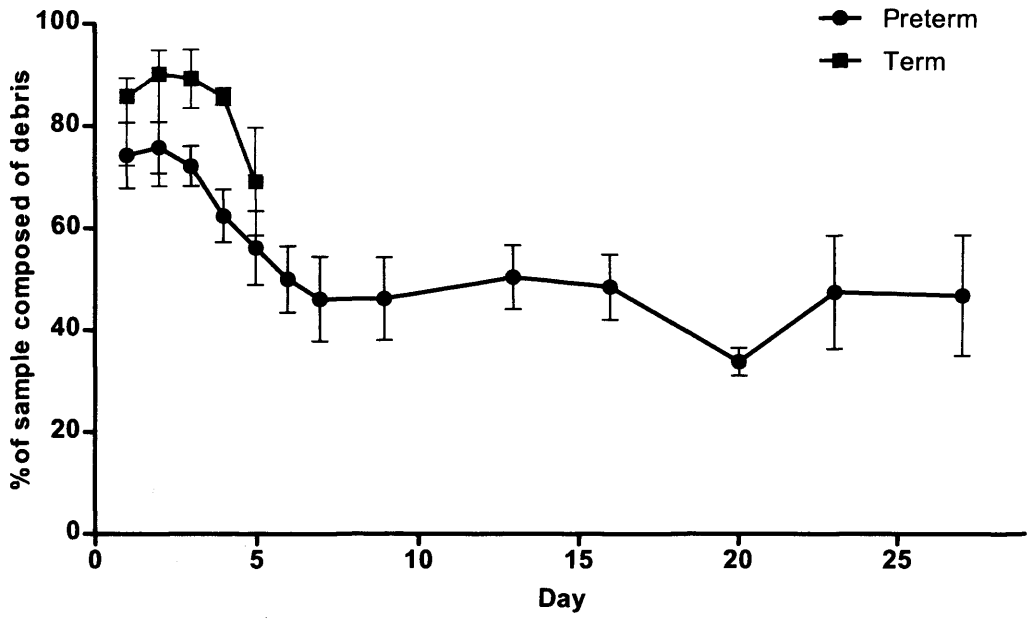


Figure 3.8 Graph showing median (and interquartile range (IQR)) percentage of BAL sample composed of debris. It can be clearly seen that the amount of debris present was highest in the first few days of sampling before decreasing to a consistently lower level after the first week of life.



A



B

Figure 3.9 Graphs showing the median percentage of BAL samples composed of debris according to (A) diagnosis and (B) gestation. Error bars represent interquartile ranges.

For the purpose of analysis of results of FACS plots for the remainder of this thesis, the debris material was gated out.

3.3.3 Total cell counts

Total cell counts obtained by haemocytometer counting were noted to be significantly greater in BAL fluid of preterm ventilated infants compared to term controls (Preterm median 1.48 x10^6 cells/ml, mean 2.24 x10^6 cells/ml, range 0-15 x10^6 cells/ml; Term median 0.69 x10^6 cells/ml, mean 0.71 x10^6 cells/ml, range 0.07 – 1.7 x10^6 cells/ml; p=0.0002, Mann-Whitney U-test, Figure 3.10B). There was no significant difference in total cell counts between infants who went on to develop CLD (mean 2.19 x 10^6 cells/ml; range x10^6 cells/ml, median 1.45 x10^6cells/ml) compared to infants whose RDS resolved (mean 2.44 x10^6 cells/ml; x10^6cells/ml, median 1.84, p=0.15, Mann-Whitney U-test, Figure 3.10A). However, both RDS and CLD groups were significantly different to term babies (Kruskal-Wallis test, p=0.0004, CLD vs term p<0.01, RDS vs term p<0.001).

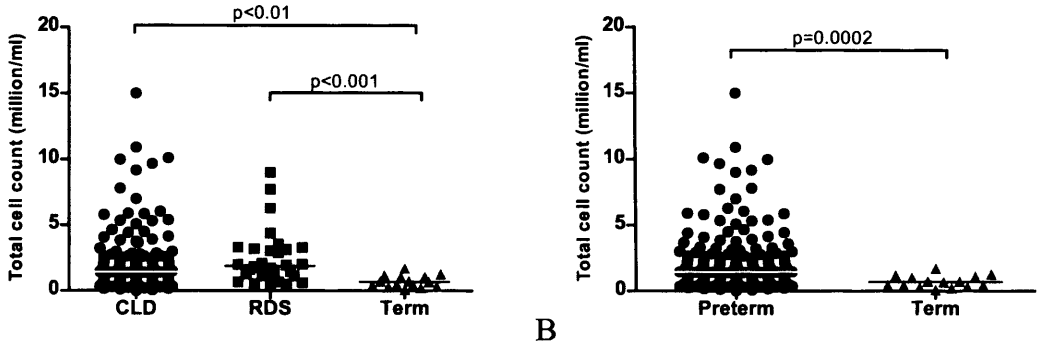


Figure 3.10 Scatterplots showing total cell count in all BAL samples by (A) diagnosis and (B) gestation. Horizontal lines represent medians.

The highest recorded (peak) total cell counts in each infant were also noted to be significantly greater in BAL fluid of preterm ventilated infants compared to term controls (Preterm median 3.86 x10^6 cells/ml, mean 4.70 x10^6 cells/ml, range 0.7-10.9 x10^6 cells/ml; Term median 1.04 x10^6 cells/ml, mean 1.033 x10^6 cells/ml, range 0.427 – 1.7 x10^6 cells/ml; p=0.0027, Mann-Whitney U-test) (Figure 3.11B). There was again no significant difference in peak total cell counts between infants who went on to develop CLD (mean 5.21 x 10^6 cells/ml; range 1.05-10.9 x10^6 cells/ml, median 5.09 x10^6 cells/ml) compared to infants whose RDS resolved (mean 3.93 x10^6 cells/ml; range 0.7-7.72 x10^6cells/ml, median 3.13 x10^6 cells/ml; p=0.332, Mann-Whitney U test) (Figure 3.11A).

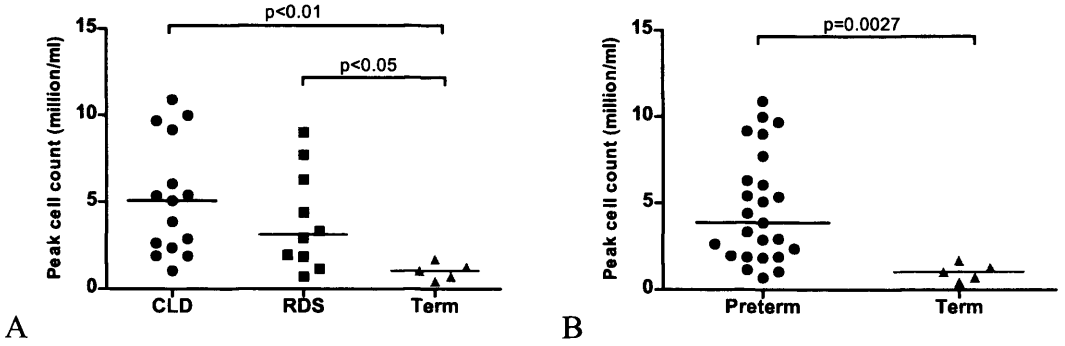


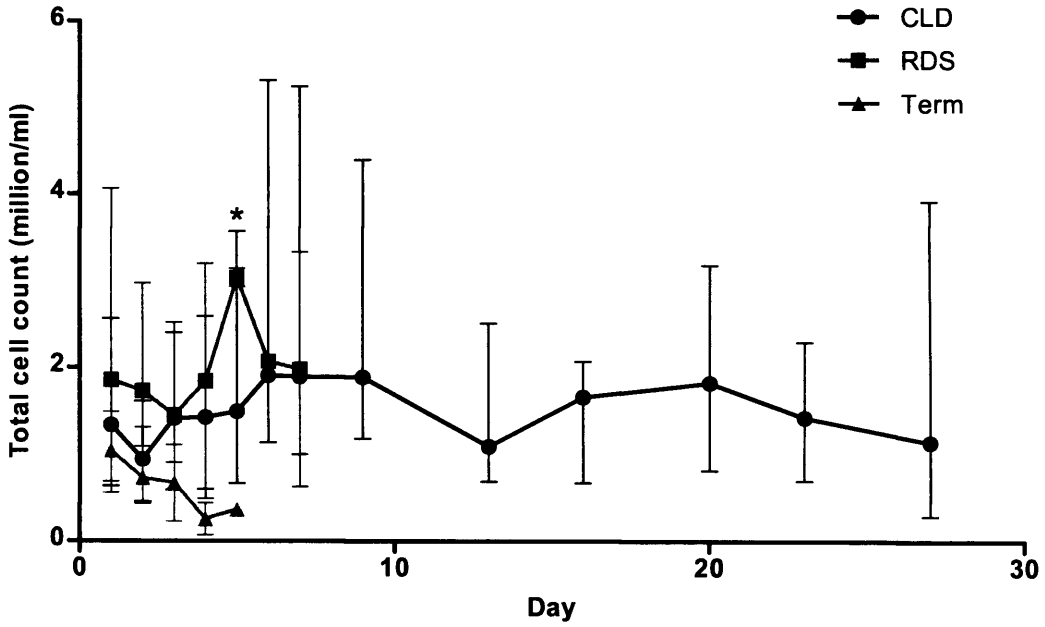
Figure 3.11 Peak cell count for all infants according to (A) diagnosis and (B) gestational group. Horizontal lines display median cell counts.

The day on which the peak cell counts occur was earliest in term babies (median day of peak count = day 1, mean 1.25 days), followed by RDS babies (median day 2, mean 2.44 days) and finally in babies who get CLD (median day 7, mean 9.5 days) (Figure 3.12). This may represent a delay in the ability to mount an inflammatory response in the group who get CLD or imply that the processes by which cells are removed from the lung are more efficient in term infants. When the cell counts for only the first 5 days of life are compared (ie. while babies from all 3 groups remain ventilated), the peak cell count in babies who get CLD occurs on a median of day 2.5 (mean 2.88), implying that, while babies who develop CLD have a peak in total cell count during the first 5 days of life, a higher peak cell count occurs after the first 5 days of life too.

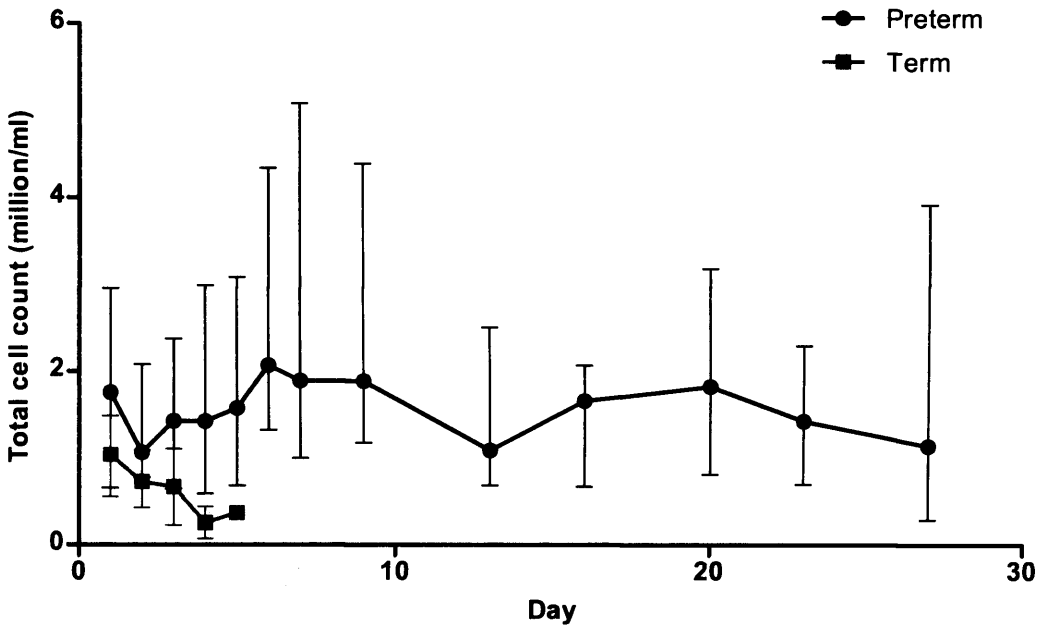
Preterm infants who get CLD have a larger number of samples due to their continuing intubation and ventilation. When looking at pooled data for the whole CLD group of babies, an apparent second rise in the total cell count, peaking around day 21 can be seen in the CLD group after the RDS and term groups have been extubated. In practice, when cell counts are measured longitudinally, episodic spikes in cell counts were observed, frequently increasing from a low baseline and then returning to baseline following such a spike. Individual patients rarely had cell counts that resemble the overall median value trends, and this can be seen in the individual patient data in figure 3.13. Factors likely to be associated with these episodic spikes will be examined in more detail later in this chapter.

When all three diagnostic groups are compared longitudinally (Figure 3.12), a significant difference between the cell counts in the three diagnostic groups can be detected on day 5 ($p=0.047$, Kruskal-Wallis test) although small numbers make comparison between the groups difficult. The trend observed is that term infants generally have lower total cell counts which fall gradually until extubation, whereas preterm infants show a marked rise in total cell count from about day 3 until the end of the first week of life.

Preterm infants have consistently higher total cell counts than their term counterparts. Possible reasons at this stage could include immaturity of regulation of cell recruitment or disposal as well as possibly a different distribution of cell types within the total which may predispose to ongoing inflammation.

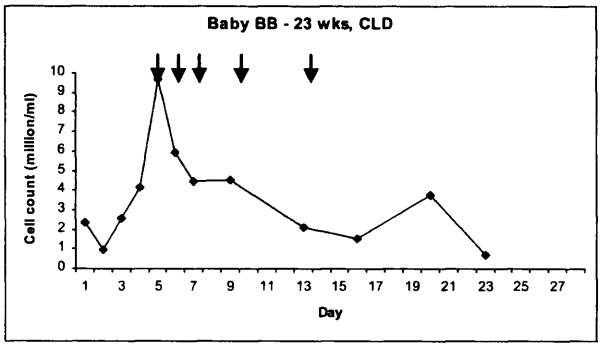


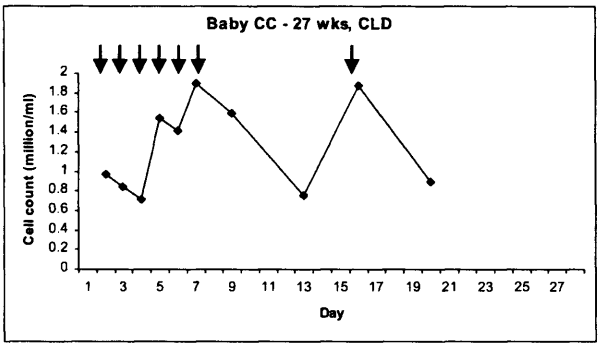
A

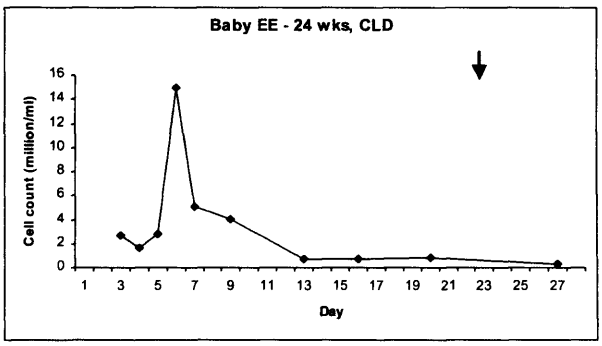


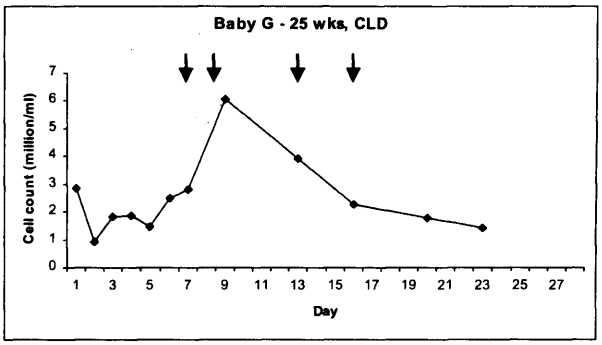
B

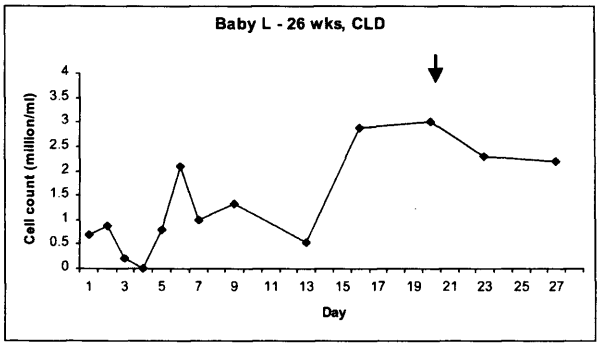
*Figure 3.12 Graphs showing longitudinal variation in median total cell count for (A) babies divided into groups based on diagnosis and (B) babies divided by gestational age. Error bars show interquartile ranges. (*p=0.047)*

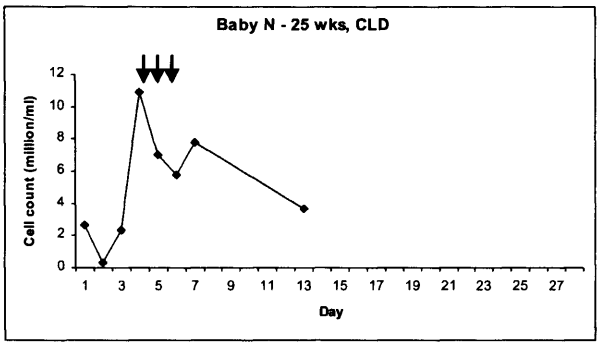

Baby BB - 23 wks, CLD
Cell count (million/ml)
Day

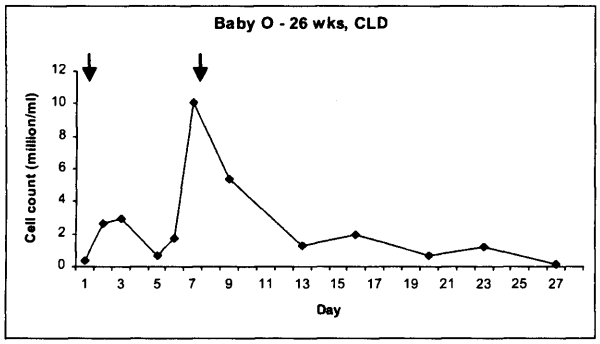

Baby CC - 27 wks, CLD
Cell count (million/ml)
Day

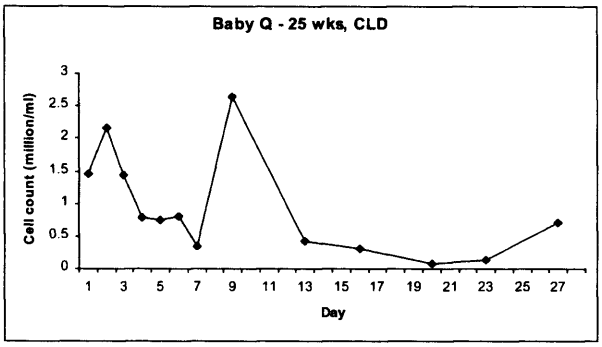

Baby EE - 24 wks, CLD
Cell count (million/ml)
Day

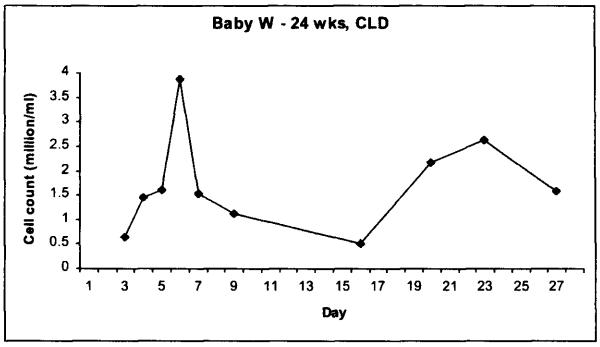

Baby G - 25 wks, CLD
Cell count (million/ml)
Day

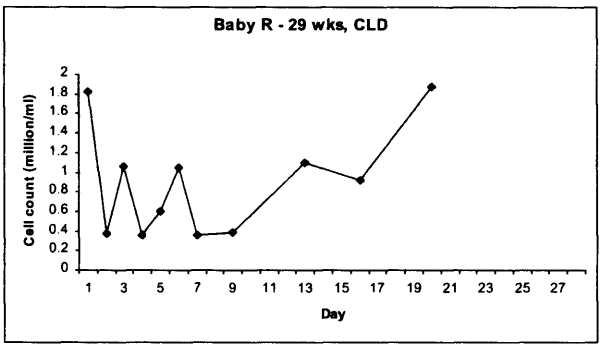

Baby L - 26 wks, CLD
Cell count (million/ml)
Day

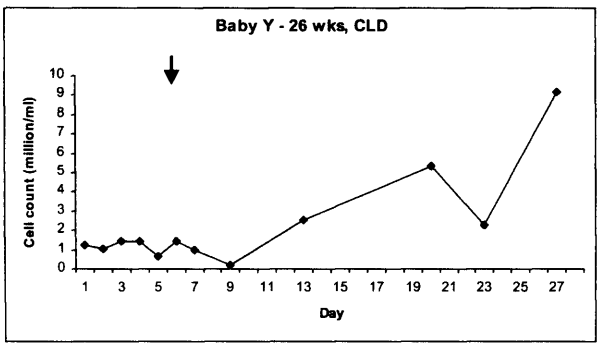

Baby N - 25 wks, CLD
Cell count (million/ml)
Day

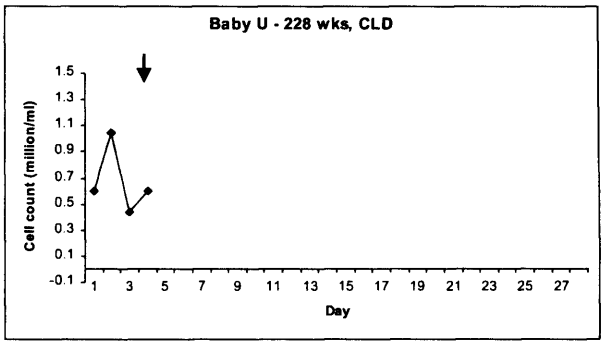

Baby O - 26 wks, CLD
Cell count (million/ml)
Day

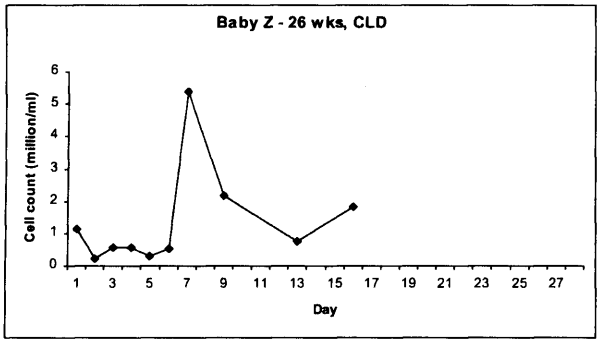

Baby Q - 25 wks, CLD
Cell count (million/ml)
Day

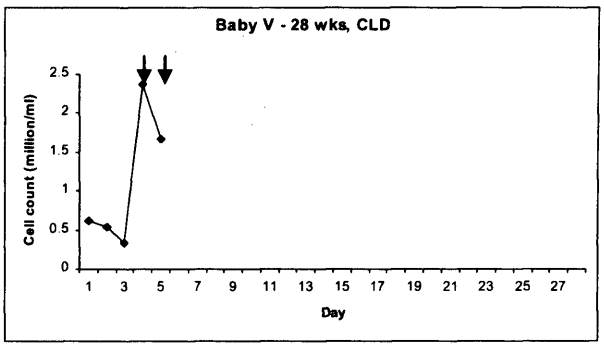

Baby W - 24 wks, CLD
Cell count (million/ml)
Day


Baby R - 29 wks, CLD
Cell count (million/ml)
Day


Baby Y - 26 wks, CLD
Cell count (million/ml)
Day


Baby U - 228 wks, CLD
Cell count (million/ml)
Day


Baby Z - 26 wks, CLD
Cell count (million/ml)
Day


Baby V - 28 wks, CLD
Cell count (million/ml)
Day

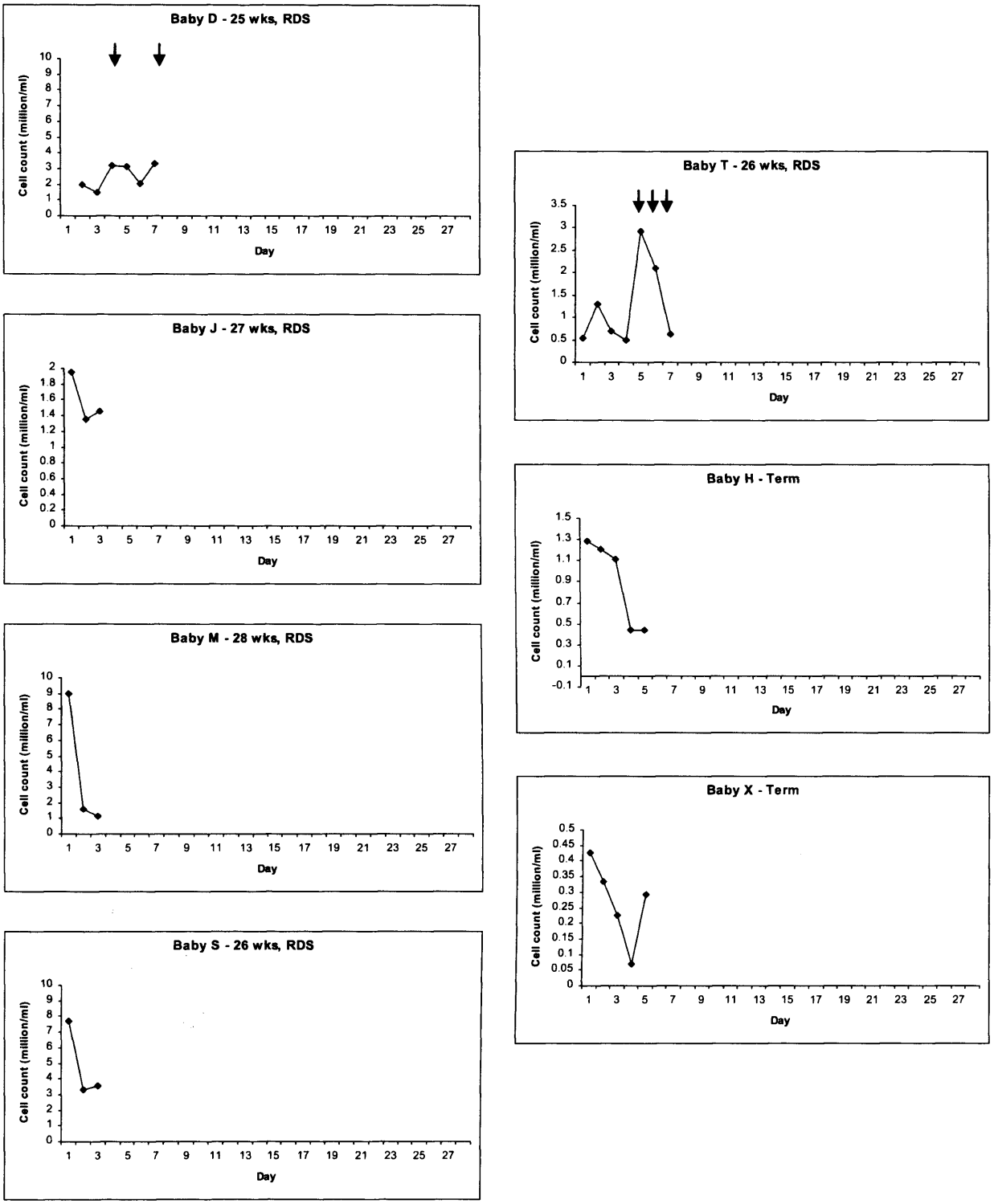
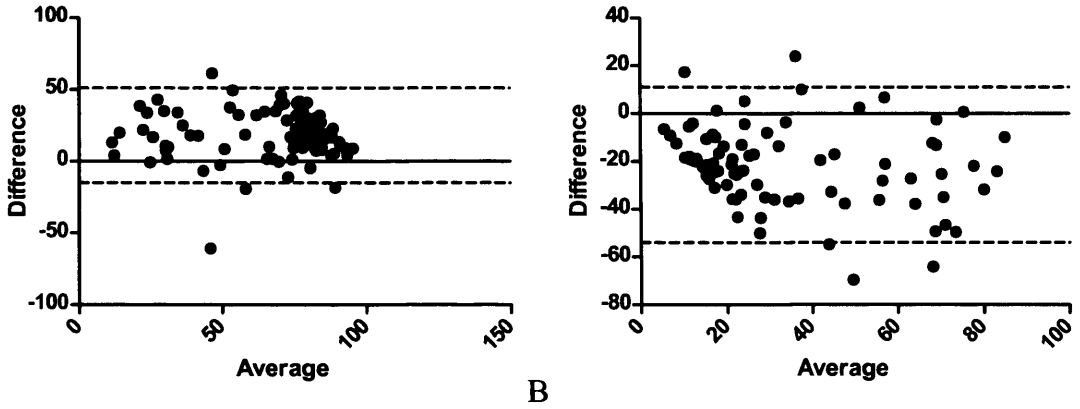


Figure 3.13 Graphs of total cell count for individual babies (only babies with 3 or more samples are represented). Late spikes in cell counts can be seen in infants ventilated over longer periods. Vertical arrow heads indicate that the BAL sample had 16S rRNA genes detected, implying the presence of microbes in the sample.

3.3.4 Differential counts

3.3.4.1 Comparison of BAL FACS data against BAL cytospin data

Differential counts of cells in BAL were obtained both by flow cytometry and by counting of cytospin preparations.



A B

Figure 3.14 Bland-Altman plots showing relationship between the differential count obtained by FACS and by microscopy of counted cytospin preparations for (A) neutrophils and (B) macrophages. Horizontal dotted lines represent 95% limits o agreement.

Overall, the cytospin preparations gave significantly higher results for the percentage of neutrophils in BAL samples (Mean cytospins 73.86; mean FACS 56.14%, $p<0.0001$, Paired t-test) but there was a reasonable level of agreement between the two measurements (Figure 3.14A). Cytospins gave significantly lower results for the percentage of macrophages in BAL samples than FACS (Mean cytospins 22.0%; mean FACS 43.31%, $p<0.0001$, paired t-test) but again there was agreement between the two observations (Figure 3.14B). Possible reasons for this are reviewed in the discussion at the end of this chapter.

As identification of cell types by flow cytometry is potentially more accurate and enables further differentiation of sub-types of cells, data acquired by flow cytometry will be presented for all following cell type data.

3.3.4.2 Neutrophil counts

Neutrophils were the predominant cell type in the majority of BAL samples, as identified by the expression of CD15 on the cell surface.

Preterm infants had significantly higher neutrophil counts than their term counterparts (Preterm mean 0.84 $x10^6$ cells/ml, median 0.26 $x10^6$ cells/ml, range 0.01-11.12 $x10^6$ cells/ml; Term mean 0.08 $x10^6$ cells/ml, median 0.04 $x10^6$ cells/ml, range 0.01-0.32 $x10^6$ cells/ml; Mann-Whitney U-test, p=0.001)(Figure 3.15B). The difference in the absolute number of neutrophils was highly significant between CLD and term infants and between RDS and term infants (CLD mean 0.84 $x10^6$ cells/ml, median 0.25 $x10^6$ cells/ml, range 0.01-11.12 $x10^6$ cells/ml; RDS mean 0.85 $x10^6$ cells/ml, median 0.62 $x10^6$ cells/ml, range 0.1-3.60 $x10^6$ cells/ml; Term mean 0.08 $x10^6$ cells/ml, median 0.04 $x10^6$ cells/ml, range 0.01-0.32 $x10^6$ cells/ml; Kruskal-Wallis test, p=0.001, CLD vs term <0.01, RDS vs term <0.001) although there was no significant difference between babies whose RDS resolved and those who developed CLD (Figure 3.15A).

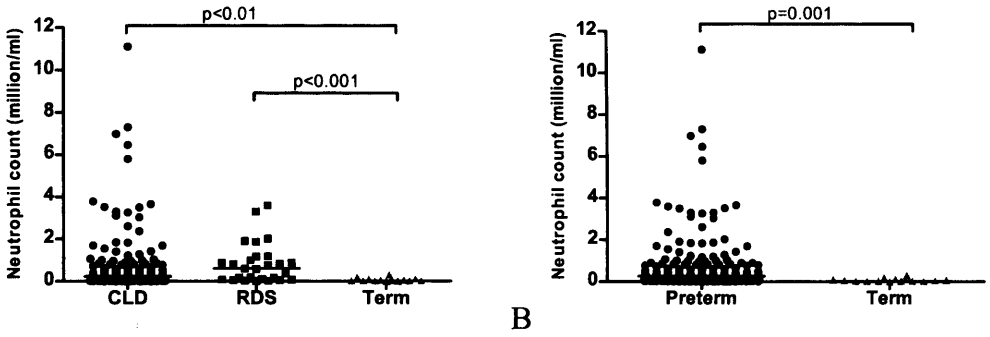


Figure 3.15 Scatterplots showing absolute neutrophil counts in BAL samples for babies according to (A) diagnosis and (B) gestation. Horizontal lines represent medians in each group.

When looking at peak values for neutrophil count, there were no significant differences between the peak neutrophil counts measured in the three diagnostic groups (Kruskal-Wallis test, p=0.089) (Figure 3.16A) or between term and preterm infants (p=0.095, Mann-Whitney U-test) (Figure 3.16B), however there was a significant difference (p<0.05) between CLD and term infants (Figure 3.16A) (CLD mean 3.64 $x10^6$ cells/ml, median 2.82 $x10^6$ cells/ml; RDS mean 1.50 $x10^6$ cells/ml, median 1.19 $x10^6$ cells/ml; term mean 0.57 $x10^6$ cells/ml, median 0.25 $x10^6$ cells/ml;

preterm mean 2.80 x10^6 cells/ml, median 1.91 x10^6 cells/ml). This suggests that overall levels of neutrophils were higher in the preterm infants but that the peak levels attained did not differ statistically.

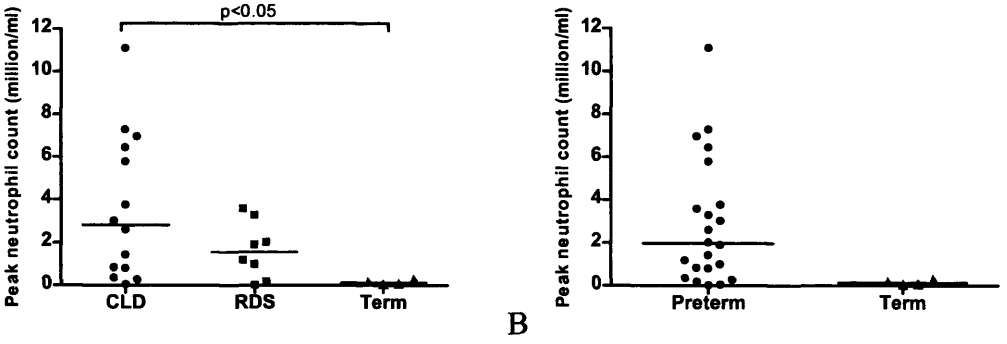
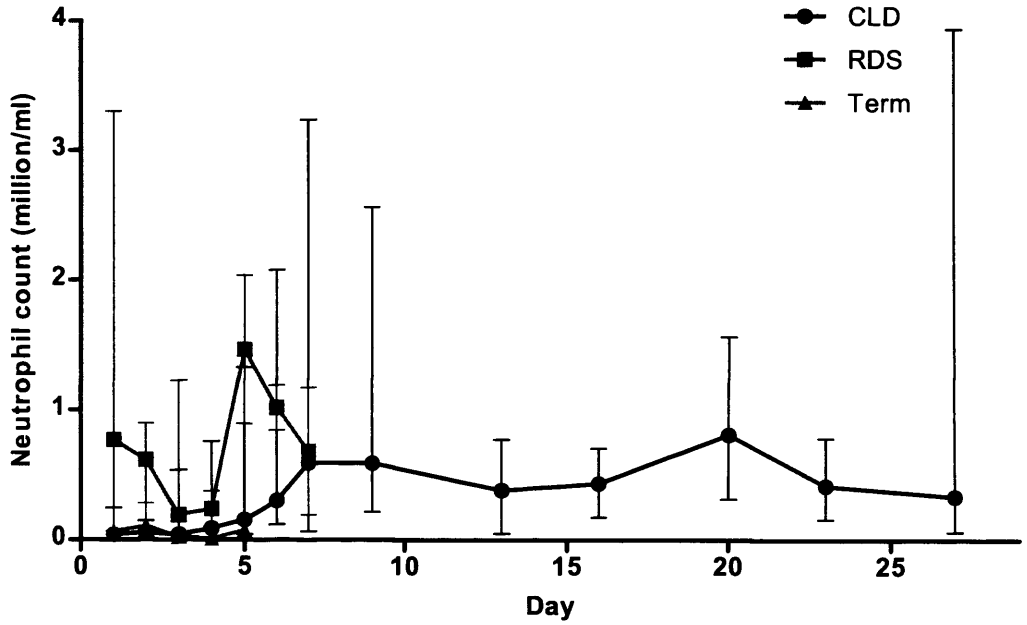


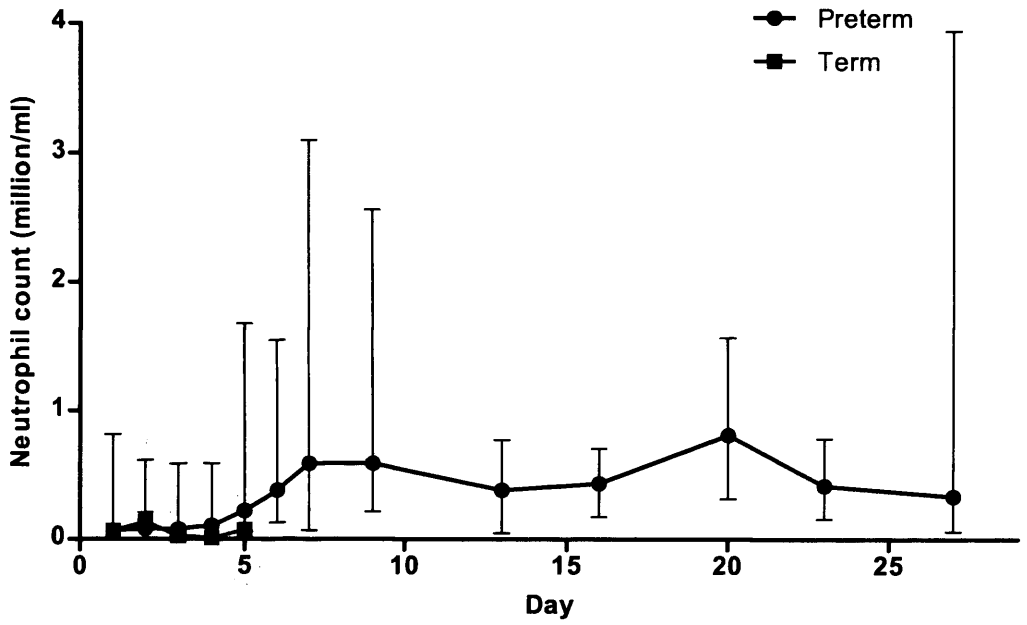
Figure 3.16 Scatterplots representing peak neutrophil counts for each infant according to (A) diagnosis and (B) gestation. Horizontal lines indicate medians.

The days on which these peaks occurred may indicate a difference in the ability of the infants to recruit inflammatory cells to the lung. Neutrophil counts achieved their peak values earliest in term infants and babies with RDS (Term mean 2.5 days median 2 days; RDS mean 2.5 days median 2 days) and much later in CLD (mean 10.1 days median 7 days) (Figure 3.17A) – in other words the peak neutrophil counts in the CLD group tend to occur after almost all of their RDS and term counterparts had been extubated.

If data for only the first 5 days of life are compared, babies who go on to get CLD show a peak at a mean of 3.3 days (median 3.5 days) while RDS and term values remain unchanged. In other words the peak of neutrophil influx is still somewhat delayed in CLD infants compared to RDS and term babies. This may be a true delay in postnatal neutrophil influx the CLD group or may be as a result of earlier (perhaps antenatal) neutrophil influx in the other two groups or simply because it takes longer for such a large number of cells to leave the circulation and migrate into the airways.



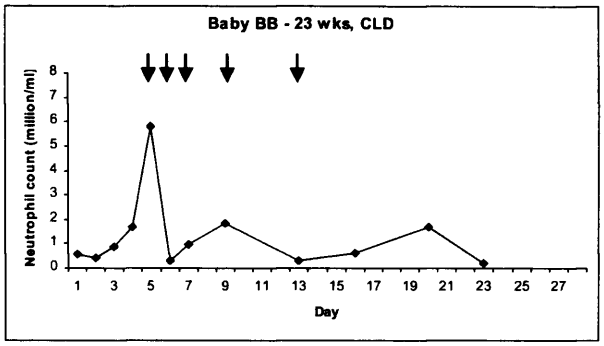
A

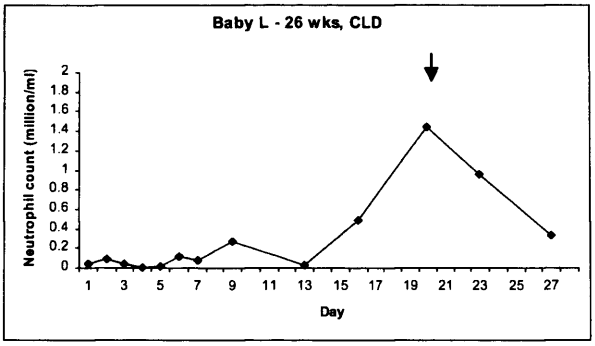


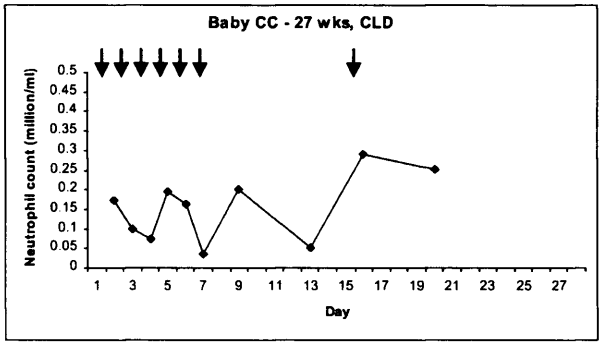
B

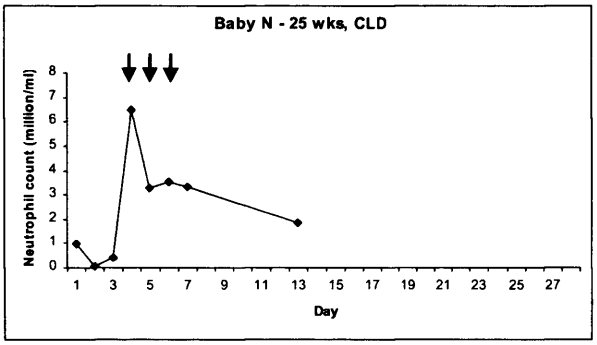
Figure 3.17 Graphs showing median (and interquartile range) neutrophil count over time according to (A) diagnostic group and (B) gestational age.

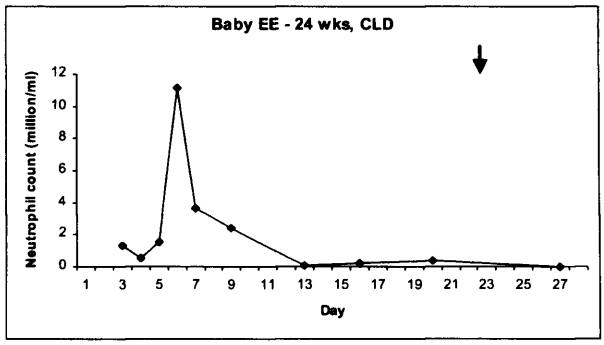
In figure 3.17(A), it can be seen that in babies whose RDS resolved, the median absolute neutrophil count was higher than either of the other groups on each day studied. This may be partly as a result of the small number of infants in the group who remained ventilated for longer periods, particularly on days 5 and 6, where the result shown on the graph represents a "median" of only two or three babies' results. For this reason, patterns of BAL fluid neutrophil counts for individual babies over the duration of their time on ventilation may be more informative than aggregated data. Data showing the BAL neutrophil counts for individual infants with 3 or more BAL samples is shown in Figure 3.18.

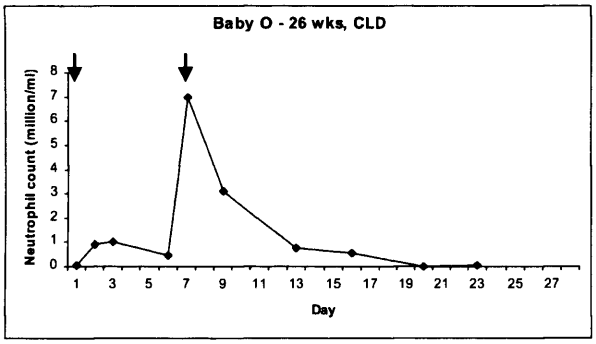

Baby BB - 23 wks, CLD
Neutrophil count (million/ml)
Day

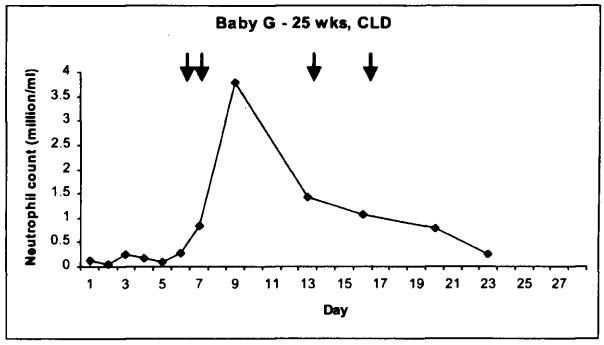

Baby L - 26 wks, CLD
Neutrophil count (million/ml)
Day

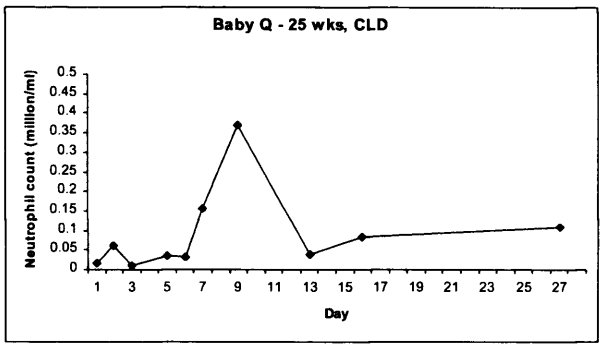

Baby CC - 27 wks, CLD
Neutrophil count (million/ml)
Day

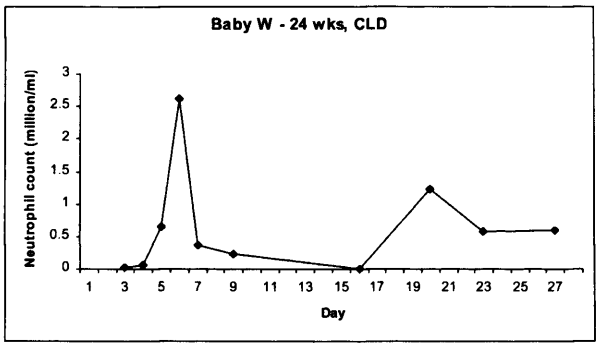

Baby N - 25 wks, CLD
Neutrophil count (million/ml)
Day

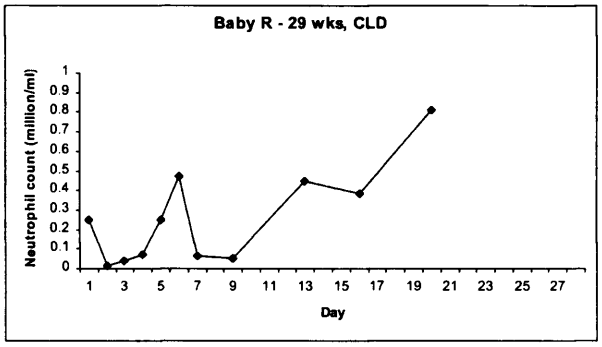

Baby EE - 24 wks, CLD
Neutrophil count (million/ml)
Day

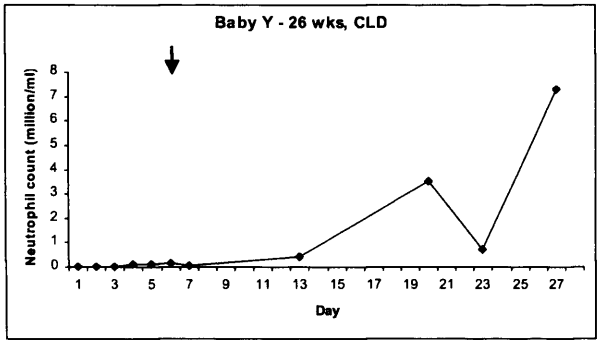

Baby O - 26 wks, CLD
Neutrophil count (million/ml)
Day

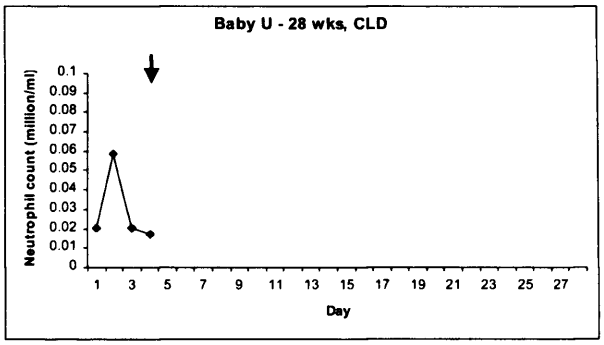

Baby G - 25 wks, CLD
Neutrophil count (million/ml)
Day

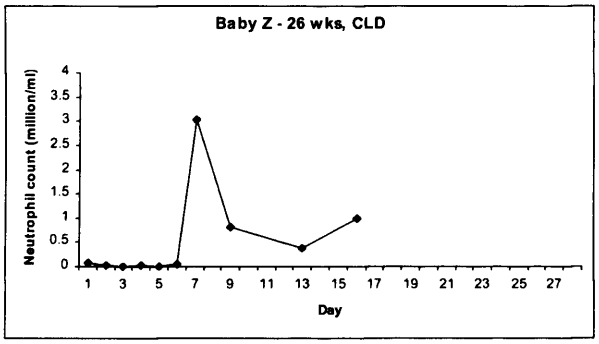

Baby Q - 25 wks, CLD
Neutrophil count (million/ml)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Day

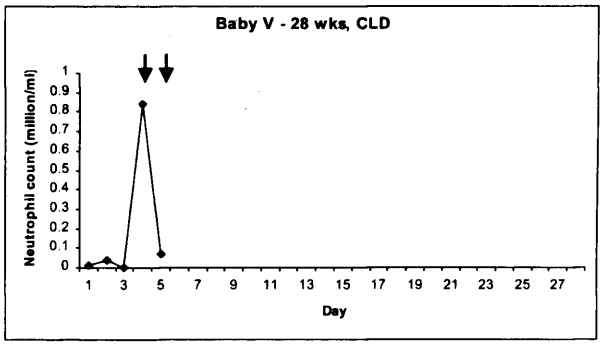

Baby W - 24 wks, CLD
Neutrophil count (million/ml)
3
2.5
2
1.5
1
0.5
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Day


Baby R - 29 wks, CLD
Neutrophil count (million/ml)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Day


Baby Y - 26 wks, CLD
Neutrophil count (million/ml)
8
7
6
5
4
3
2
1
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Day


Baby U - 28 wks, CLD
Neutrophil count (million/ml)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Day


Baby Z - 26 wks, CLD
Neutrophil count (million/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Day


Baby V - 28 wks, CLD
Neutrophil count (million/ml)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Day

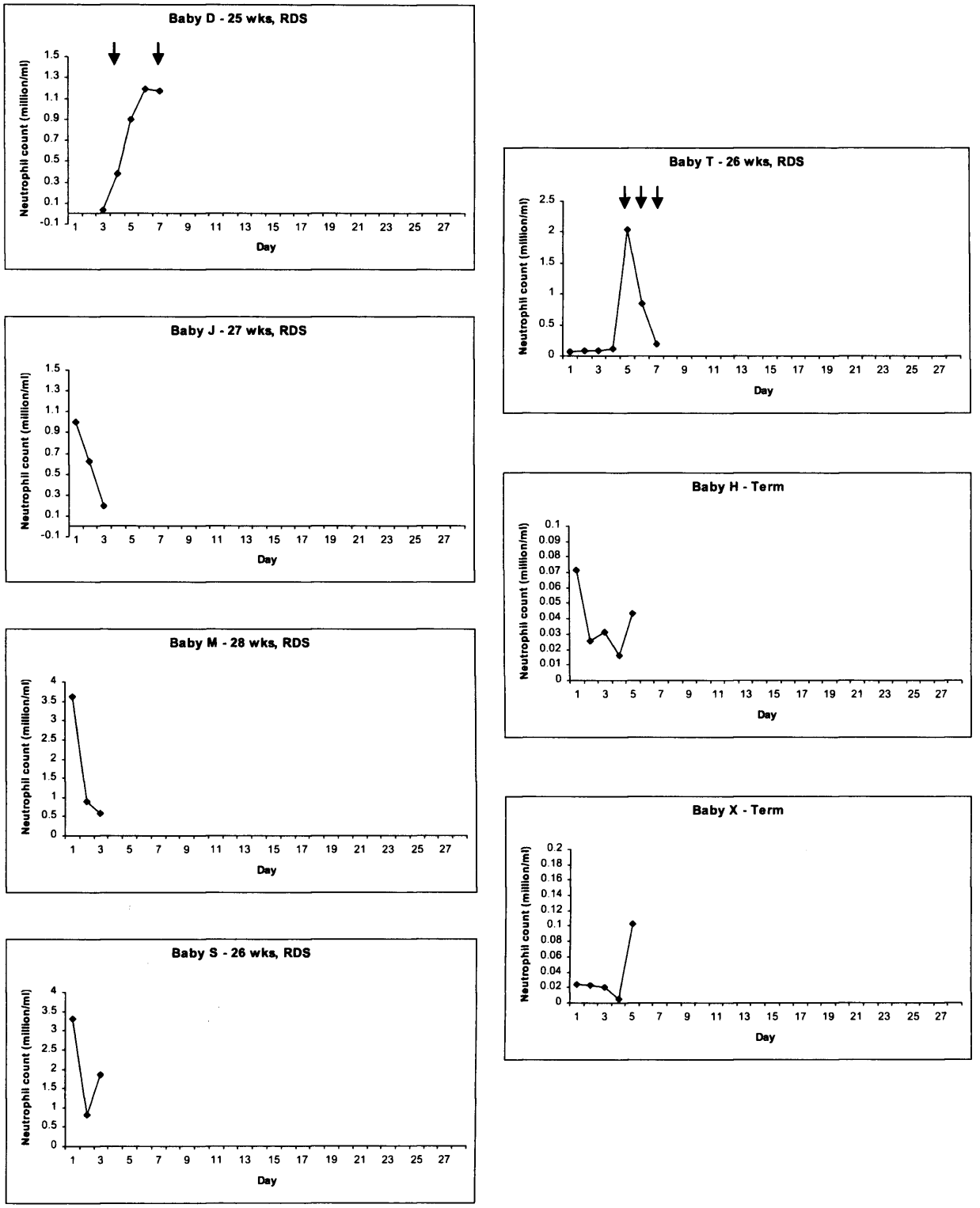


Figure 3.18 Absolute neutrophil count (as identified by flow cytometry) for all infants with 3 or more BAL samples taken. Arrows represent days on which microbial DNA was isolated from the BAL.

3.3.4.3 Neutrophil cell surface markers

Neutrophils are the first line of defence in the innate immune system. They express a number of molecules on the cell surface to enable them to respond to foreign material, which include CD14, TLR2 and TLR4. There were fewer samples assessed for these markers as there were frequently insufficient cells available from the BAL sample to allow for the additional flow cytometry analysis which these markers required. Of the 207 samples collected, 80 had neutrophil CD14 assessed and 87 had TLR 2 and 4 assessed. I attempted to assess the differences in these cell surface markers in relation to gestation and diagnosis as well as any changes in the markers over the duration of ventilation.

3.3.4.3.1 CD14 expression on neutrophils

Quiescent adult neutrophils express low levels of CD14 on the cell surface (Barth et al., 2001, Alexis et al., 2001). Eighty of the 207 BAL samples had sufficient cells present in order to look more closely at CD14 expression on neutrophils. In the BAL samples analysed, I saw that some of the most preterm infants had a large proportion of the neutrophil population expressing high levels of CD14, particularly on the first 2 days of life (Figure 3.20 babies BB, CC and G) compared to term infants (Figure 3.20 babies H and X). There was no significant difference in the mean CD14 expression on neutrophils between term and preterm infants ($p=0.63$, term mean 41.43%, median 29.24%, range 3.5-95.1%; preterm mean 36.52%, median 24.08%, range 0.2-97.2%). There was no significant difference between the groups on individual days or between term and preterm infants. There were insufficient data to separate infants with RDS from those who developed CLD.

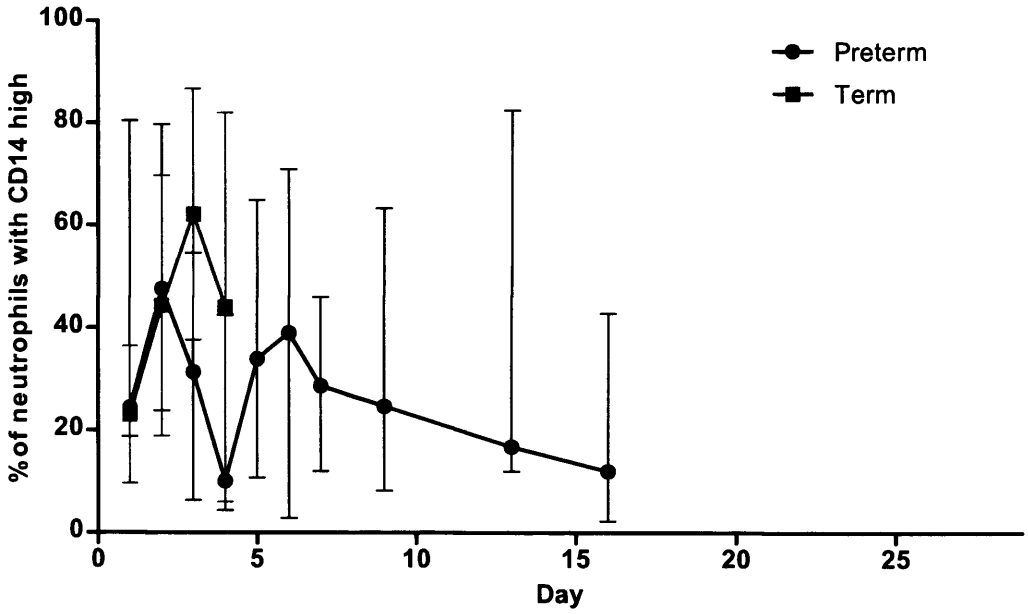
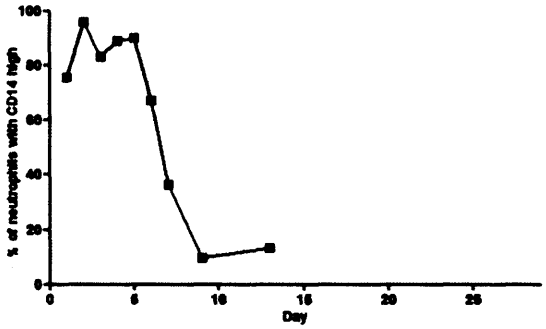
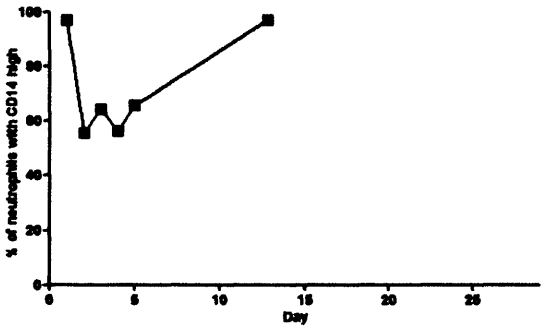


Figure 3.19 Graph showing median percentage of neutrophils with high CD14 expression in term and preterm infants. Error bars show interquartile ranges.

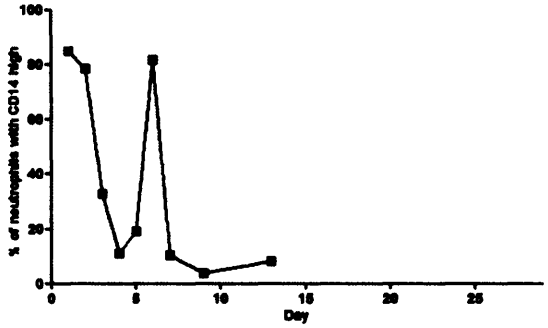
Baby BB – 23 wks, CLD



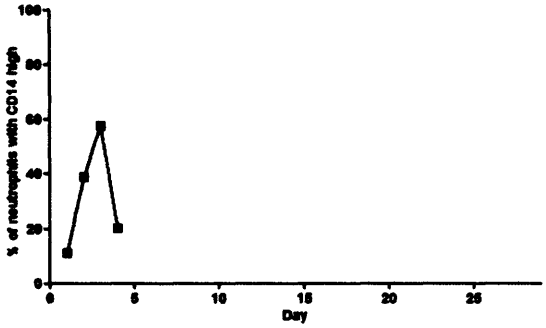
Baby G – 25 wks, CLD



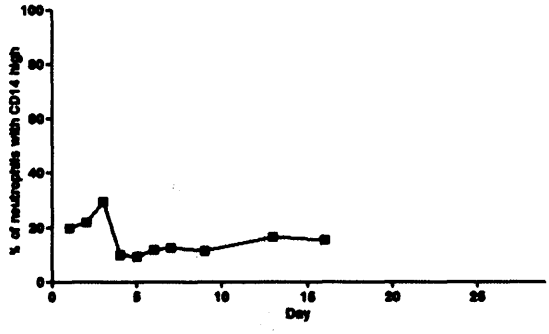
Baby CC – 27 wks, CLD



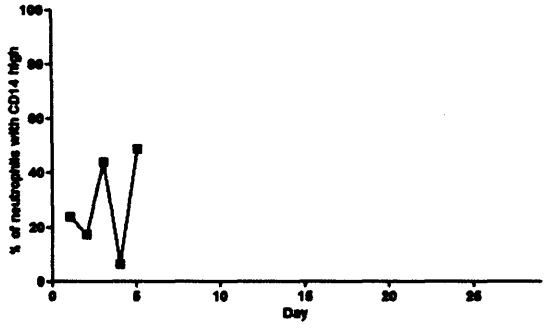
Baby U – 28 wks, CLD



Baby EE – 24 wks, CLD



Baby V – 28 wks, CLD



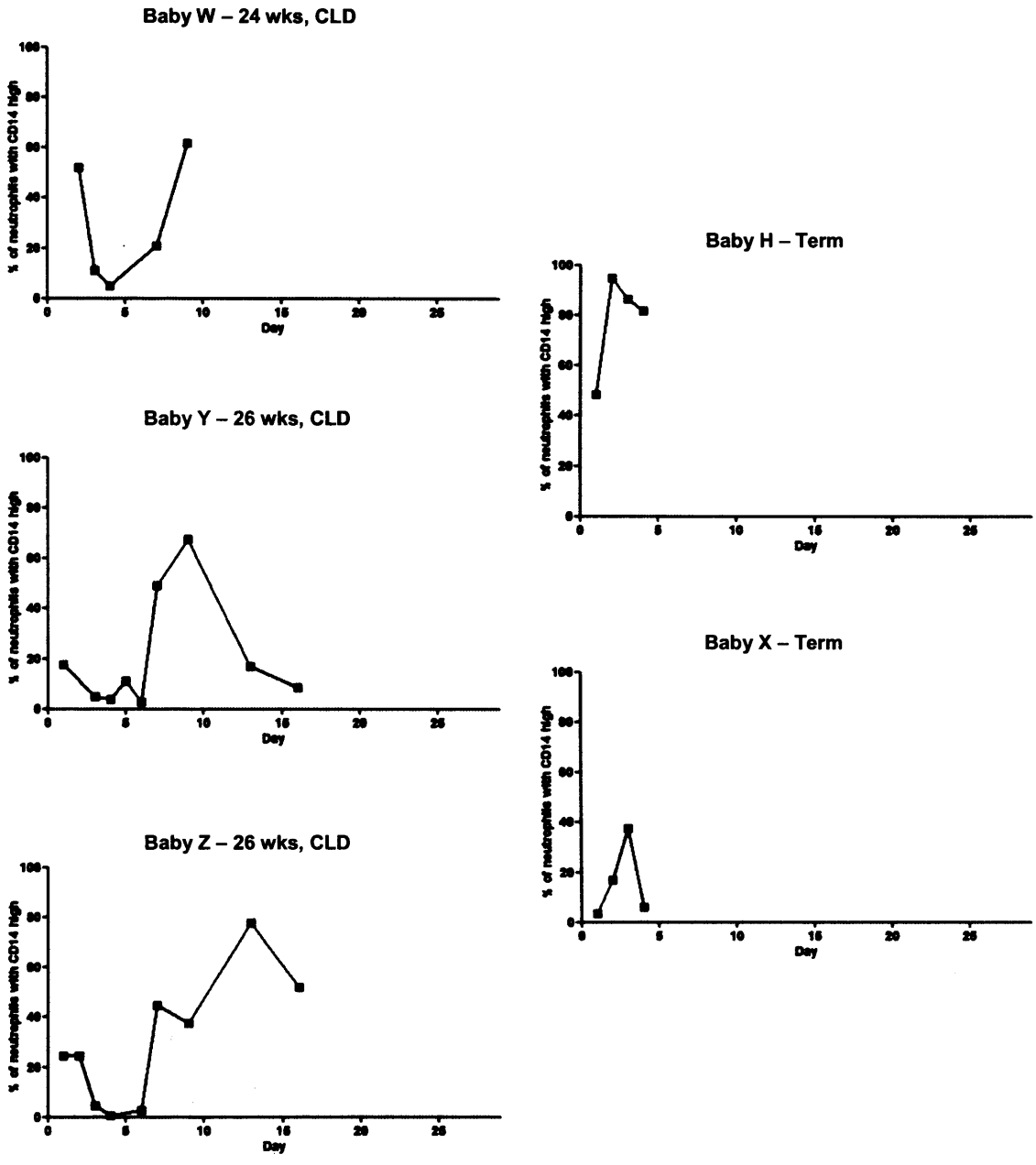


Figure 3.20 Graphs showing the percentage of neutrophils with high CD14 expression in infants with 3 or more BAL samples.

3.3.4.3.2 TLR 2 and 4 expression on neutrophils

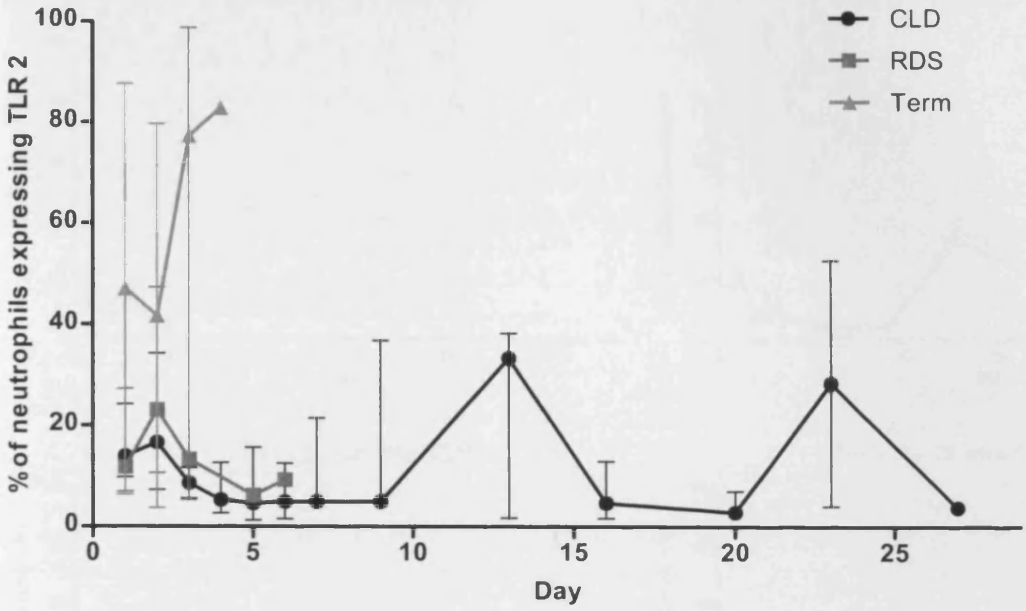
TLR 2 and 4 are both expressed in small amounts on quiescent adult neutrophils (Koller et al., 2008, Harter et al., 2004) and in lower amounts in studies on neutrophils isolated from umbilical cord blood samples (Viemann et al., 2005, Sadeghi et al., 2007) and are important for pathogen recognition by the neutrophil. No published data on TLR expression in neonatal BAL samples were found.

There was sufficient BAL sample available to study TLR expression on neutrophils in 87 of the 207 BAL samples. The percentage of BAL neutrophils expressing TLR 2 and TLR 4 was small, with TLR 2 expression slightly higher than TLR4 and both TLR 2 and TLR 4 appeared to change in their expression in parallel with one another from sample to sample.

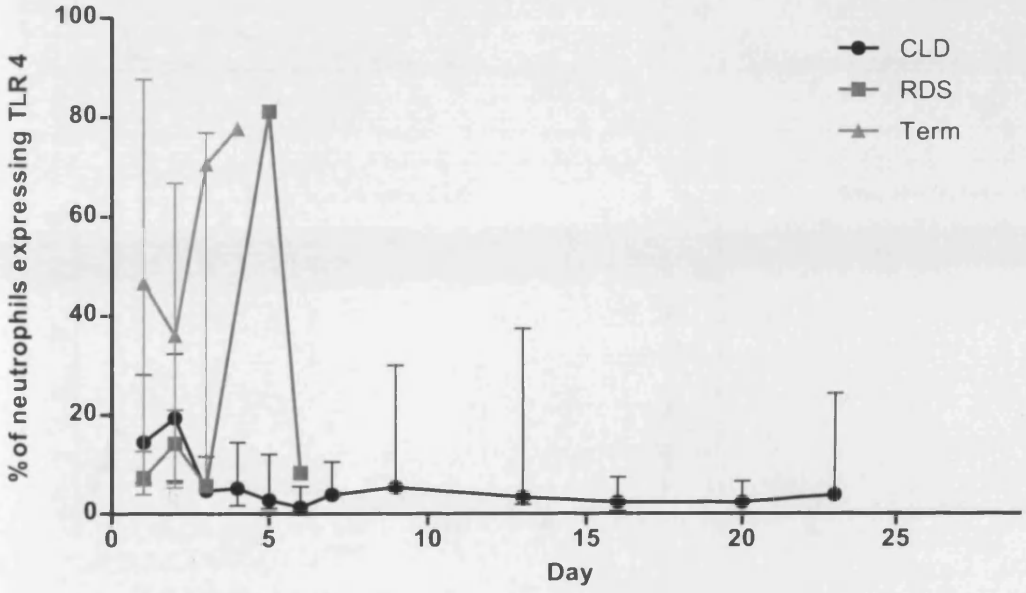
Overall the percentage of neutrophils showing detectable TLR 2 expression in term infants was significantly more than in preterm infants even in this small sample ($p=0.048$, term mean 47.66%, median 59.05%, range 2.8-87.7%; preterm mean 15.0%, median 8.1%, range 0.25-98.89%) (Figure 3.21A). There was no significant difference between infants with RDS and those who developed CLD ($p=0.159$), nor was there any difference in TLR 2 expression between the groups on individual days.

The percentage of neutrophils expressing TLR 4 was also significantly higher in term infants than their preterm counterparts ($p=0.041$, term mean 43.89%, median 51.1%, range 1.77-87.7%; preterm mean 11.89%, median 5.63%, range 0-81.2%) (Figure 3.21B), but again there was no significance between infants with RDS and those who developed CLD nor between the groups on individual days.

Together these data may indicate that preterm neutrophils may have a reduced ability to recognise pathogens in the lung, which may allow the pathogen to persist, rather than be cleared and potentiate the inflammatory response within the lung.



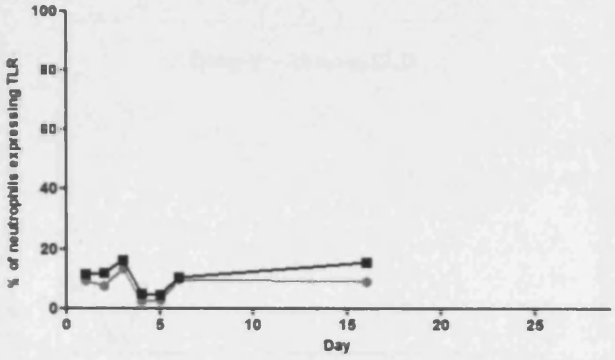
A



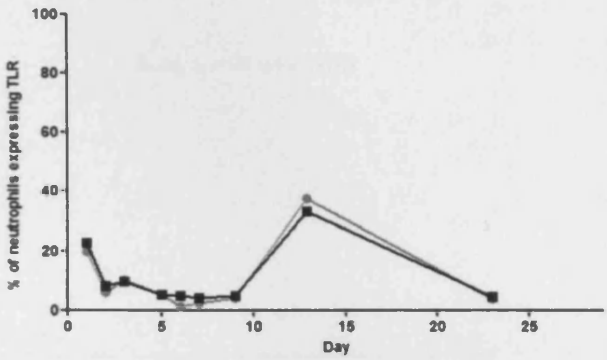
B

Figure 3.21 Graph showing median percentage of neutrophils with high (A) TLR 2 and (B) TLR 4 expression in term and preterm infants. Error bars show interquartile ranges.

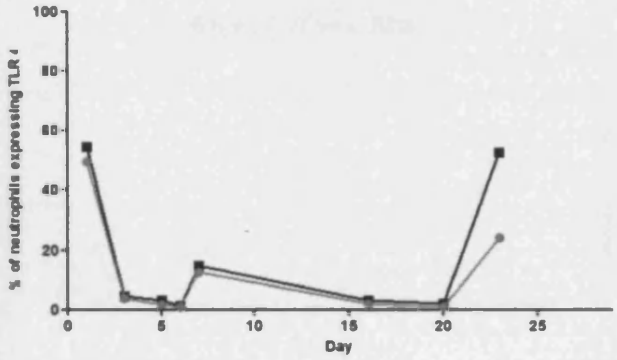
Baby EE – 24 wks, CLD



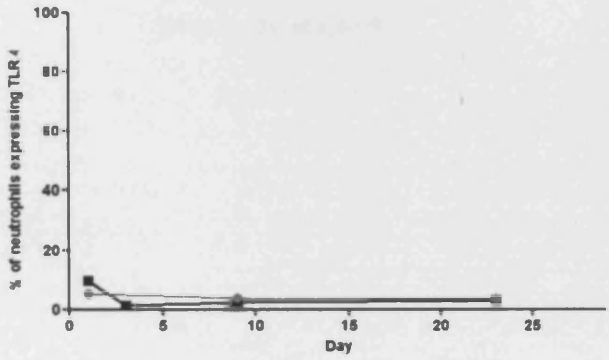
Baby O – 26 wks, CLD



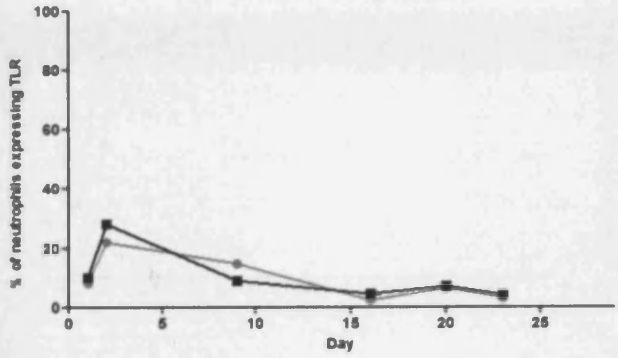
Baby G – 25 wks, CLD



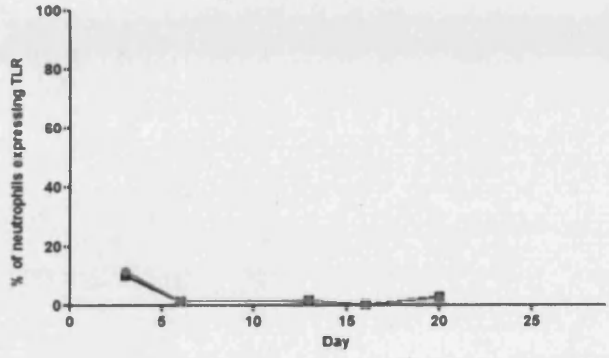
Baby Q – 25 wks, CLD



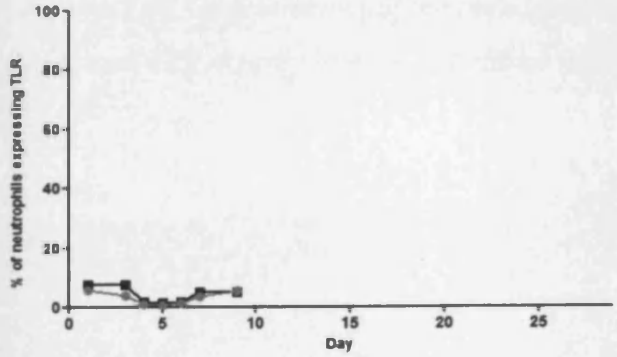
Baby L – 26 wks, CLD



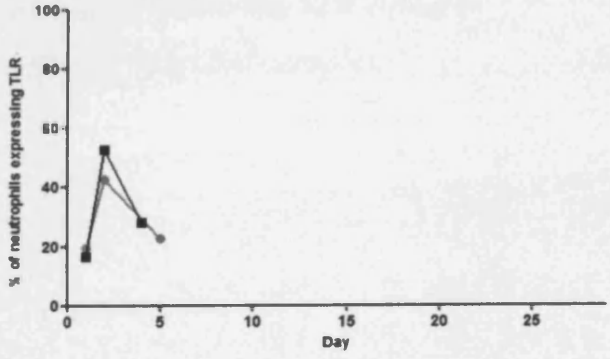
Baby R – 29 wks, CLD



Baby N – 25 wks, CLD



Baby U – 28 wks, CLD



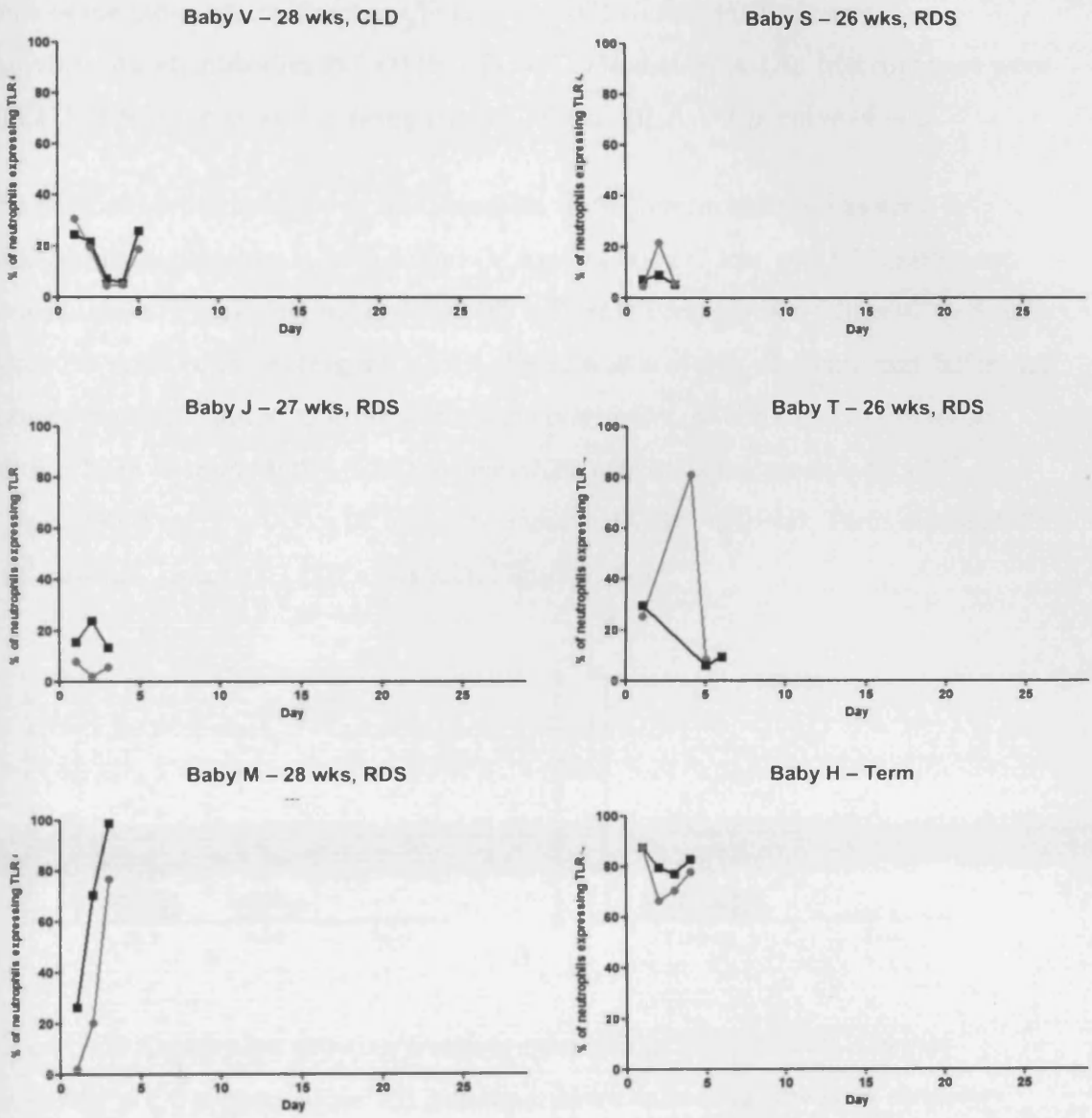


Figure 3.22 Graphs showing the percentage of neutrophils expressing TLR 2 (black line) and TLR 4 (grey line) in individual infants with 3 or more BAL samples.

3.3.4.4 Macrophages

Cells of the monocyte/macrophage lineage (MNC) were identified using combinations of antibodies to CD11b, CD14, CD36 and HLA-DR. Macrophages were all CD11b positive as well as being either CD36 or HLA-DR positive or both.

The number of macrophages in BAL samples from preterm infants was very significantly higher than in term babies (Mann-Whitney U-test, $p<0.0001$) (Preterm median 0.29 $x10^6$ cells/ml, mean 0.48 $x10^6$ cells/ml; Term median 0.05 $x10^6$ cells/ml, mean 0.07 $x10^6$ cells/ml) (Figure 3.23B). There was however no significant difference between the CLD and RDS groups (Kruskal-Wallis test, $p<0.0001$, CLD vs term <0.001, RDS vs term <0.01) (CLD median 0.29 $x10^6$ cells/ml, mean 0.49 $x10^6$ cells/ml; RDS median 0.31 $x10^6$ cells/ml, mean 0.43 $x10^6$ cells/ml; Term median 0.05 $x10^6$ cells/ml, mean 0.07 $x10^6$ cells/ml) (Figure 3.23A).

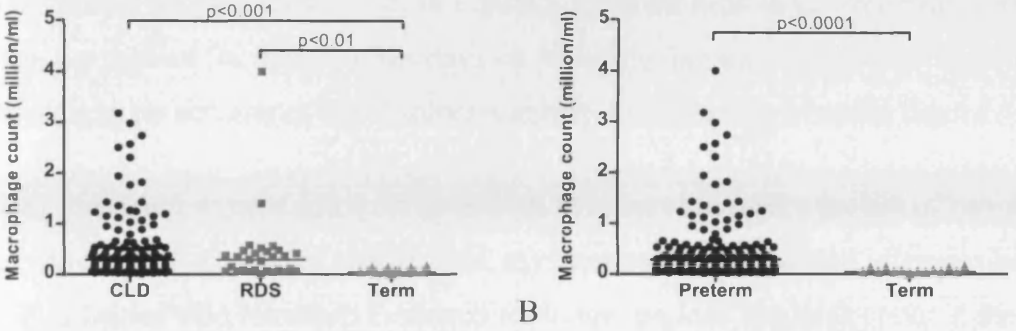


Figure 3.23 Scatterplots showing absolute macrophage count in BAL samples according to (A) diagnosis and (B) gestation. Horizontal lines represent medians.

When peaks in the macrophage count are assessed, there is a significant difference between the peak number present in term and preterm infants (Mann-Whitney U-test, $p=0.021$) (Term mean 0.13 $x10^6$ cells/ml, median 0.13 $x10^6$ cells/ml; preterm mean 1.34 $x10^6$ cells/ml, median 1.07 $x10^6$ cells/ml) (Figure 3.24B). Also, when comparing the different diagnostic groups, there is a statistically significant difference between the peak macrophage count in term infants and those who get CLD ($p<0.05$) but not between RDS and CLD or between RDS and term babies (Term mean 0.13 $x10^6$ cells/ml, median 0.13 $x10^6$ cells/ml; RDS mean 0.93 $x10^6$ cells/ml, median 0.49 $x10^6$ cells/ml; CLD mean 1.58 $x10^6$ cells/ml, median 1.60 $x10^6$ cells/ml; Kruskal-Wallis test, $p=0.023$) (Figure 3.24A).

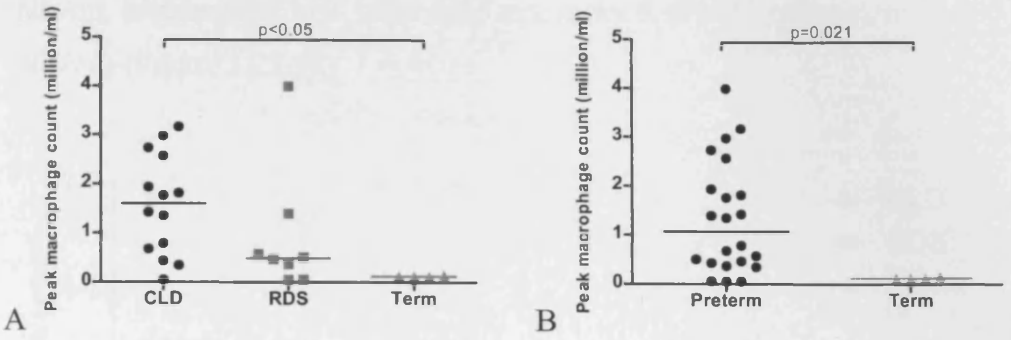


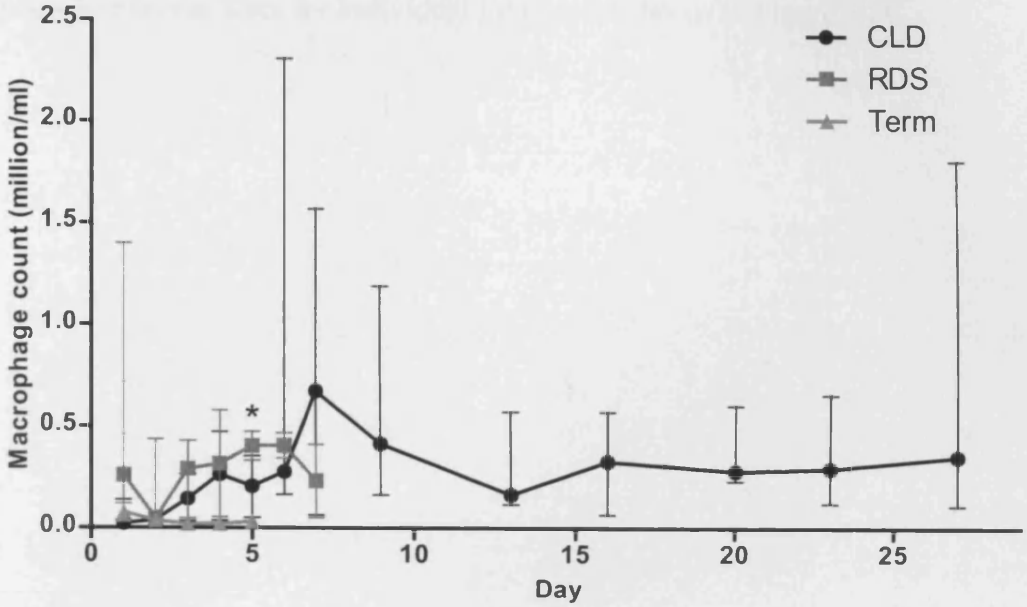
Figure 3.24 Scatterplots showing peak macrophage counts for each infant based on (A) diagnosis and (B) gestation. Horizontal lines represent medians.

The days on which peak macrophage counts occurred differed in the three groups; with term babies experiencing their highest macrophage counts on day 1 (median 1 day, mean 1.25 days), babies with RDS a little later (median 2 days, mean 2.4 days) and CLD even further delayed (median 7 days, mean 9.1 days). These days do not agree exactly with the data shown in Figure 3.25 as the peak days were calculated by taking the median (or mean) of the days on which the highest count occurred in each baby, taking no account of the absolute number of macrophages on that day.

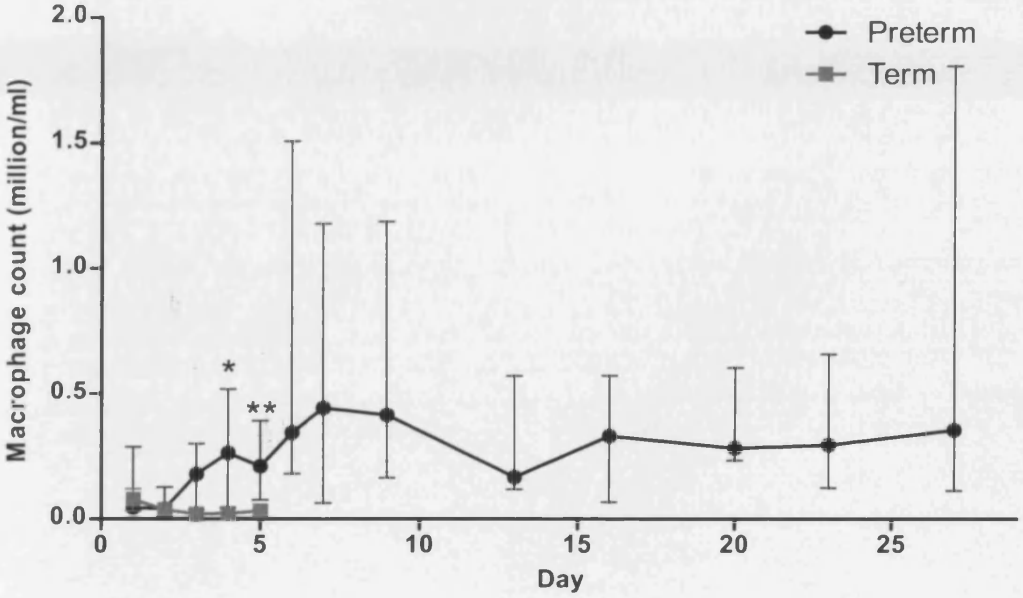
If only the first 5 days of life were reviewed, in order to compare groups of babies who were all still ventilated and exclude any later macrophage count increases in the group of babies who remained intubated for longer periods, the peak count in the CLD group was experienced after an average of 4.1 days (median 4 days), which is still later than the other two groups. This may imply a delayed monocyte influx in the CLD group relative to the other two groups or simply a longer time being required to reach the higher count, similar to that seen in neutrophil influx.

When viewed longitudinally, there is a significant difference among the 3 groups on day 5 (Kruskal–Wallis test, p=0.049) (Figure 3.25B) but numbers in each group are too small to make further comparisons between the groups. When term and preterm infants are compared, preterm infants have significantly higher macrophage counts on day 4 and day 5 than their term counterparts (Mann-Whitney U-test, p=0.019 for day 4; Preterm mean 0.37 $x10^6$ cells/ml, median 0.27 $x10^6$ cells/ml; Term mean 0.03 $x10^6$ cells/ml, median 0.03 $x10^6$ cells/ml and p=0.010 for day 5; Preterm mean 0.56 $x10^6$

cells/ml, median 0.35 x10^6 cells/ml; Term mean 0.05 x10^6 cells/ml, median 0.04 x10^6 cells/ml) (Figure 3.25A).



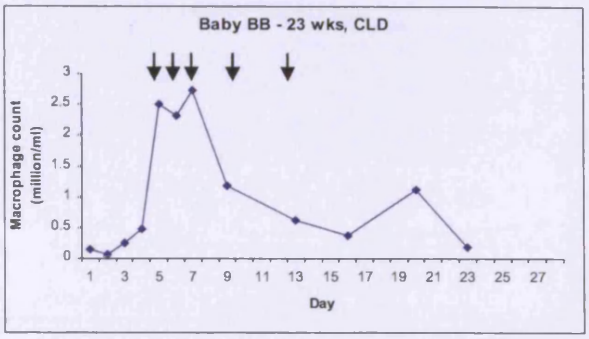
A

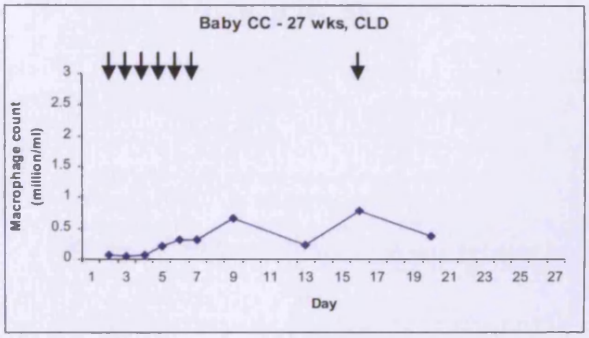


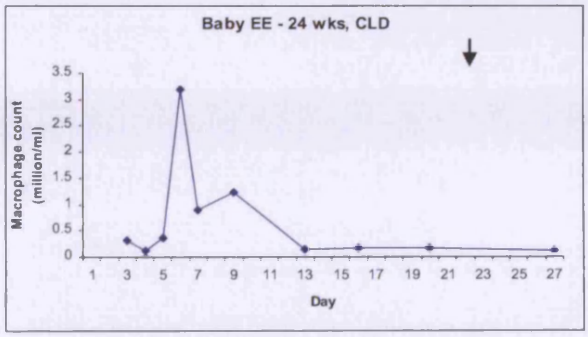
B

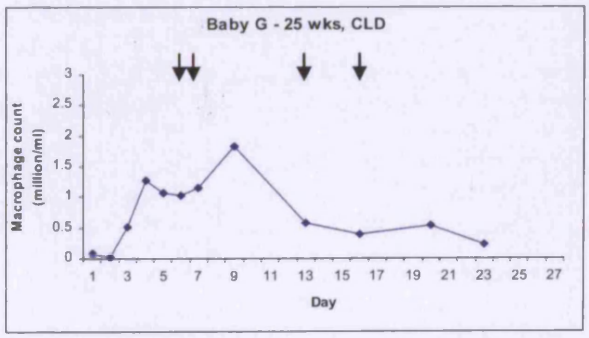
Figure 3.25 Graphs representing median (and IQR) macrophage count over the duration of the period of ventilation in all infants, according to (A) diagnosis (p=0.049) and (B) gestation (* p=0.019, ** p=0.010).*

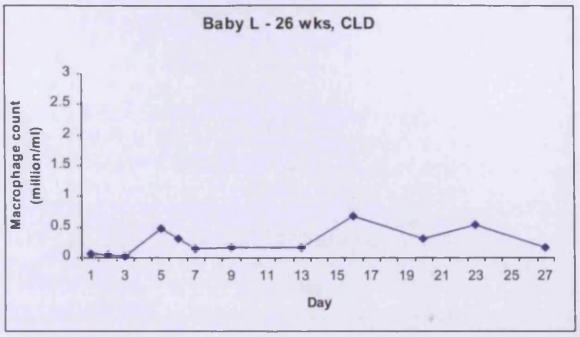
Once again, similar to neutrophil counts, aggregated data for groups of babies tend to obscure the "spikes" in macrophage count which occur in individual infants, when cell counts rise rapidly over a few days from a relatively low baseline and then return to baseline levels. Data for individual infants are shown in Figure 3.26.

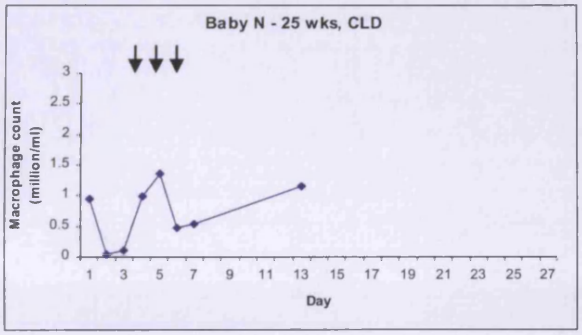

Baby BB - 23 wks, CLD
Macrophage count (million/ml)
Day

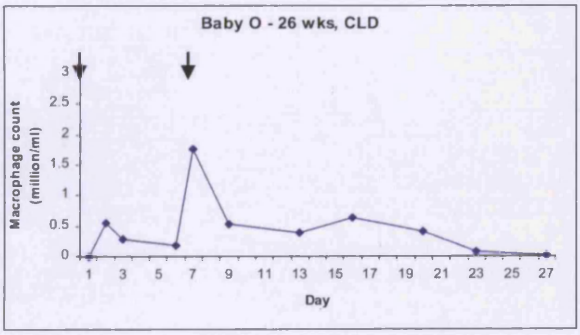

Baby CC - 27 wks, CLD
Macrophage count (million/ml)
Day

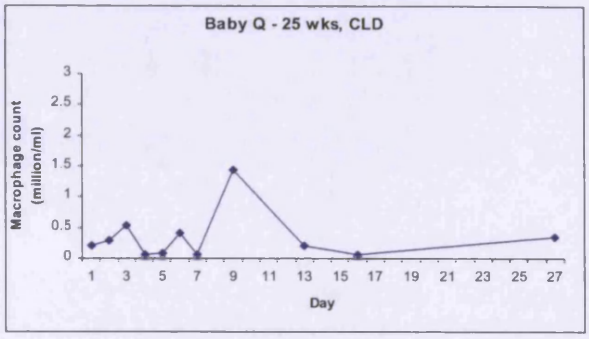

Baby EE - 24 wks, CLD
Macrophage count (million/ml)
Day

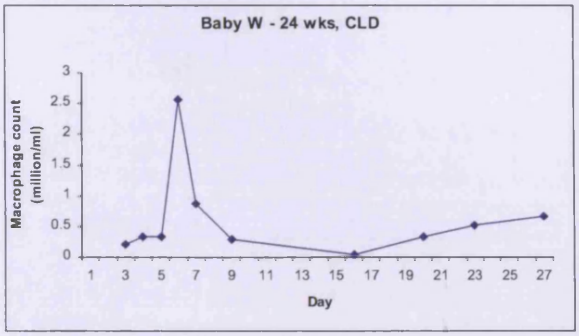

Baby G - 25 wks, CLD
Macrophage count (million/ml)
Day

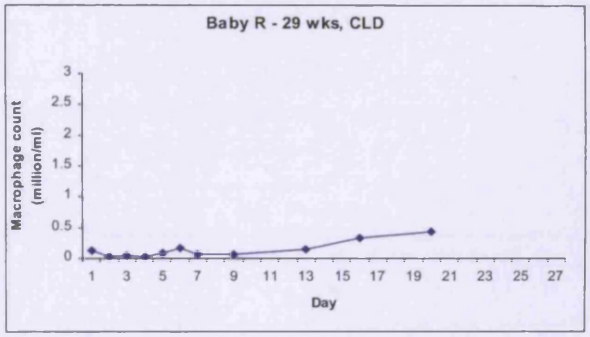

Baby L - 26 wks, CLD
Macrophage count (million/ml)
Day

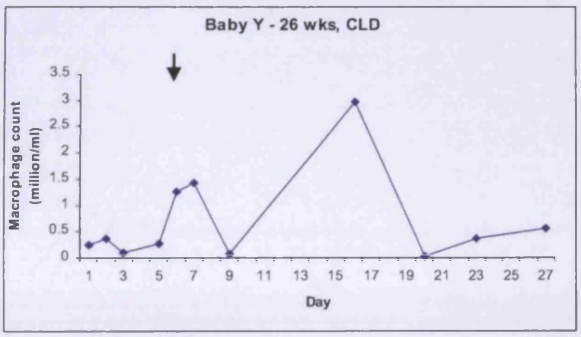

Baby N - 25 wks, CLD
Macrophage count (million/ml)
Day

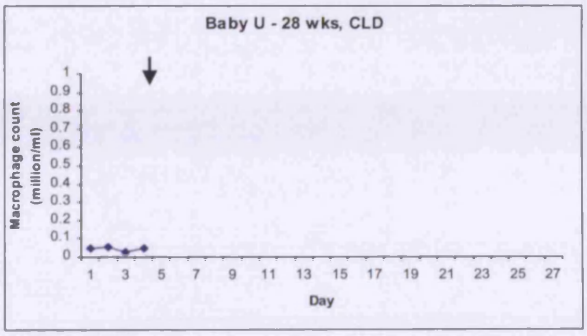

Baby O - 26 wks, CLD
Macrophage count (million/ml)
Day

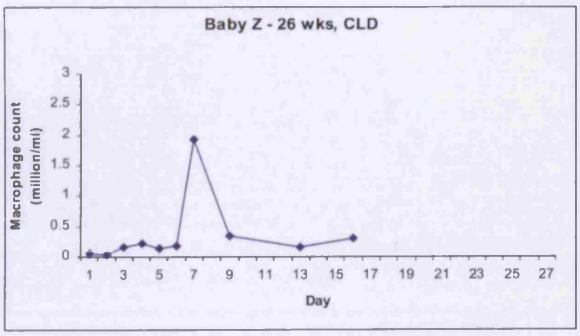

Baby Q - 25 wks, CLD
Macrophage count (million/ml)
Day

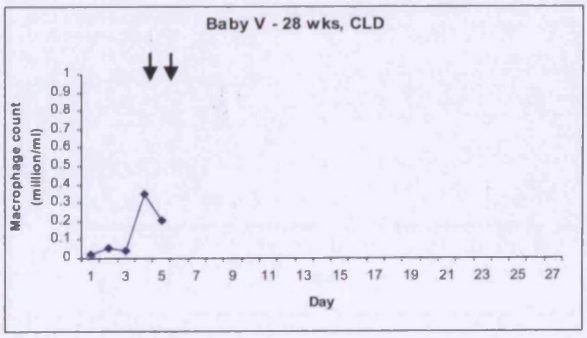

Baby W - 24 wks, CLD
Macrophage count (million/ml)
Day


Baby R - 29 wks, CLD
Macrophage count (million/ml)
Day


Baby Y - 26 wks, CLD
Macrophage count (million/ml)
Day


Baby U - 28 wks, CLD
Macrophage count (million/ml)
Day


Baby Z - 26 wks, CLD
Macrophage count (million/ml)
Day


Baby V - 28 wks, CLD
Macrophage count (million/ml)
Day

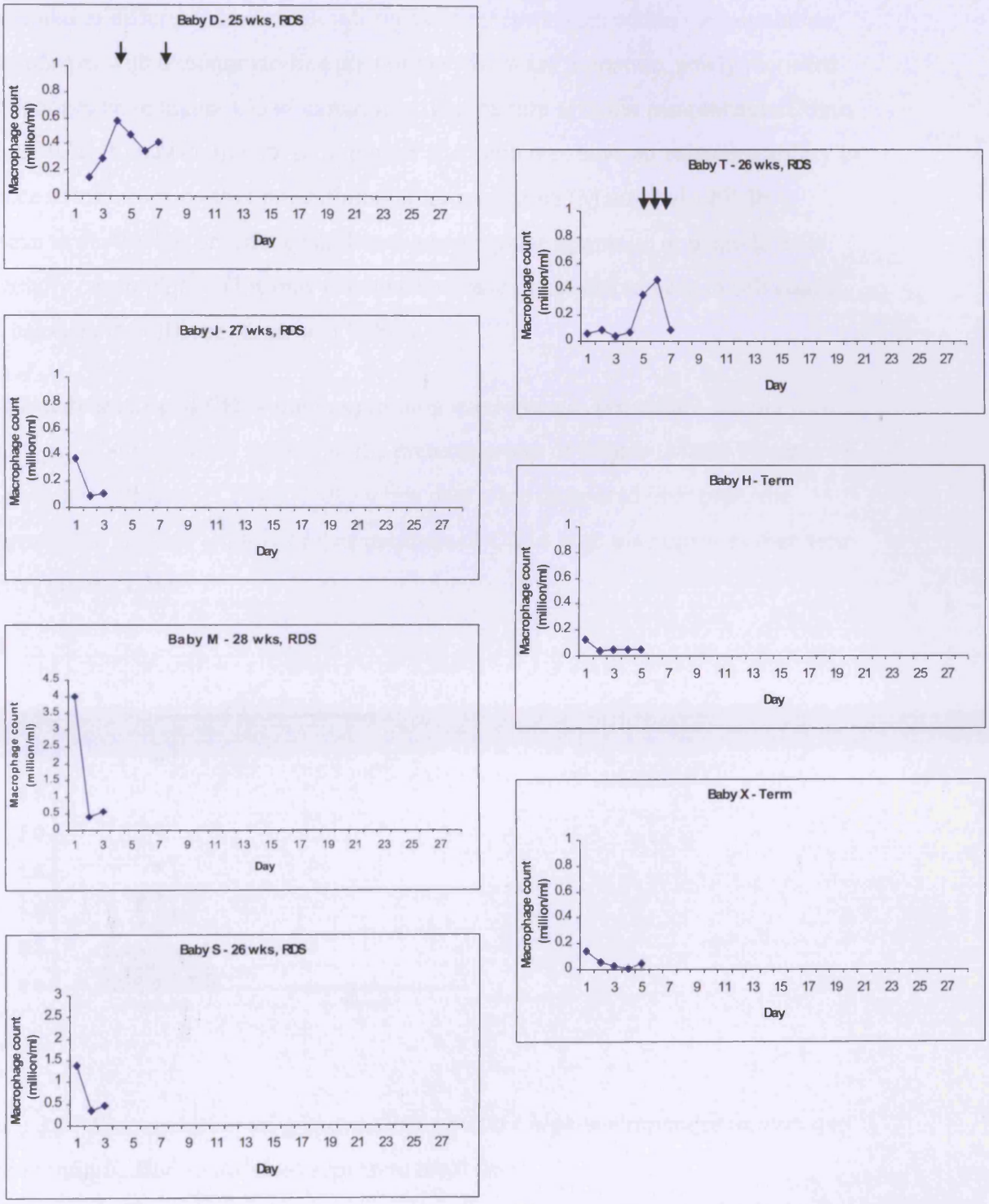


Figure 3.26 Graphs showing absolute macrophage count over time in BAL from all babies with 3 or more BAL samples. Vertical arrows represent samples in which 16S rRNA genes were detected.

3.3.4.5 Sub-types of macrophages

I attempted to differentiate various sub-types of macrophages within the population. Macrophages with a monocyte-like phenotype which are immature, newly recruited macrophages have higher CD14 expression than mature alveolar macrophages (Maus et al., 2002a). It may be that these immature macrophages have an enhanced ability to produce cytokines and other pro-inflammatory mediators (Maus et al., 2002b, Rosseau et al., 2000a) or reduced ability to phagocytose apoptotic or necrotic cells (especially neutrophils). This may account for some of the differences in cell counts seen between the different groups of babies.

The median number of CD14 high expressing macrophages (immature, monocyte-like phenotype) is significantly greater in the preterm group of infants (Mann-Whitney U-test, p=0.0002) (Figure 3.27) and also when data were compared over time, the preterm group showed slightly higher numbers of CD14 high macrophages than term infants (Figure 3.28).

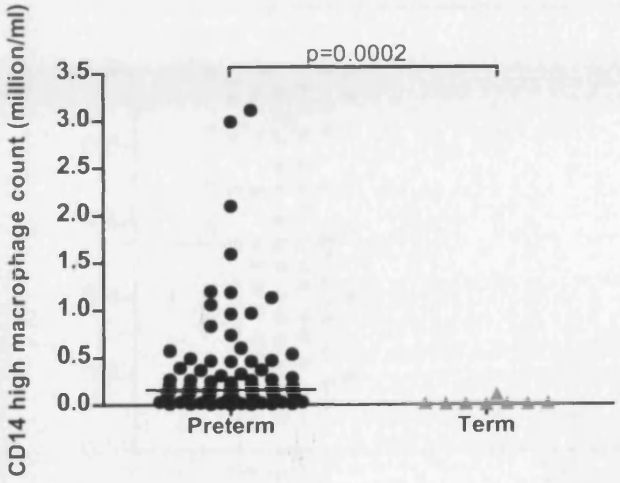


Figure 3.27 Scatterplot showing the number of CD14 high macrophages in term and preterm infants. Horizontal lines represent medians.

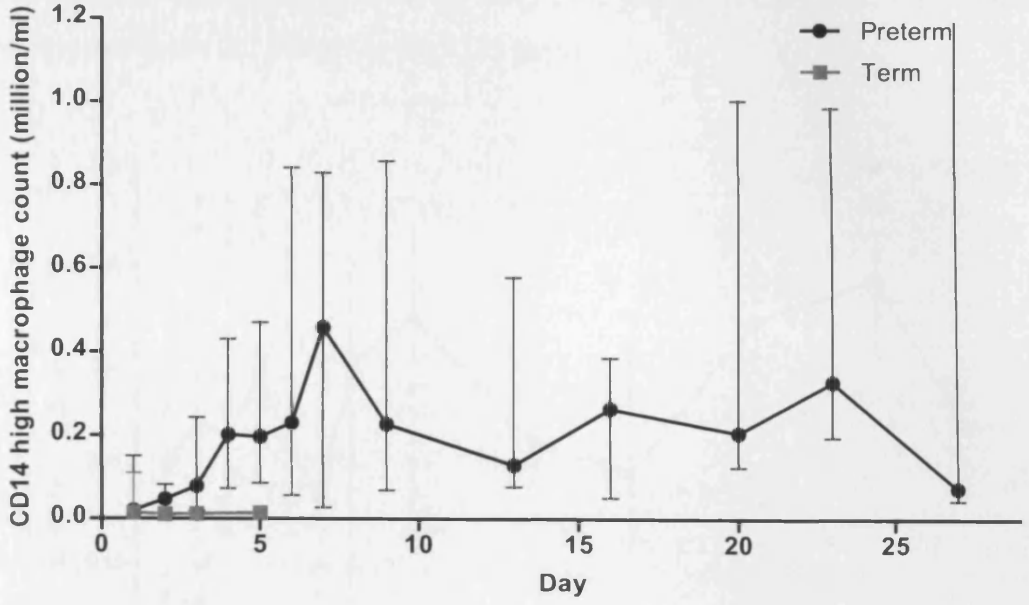


Figure 3.28 Median immature (CD14 high) macrophage count over time in term and preterm infants. Error bars represent interquartile ranges.

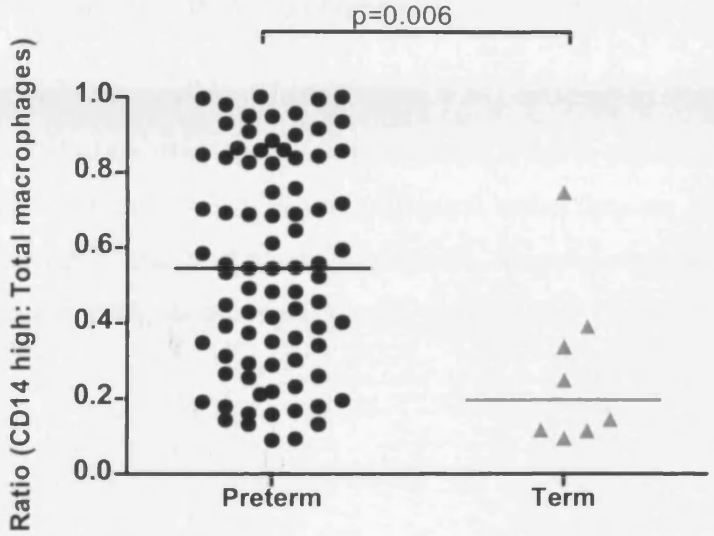


Figure 3.29 Scatterplot showing the ratio of CD14 high macrophages to the total macrophage count. Horizontal lines represent medians.

The immature macrophage phenotype was seen in a larger proportion of the total macrophage population in babies of preterm gestations (Mann-Whitney U-test, p=0.006) (Preterm median 0.55, mean 0.56; Term median 0.20, mean 0.28) (Figure

3.29). It was not possible to compare diagnostic groups as there were insufficient data on this parameter for babies in the RDS group.

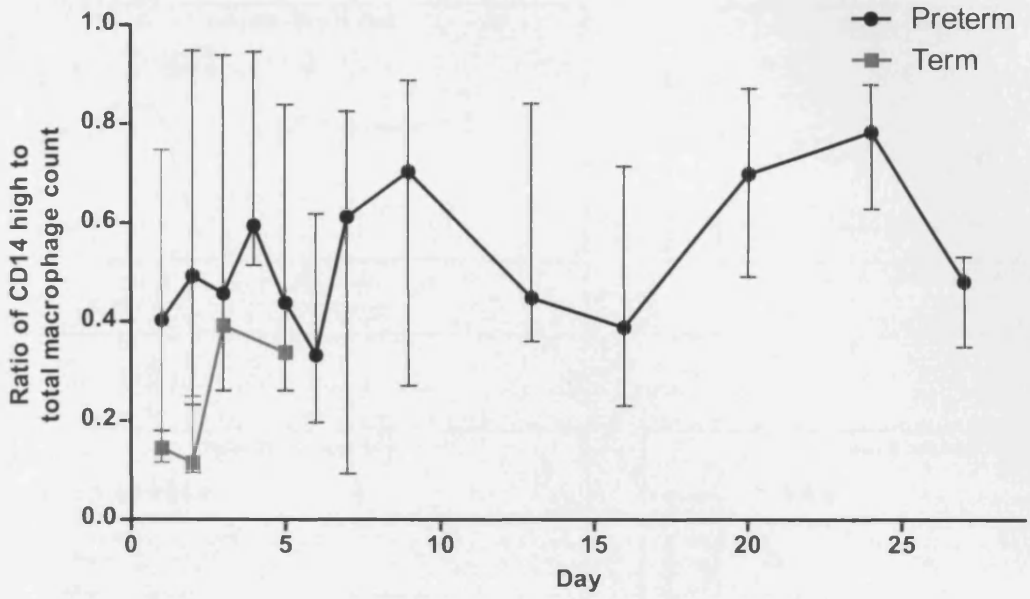
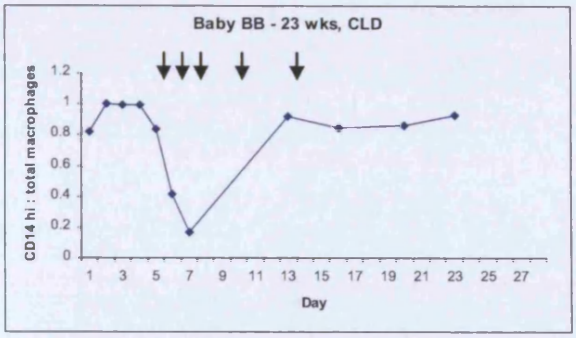
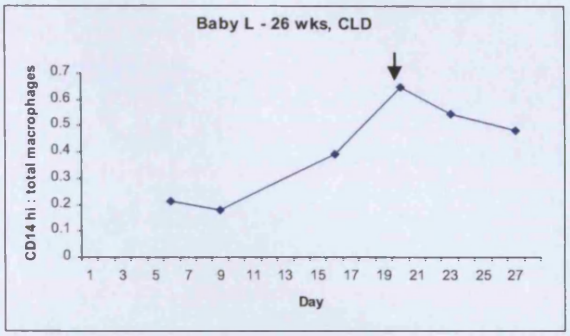
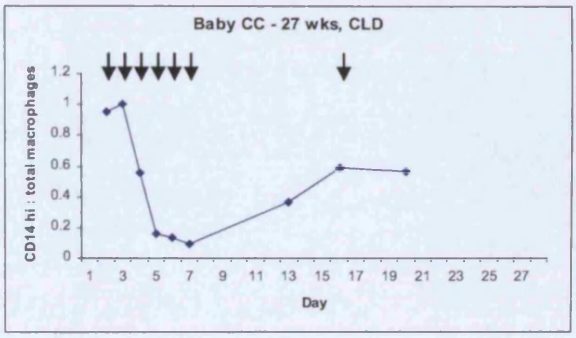


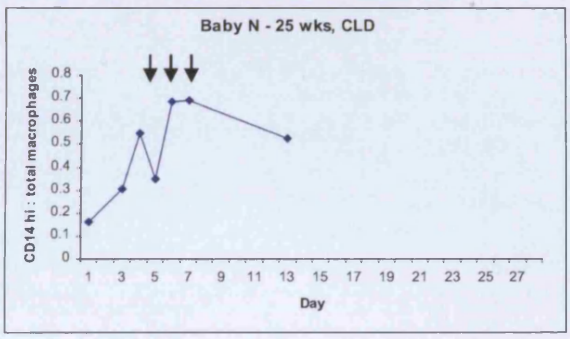
Figure 3.30 Graph showing ratio of immature: total macrophages in BAL from term and preterm infants. (Median and IQR shown)

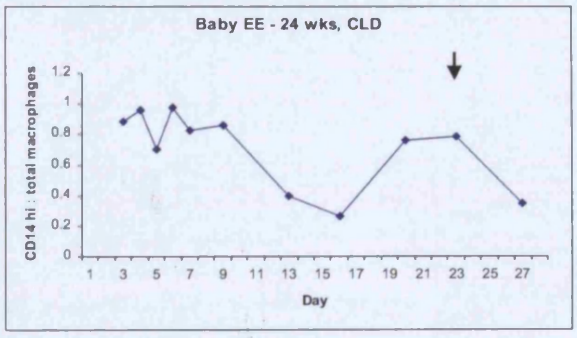
Looking at median values (Figure 3.30) once again obscures the individual variation in immature macrophage counts which is more easily seen in data from individual babies (Figure 3.31). When individual infant data are reviewed, it can be seen that in a number of the most premature infants, immature macrophages frequently make up almost 100% of the macrophage population.

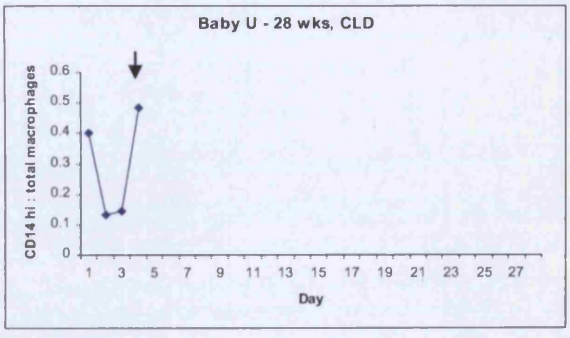

Baby BB - 23 wks, CLD
CD14 hi : total macrophages
Day


Baby L - 26 wks, CLD
CD14 hi : total macrophages
Day


Baby CC - 27 wks, CLD
CD14 hi : total macrophages
Day


Baby N - 25 wks, CLD
CD14 hi : total macrophages
Day


Baby EE - 24 wks, CLD
CD14 hi : total macrophages
Day


Baby U - 28 wks, CLD
CD14 hi : total macrophages
Day

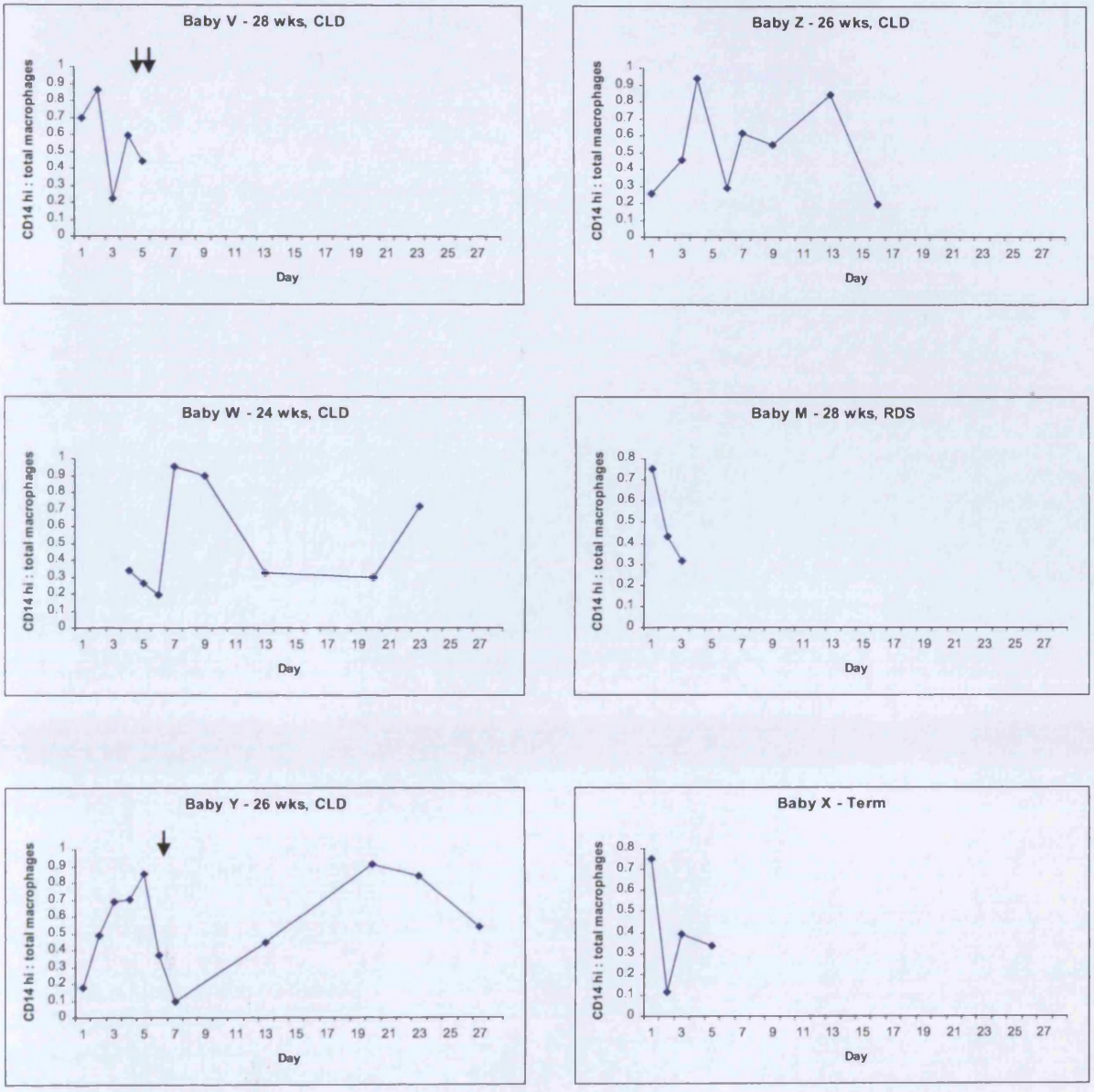


Figure 3.31 Graphs showing immature (CD 14 high): total macrophage count ratios in individual infants who had 3 or more samples in which CD14 on macrophages was measured. Vertical arrows again represent samples in which 16S rRNA genes were detected.

Other subtypes of macrophage were also identified. CD36 may confer the ability to phagocytose apoptotic neutrophils and may thus be a limiting factor in the clearance of apoptotic neutrophils. In keeping with the higher total macrophage counts in preterm infants, the number of CD36 + cells was significantly higher in these babies too (Preterm mean 0.314 $x10^6$ cells/ml, median 0.163 $x10^6$ cells/ml; Term mean 0.039 $x10^6$ cells/ml, median 0.039 $x10^6$ cells/ml; Mann-Whitney U-test, $p<0.0001$) (Figure 5.32).

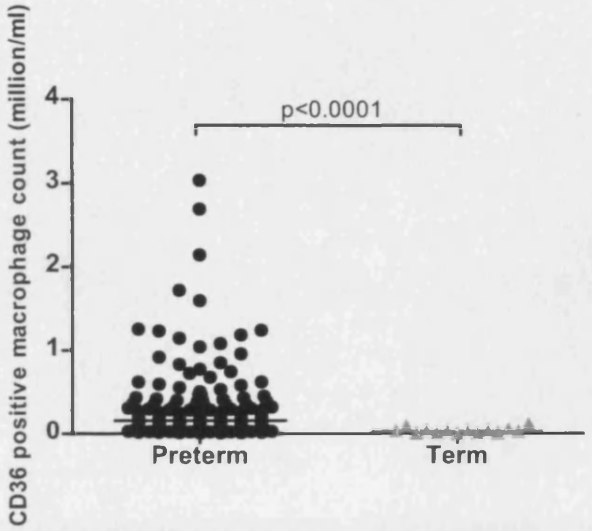
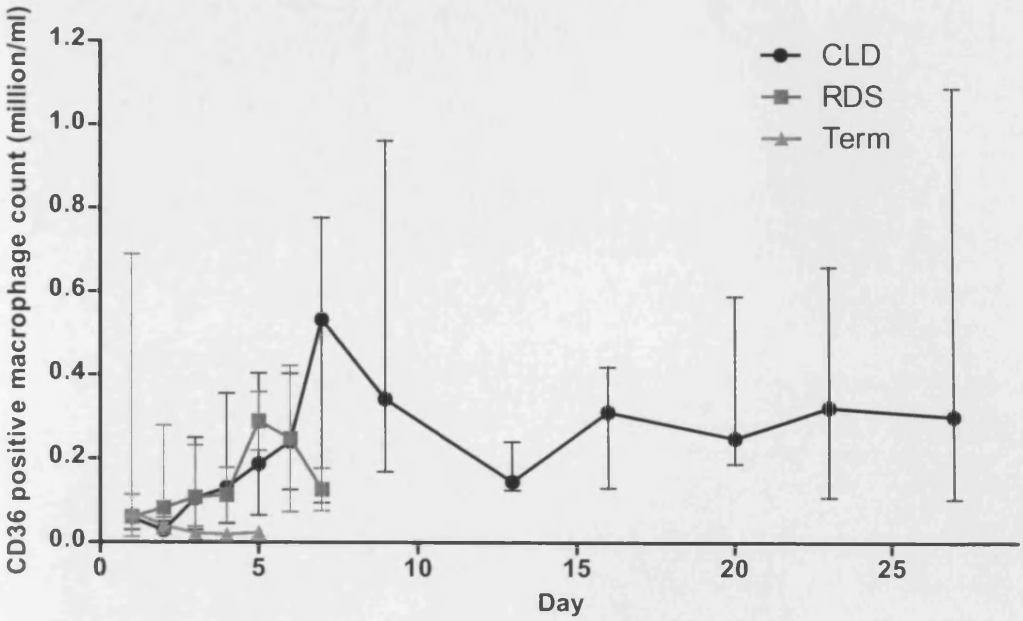
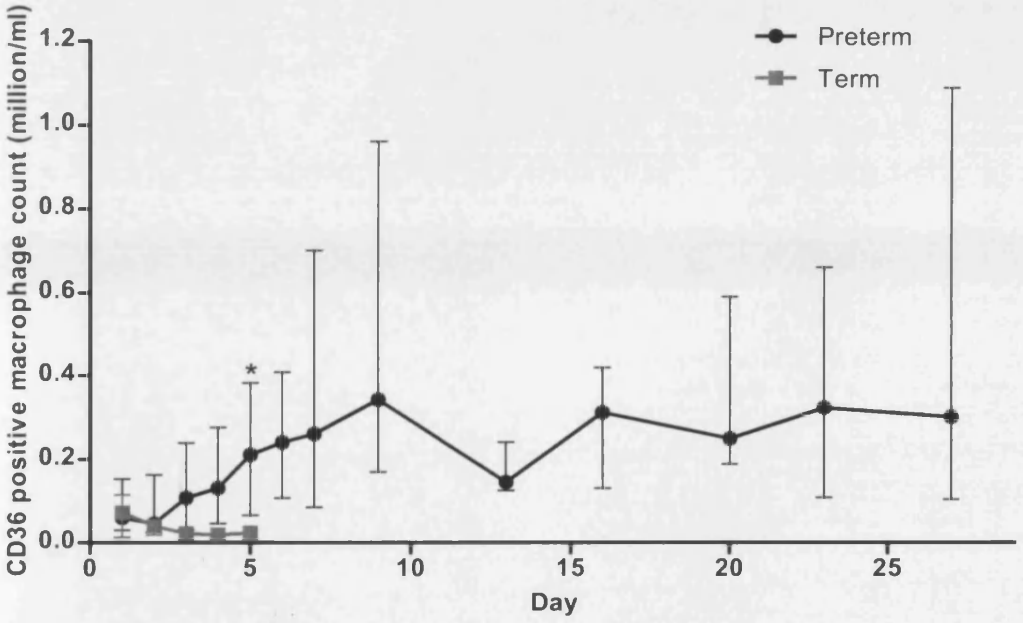


Figure 3.32 Scatterplot showing the number of CD36 positive cells in term and preterm infants. Horizontal lines represent medians.

When looking at the data longitudinally, there was a statistically significantly higher number of CD36 positive macrophages in preterm infants on day 5 (Mann-Whitney U-test, $p=0.0333$) (Term mean 0.023 $x10^6$ cells/ml, median 0.02 $x10^6$ cells/ml; preterm mean 0.35 $x10^6$ cells/ml, median 0.21 $x10^6$ cells/ml) (Figure 5.33B).



A



B

Figure 3.33 Graphs showing fluctuations in median CD36 positive macrophage count over time in babies by (A) diagnosis and (B) by gestation. (p=0.033)*

However, when the number of CD36 + cells was analysed as a proportion of the total number of macrophages, there was no significant difference in the proportion of macrophages that were CD36 + in term and preterm infants (Figure 5.34). There were also no differences in the ratio of CD36 + macrophages to total macrophage count when compared over time (Figure 3.35)

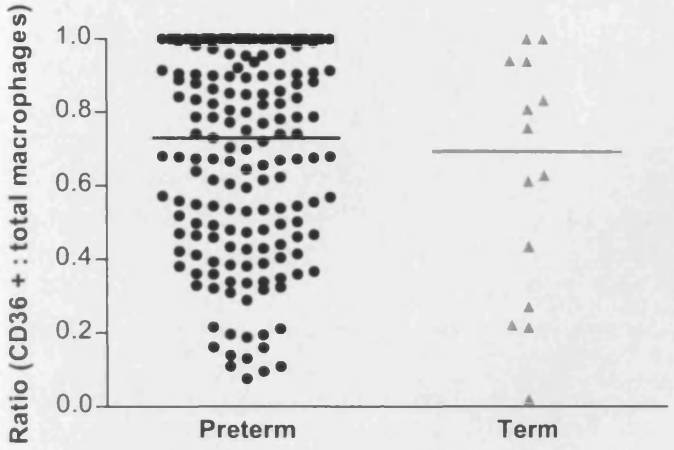
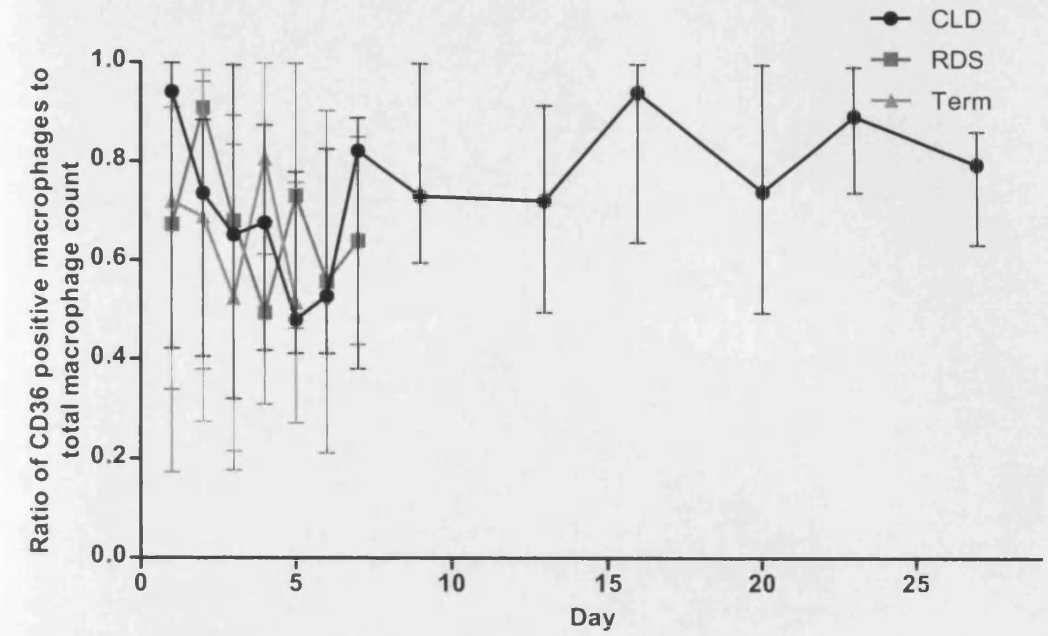
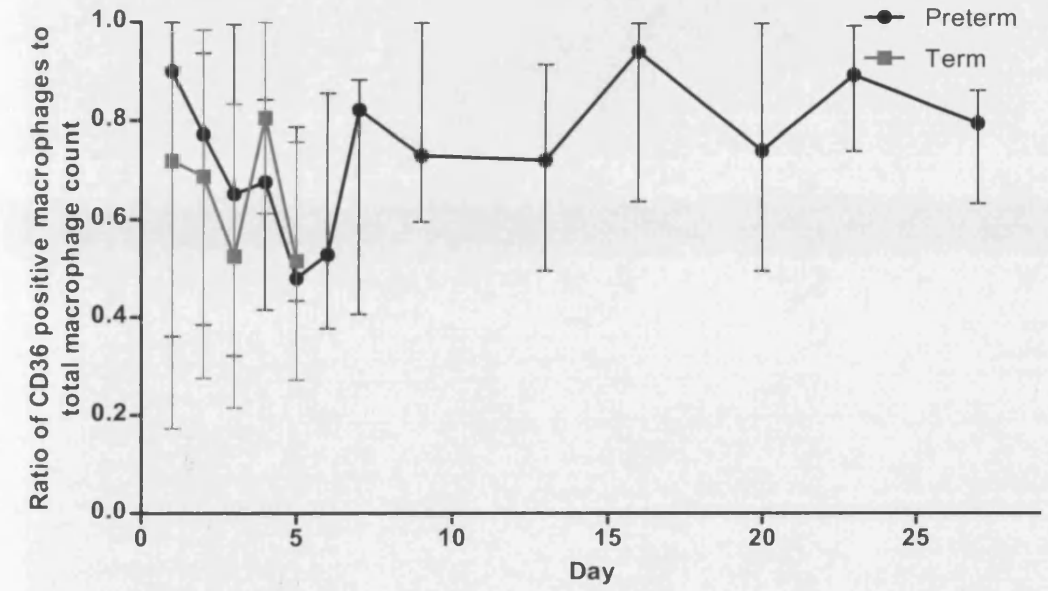


Figure 3.34 Scatterplot showing the ratio of CD36 positive macrophages to the total macrophage count in term and preterm infants. Horizontal lines represent medians.



A



B

Figure 3.35 Graphs showing ratio of CD36 positive: total macrophages in BAL displayed by a (A) diagnosis and (B) gestation.

HLA-DR is expressed at low levels by monocytes and at higher levels by alveolar macrophages (Hallwirth et al., 2004). Higher numbers of cells with higher HLA-DR expression may indicate the presence of mature alveolar macrophages in the airways. Again, as a result of higher overall macrophage numbers, there were statistically more HLA-DR strongly positive macrophages present in preterm infants (p=0.0005) (Figure 3.36).

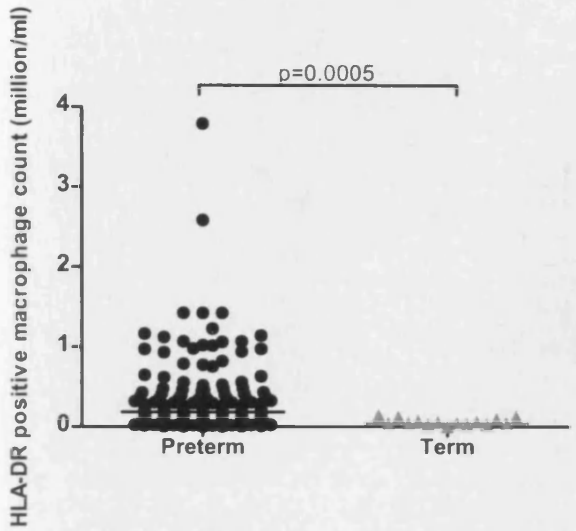
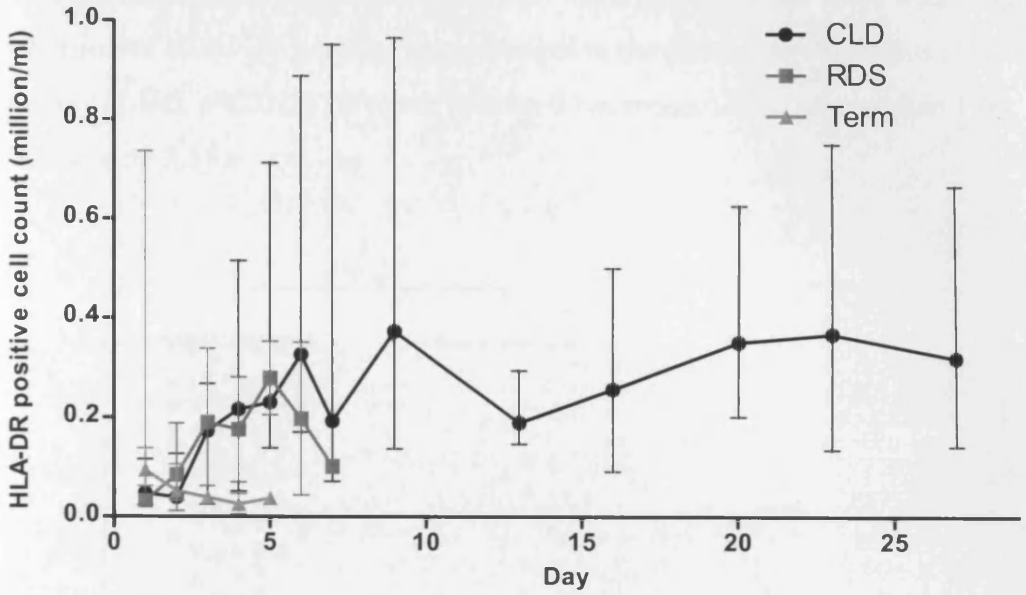


Figure 3.36 Scatterplot showing the number of HLA-DR positive cells in term and preterm infants. Horizontal lines represent medians, (p=0.0005)

When viewed longitudinally, there was a significant difference in cell numbers between term and preterm infants on day 5 (Mann-Whitney U-test, p=0.015) (Term mean 0.037 $x10^6$ cells/ml, median 0.037 $x10^6$ cells/ml; preterm mean 0.382 $x10^6$ cells/ml, median 0.23 $x10^6$ cells/ml) (Figure 3.37).



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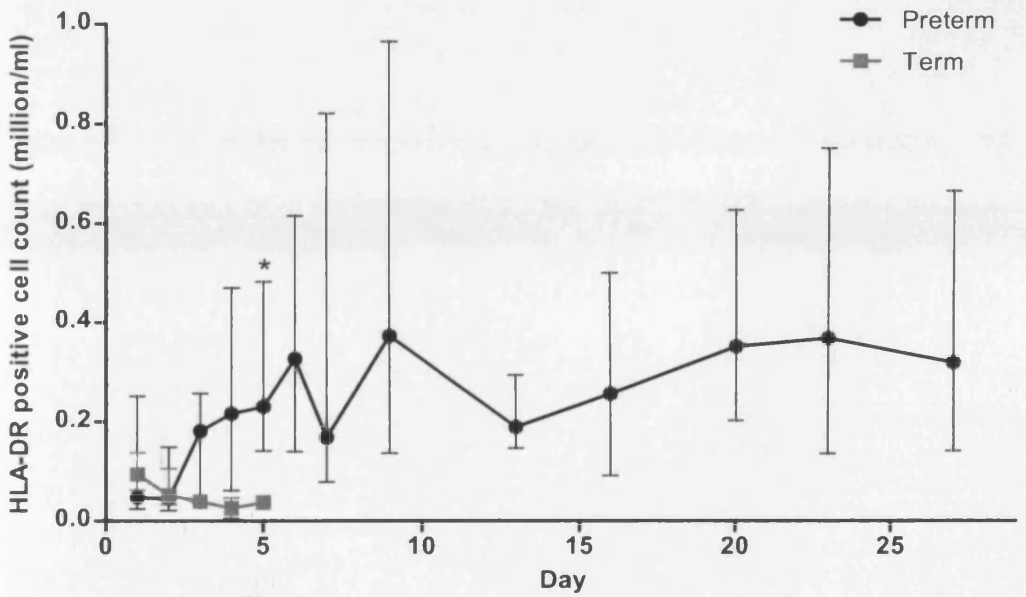


B

Figure 3.37 Graphs showing median HLA-DR positive macrophage count according to (A) diagnosis and (B) gestation. (p=0.015)*

When considered as a proportion of the total macrophage count, there was a higher proportion of HLA-DR positive macrophages in the term group of babies (Mann-Whitney U-test, p=0.013) (Preterm median 0.83, mean 0.74; Term median 1.00, mean 0.91) (Figure 3.38).

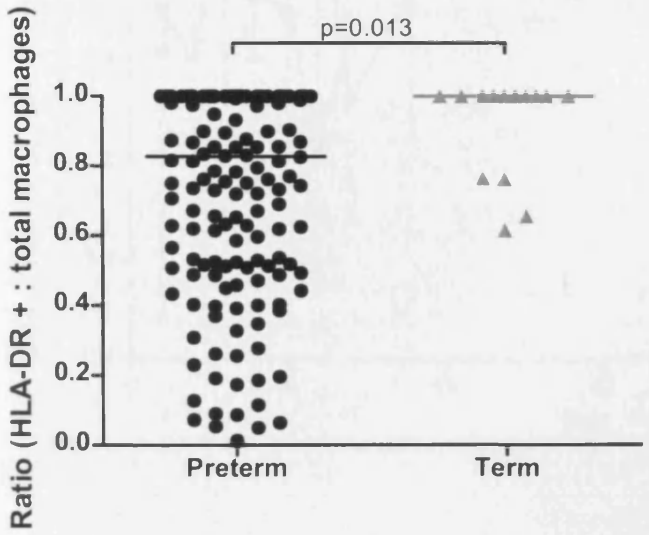
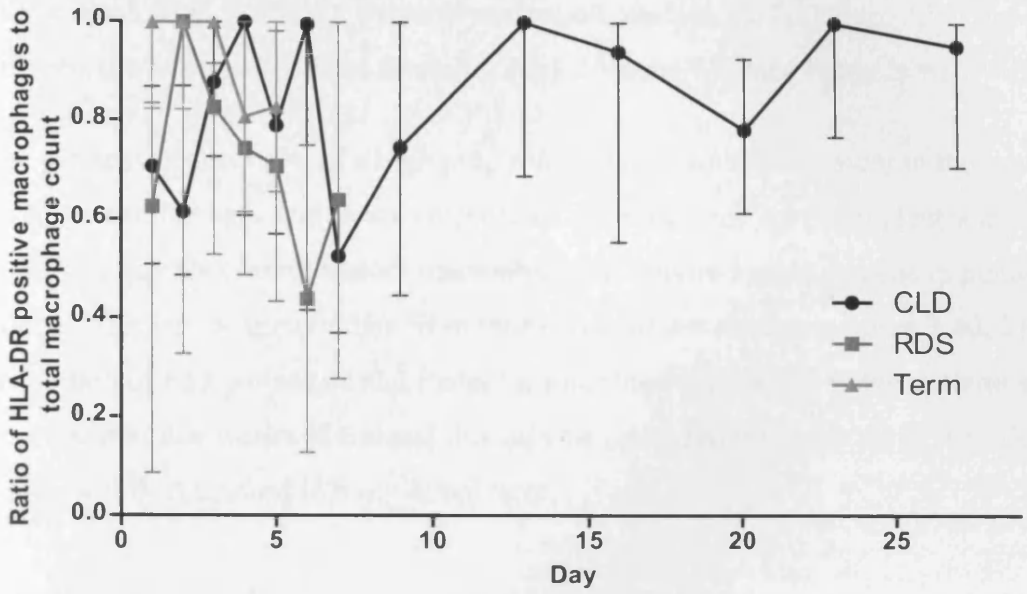


Figure 3.38 Scatterplot showing ratio of mature (HLA-DR positive macrophages): total macrophage count. Horizontal lines represent medians.



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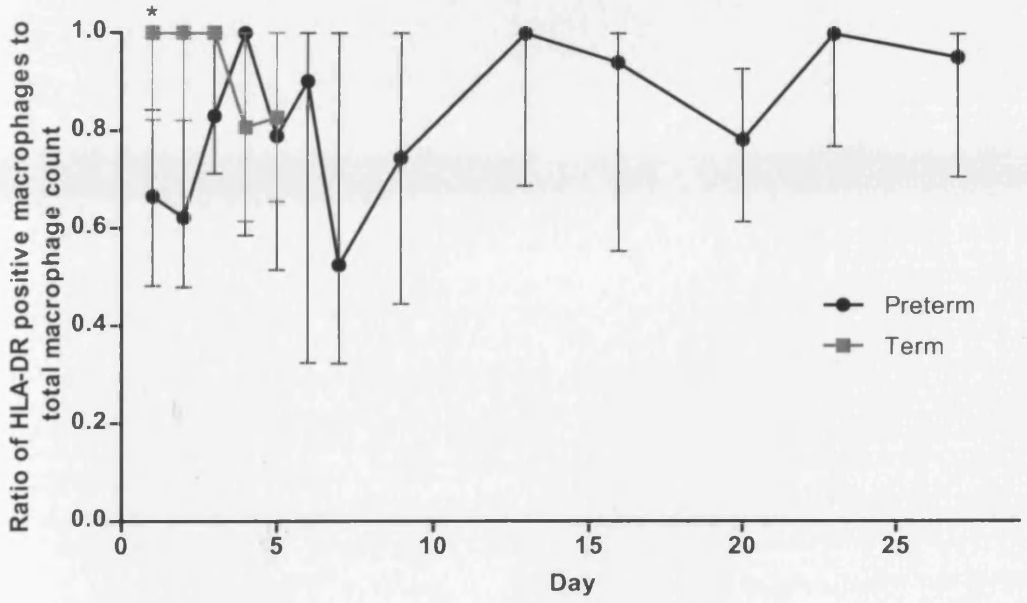
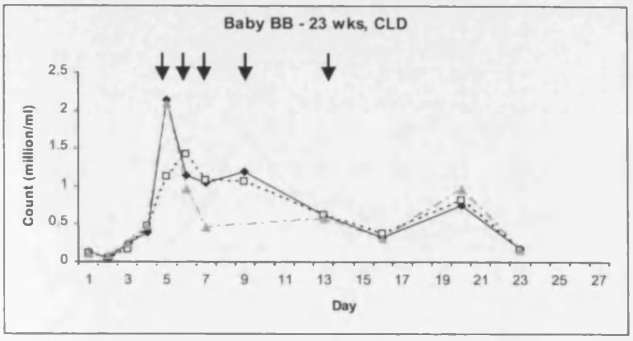


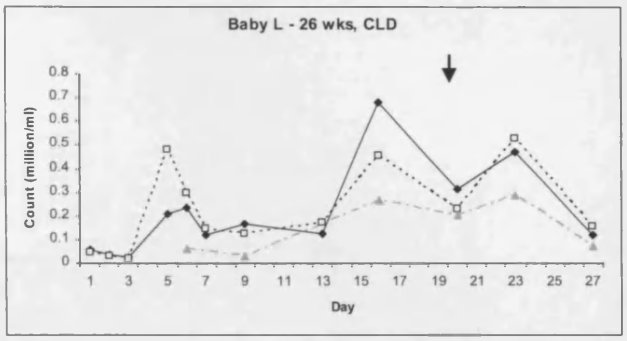
B

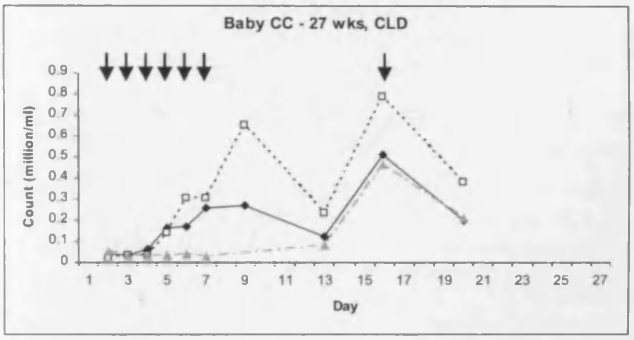
Figure 3.39 Graphs showing fluctuations in ratio of mature (HLA-DR positive): total macrophages in BAL according to (A) diagnosis and (B) gestation. (p=0.017)*

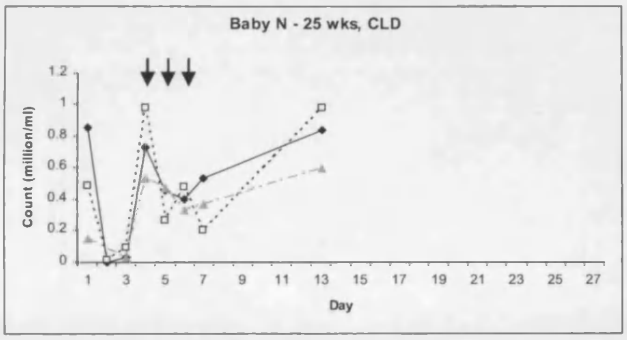
On day 1 there is a significant difference in ratio of HLA-DR positive macrophages to total macrophages between term and preterm infants (Mann-Whitney U-test, p=0.017) (Term mean 0.94, median 1; preterm mean 0.60, median 0.67) (Figure 3.39) but the difference was not significant thereafter (day 2 Mann-Whitney U-test, p=0.0947).

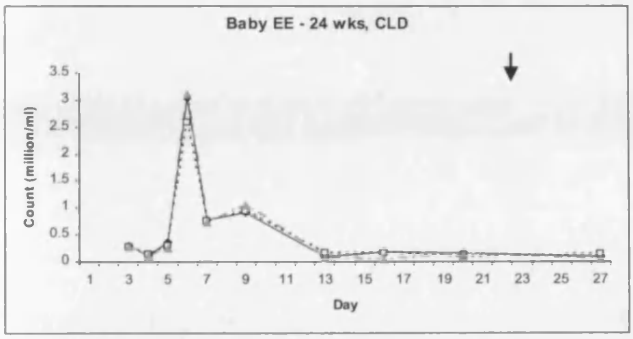
The pattern appears to be of a high proportion of macrophages present in the form of alveolar macrophages and lower proportions of monocyte-like macrophages in term infants initially but fewer mature macrophages and more immature cells in preterm infants. This can be seen in data from individual infants shown in figure 3.40. The proportion of both immature and mature macrophages fluctuates in the preterm group over the first few weeks of life and this may be related to the presence of infection which will be discussed in more detail later.

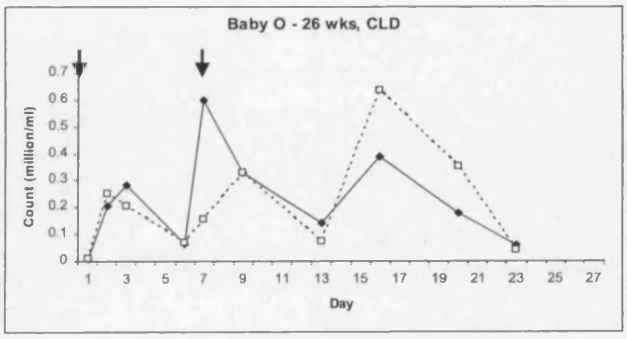

Baby BB - 23 wks, CLD
Count (million/ml)
Day

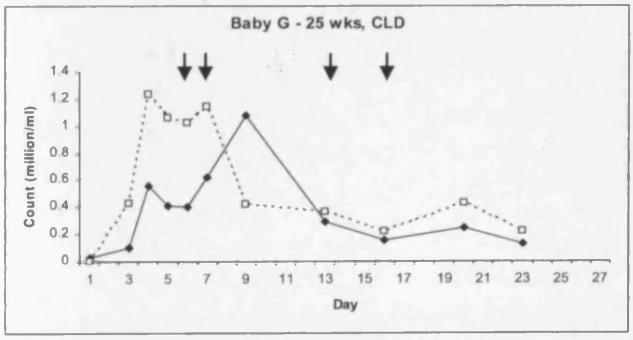

Baby L - 26 wks, CLD
Count (million/ml)
Day

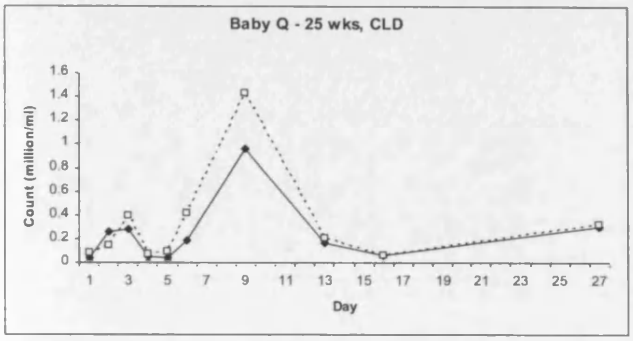

Baby CC - 27 wks, CLD
Count (million/ml)
Day

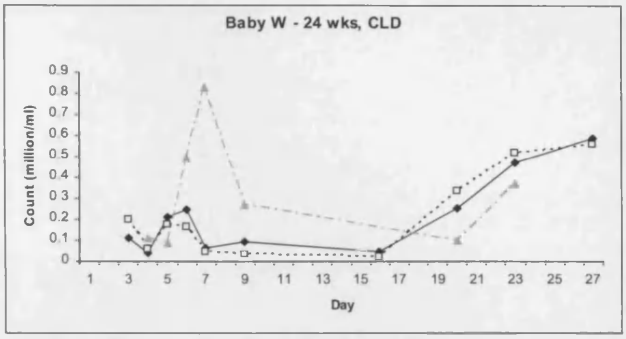

Baby N - 25 wks, CLD
Count (million/ml)
Day

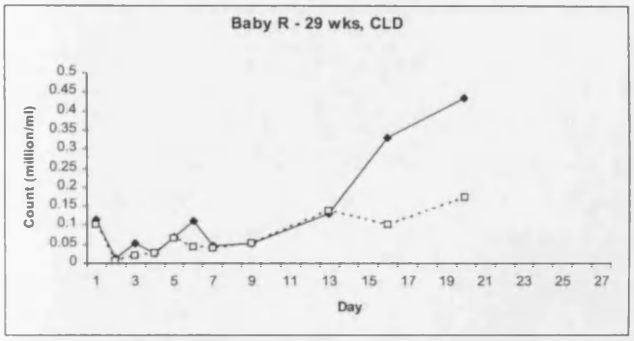

Baby EE - 24 wks, CLD
Count (million/ml)
Day

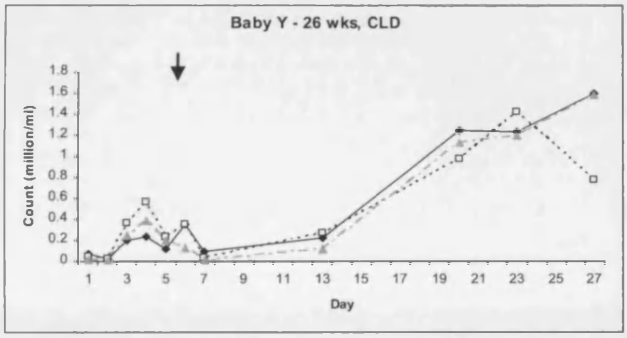

Baby O - 26 wks, CLD
Count (million/ml)
Day

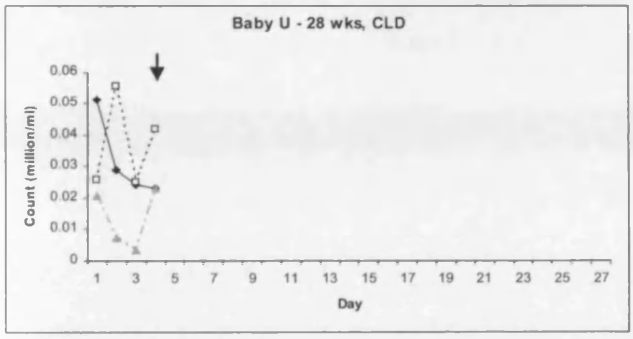

Baby G - 25 wks, CLD
Count (million/ml)
Day

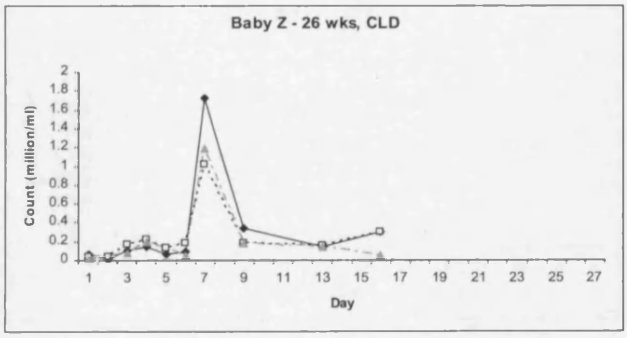

Baby Q - 25 wks, CLD
Count (million/ml)
Day

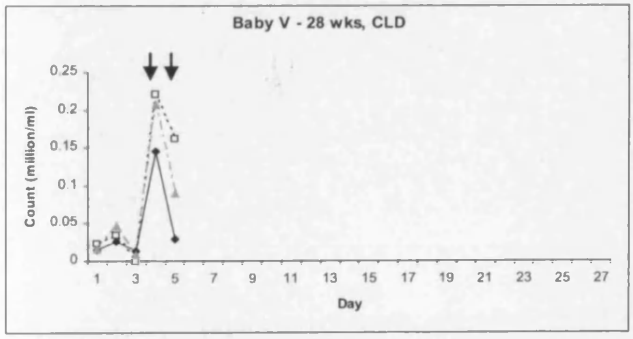

Baby W - 24 wks, CLD
Count (million/ml)
Day

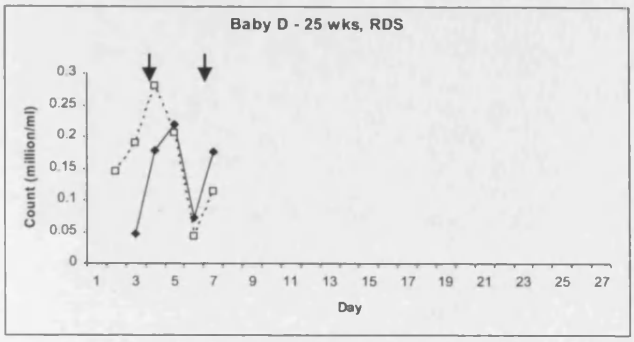

Baby R - 29 wks, CLD
Count (million/ml)
Day

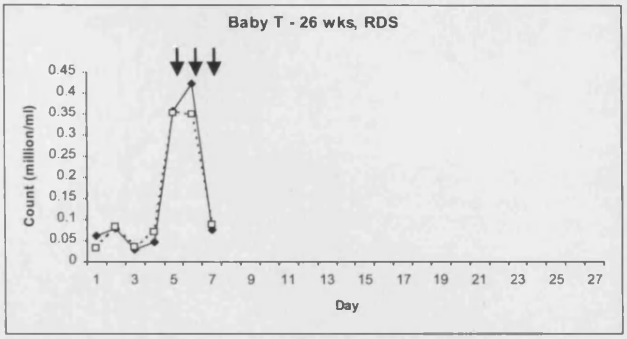

Baby Y - 26 wks, CLD
Count (million/ml)
Day

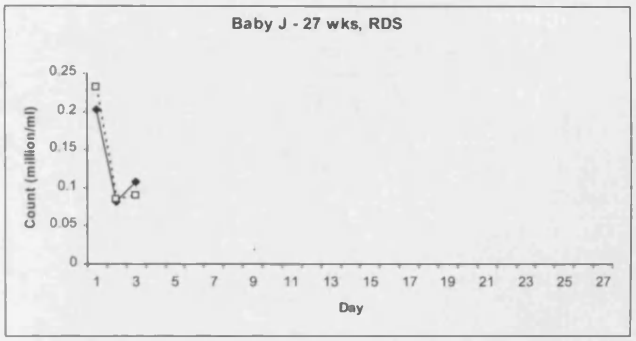

Baby U - 28 wks, CLD
Count (million/ml)
Day

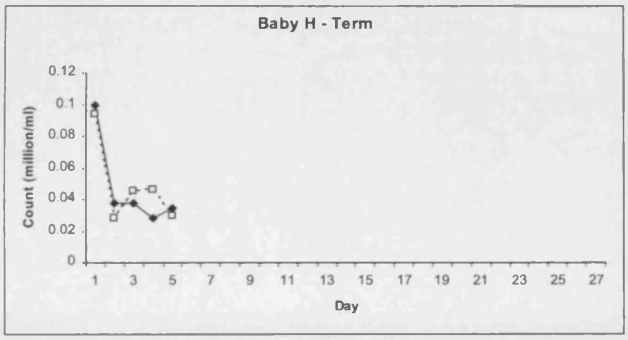

Baby Z - 26 wks, CLD
Count (million/ml)
Day

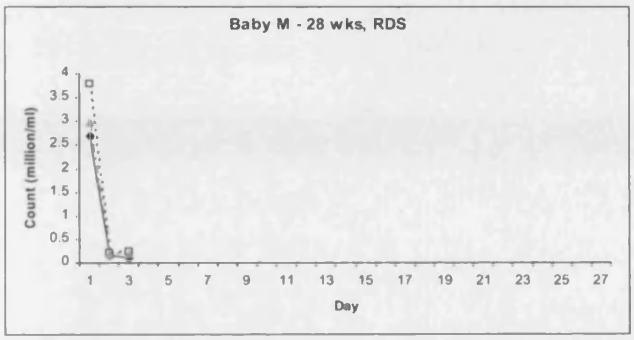

Baby V - 28 wks, CLD
Count (million/ml)
Day

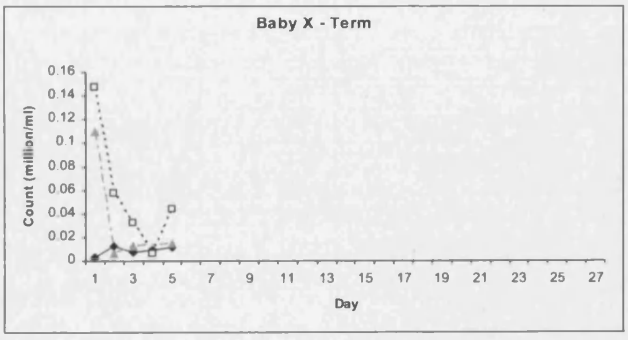












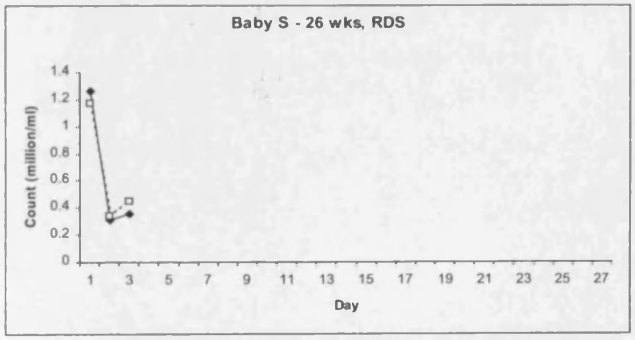


Figure 3.40 Graphs showing macrophage markers (CD14 – broken line and grey triangles, CD36-solid black line and boxes, HLA-DR – dotted line and open boxes) for individual infants with 3 or more BAL samples over the course of their ventilation period.

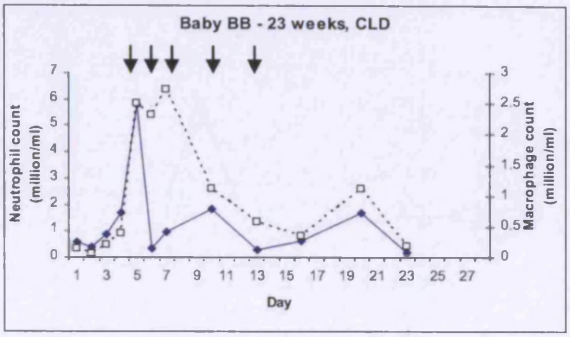
3.3.5 Relationship of neutrophils to macrophages

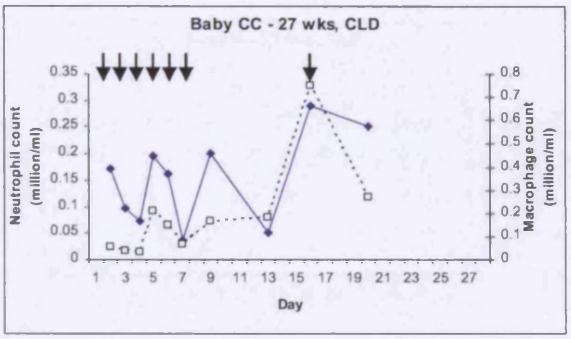
As described in section 3.3.4.2 and 3.3.4.3, neutrophil and macrophage counts both reach their peak values on later days in CLD babies than in either of the other groups. Neutrophil and macrophage numbers also reach their highest levels on the same day of life in each group.

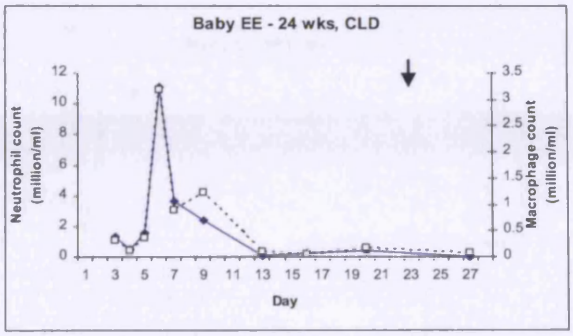
	CLD	RDS	Term
Median neutrophil peak day	7 (3.5)	2	2
Median macrophage peak day	7 (4)	2	1

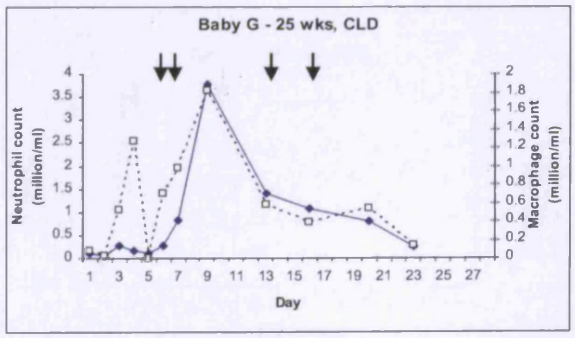
Table 3.2 Median days on which peak neutrophil and macrophage counts occurred. Figures in brackets indicate on which of the first 5 days (median) the peak cell counts occurred in CLD babies, in order to obtain a fairer comparison with RDS and term babies who were only ventilated for around 5 days.

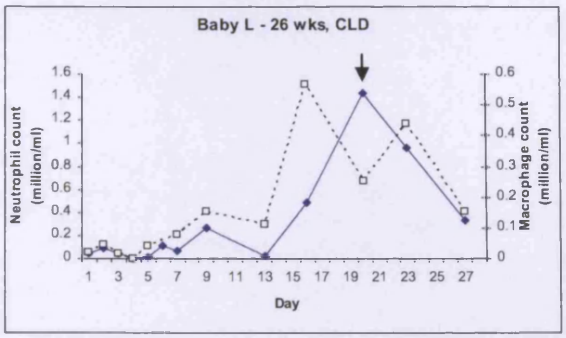
When data for individual babies are viewed, it can be seen that neutrophil counts and macrophage counts tend to increase simultaneously, rather than consecutively. These increases in cell counts may be associated with infection and this will be examined in more detail later.

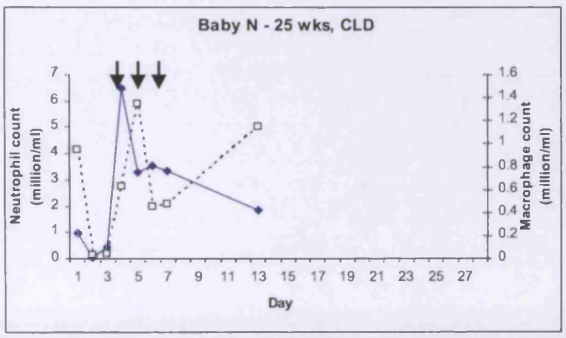

Baby BB - 23 weeks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

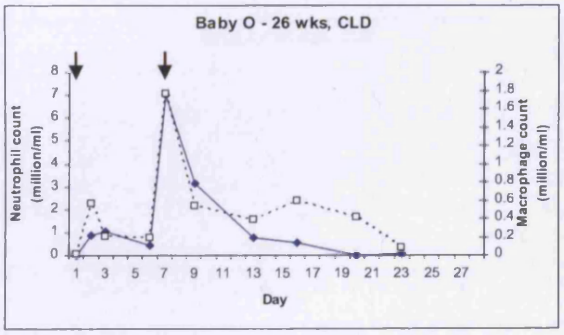

Baby CC - 27 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

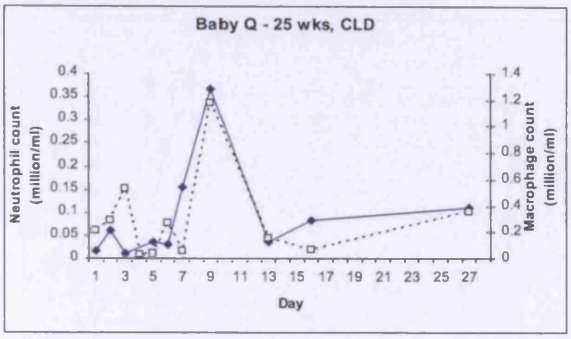

Baby EE - 24 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

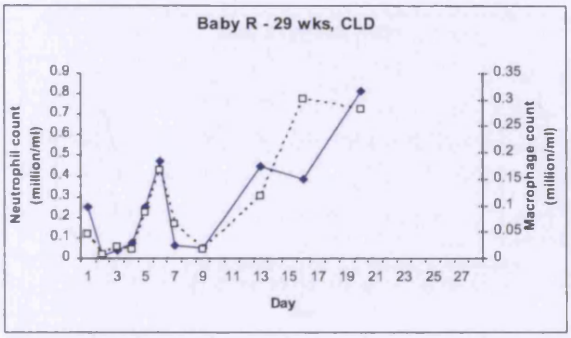

Baby G - 25 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

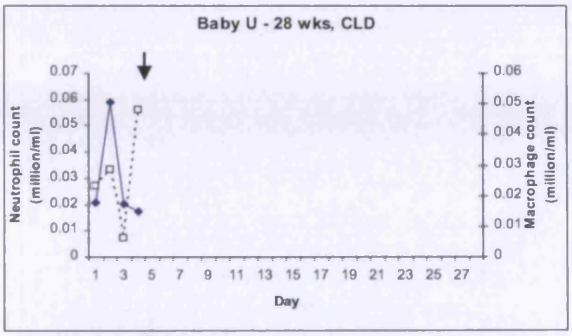

Baby L - 26 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

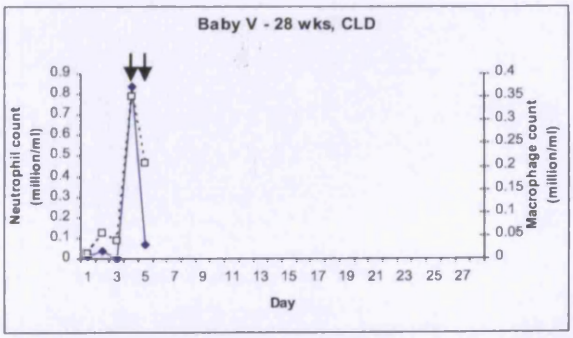

Baby N - 25 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

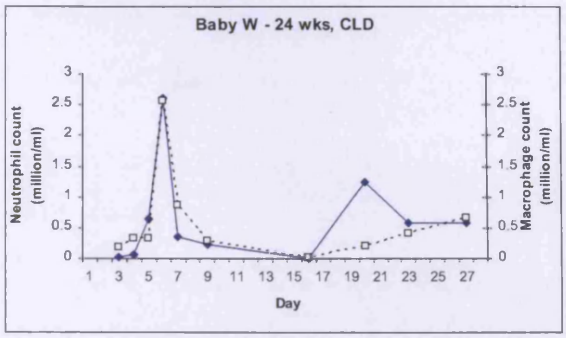

Baby O - 26 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

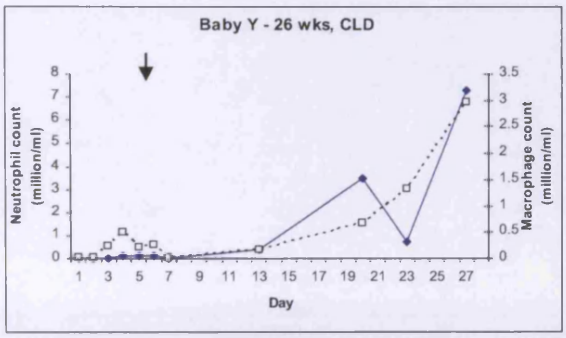

Baby Q - 25 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

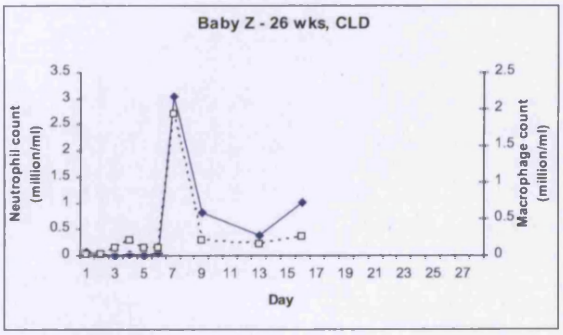

Baby R - 29 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

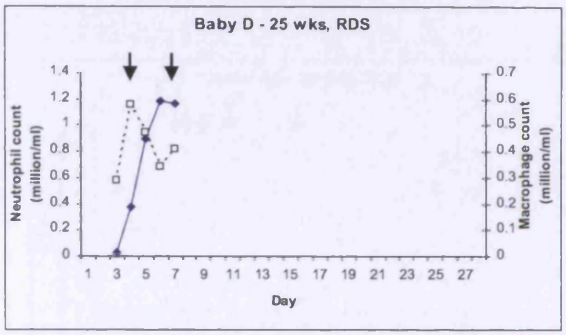

Baby U - 28 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

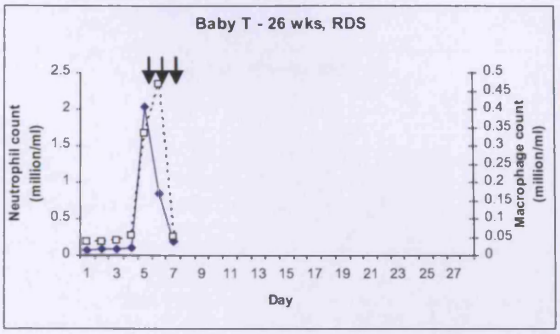

Baby V - 28 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

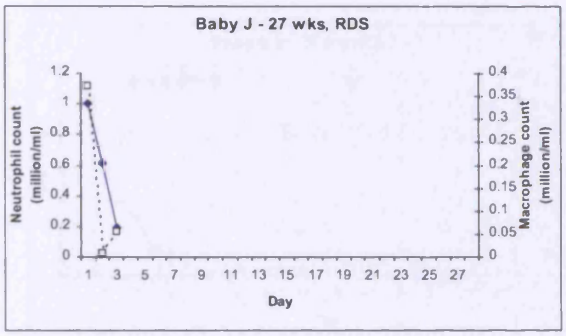

Baby W - 24 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

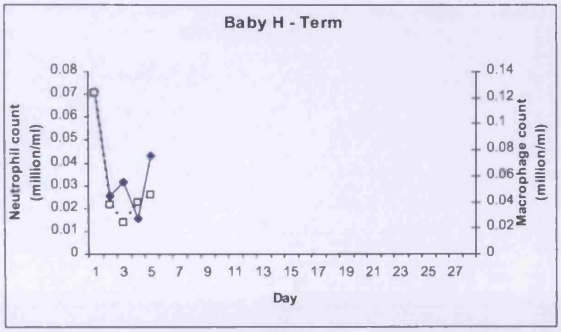

Baby Y - 26 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

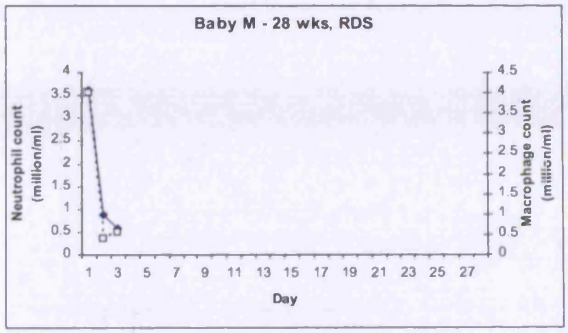

Baby Z - 26 wks, CLD
Neutrophil count (million/ml)
Macrophage count (million/ml)
Day

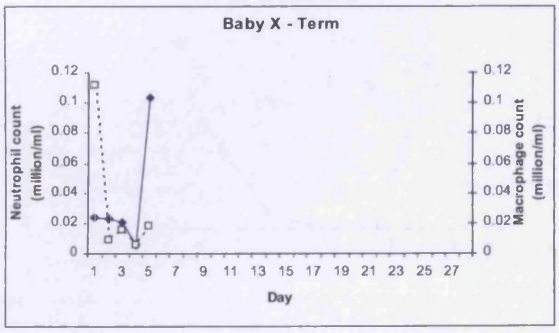












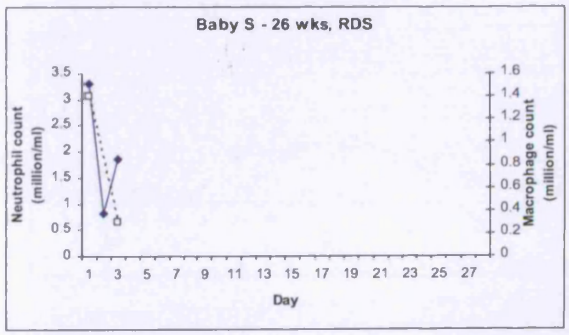
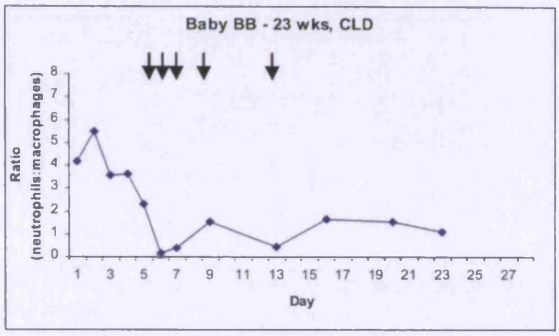
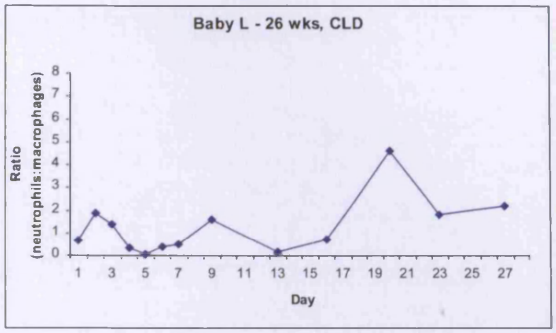
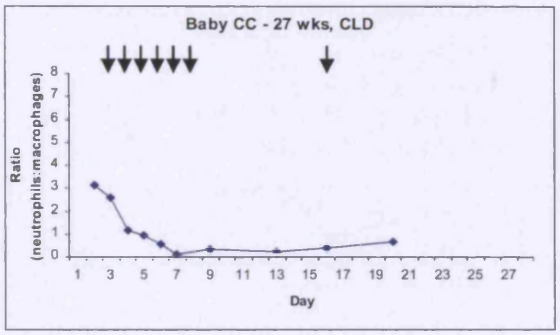
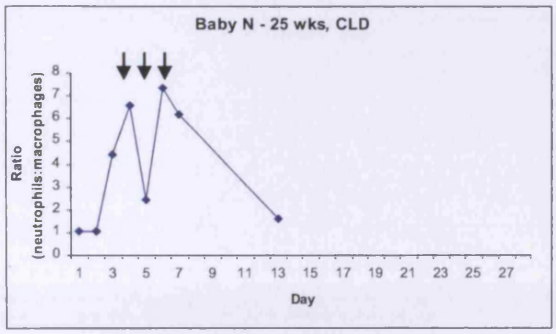


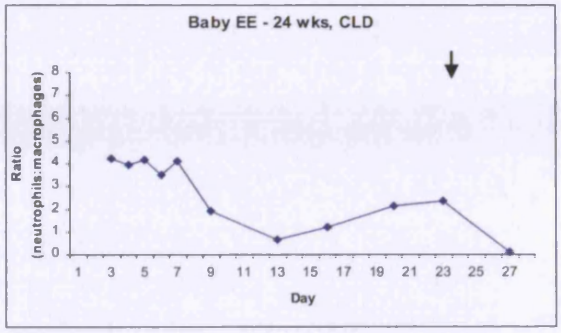
Figure 3.41 Graphs showing relationship between neutrophil count (solid line) and macrophage count (dotted line) for individual babies with 3 or more BAL samples.

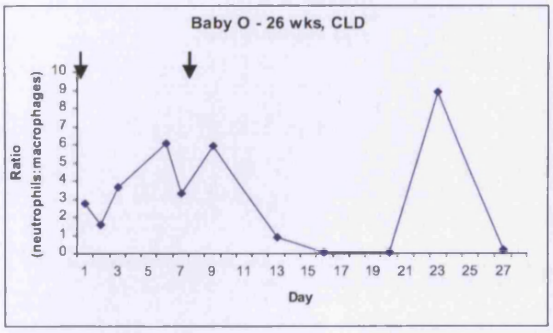

Baby BB - 23 wks, CLD
Ratio (neutrophils:macrophages)
Day

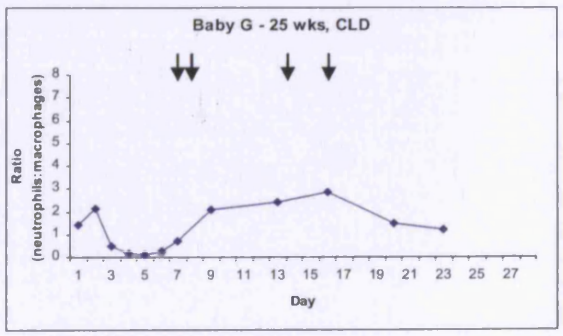

Baby L - 26 wks, CLD
Ratio (neutrophils:macrophages)
Day

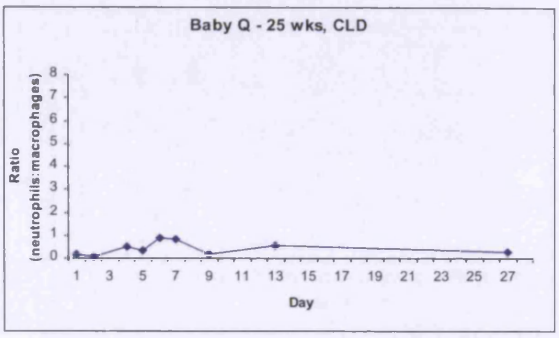

Baby CC - 27 wks, CLD
Ratio (neutrophils:macrophages)
Day

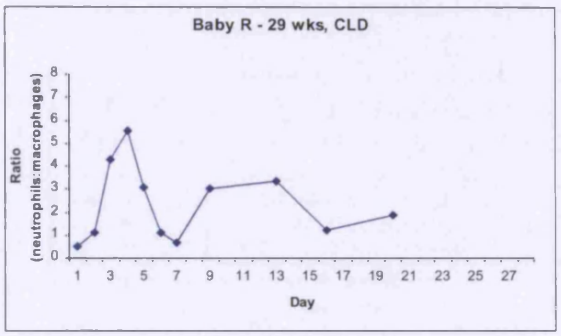

Baby N - 25 wks, CLD
Ratio (neutrophils:macrophages)
Day

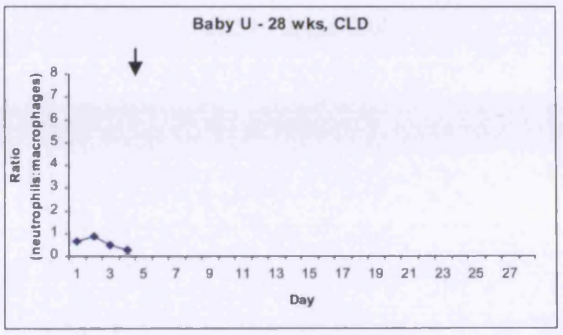

Baby EE - 24 wks, CLD
Ratio (neutrophils:macrophages)
Day

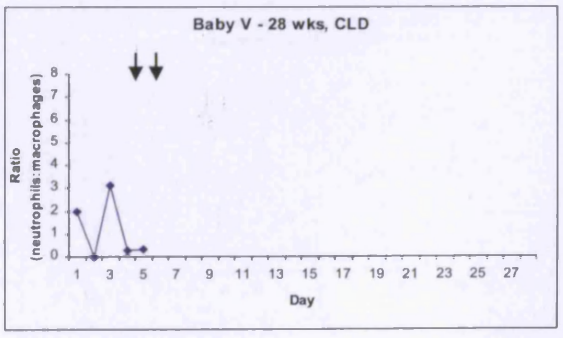

Baby O - 26 wks, CLD
Ratio (neutrophils:macrophages)
Day

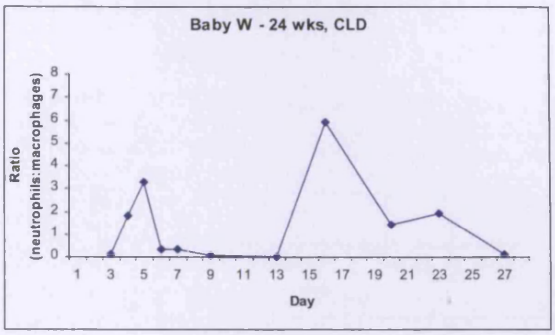

Baby G - 25 wks, CLD
Ratio (neutrophils:macrophages)
Day

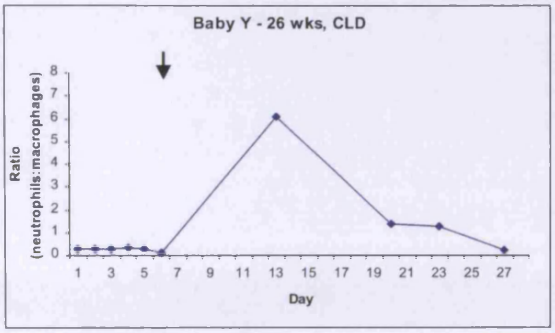

Baby Q - 25 wks, CLD
Ratio (neutrophils:macrophages)
Day

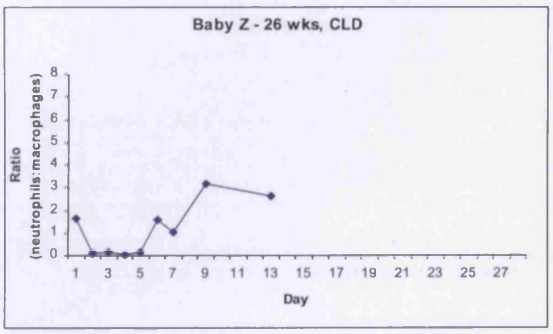

Baby R - 29 wks, CLD
Ratio (neutrophils:macrophages)
Day

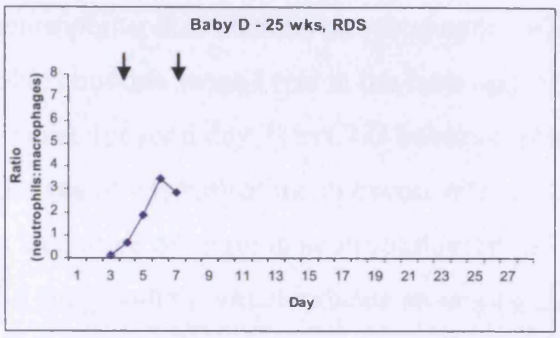

Baby U - 28 wks, CLD
Ratio (neutrophils:macrophages)
Day

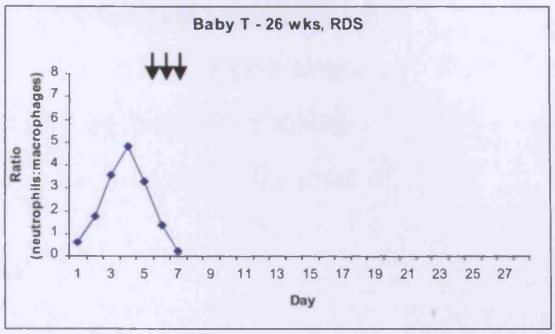

Baby V - 28 wks, CLD
Ratio (neutrophils:macrophages)
Day

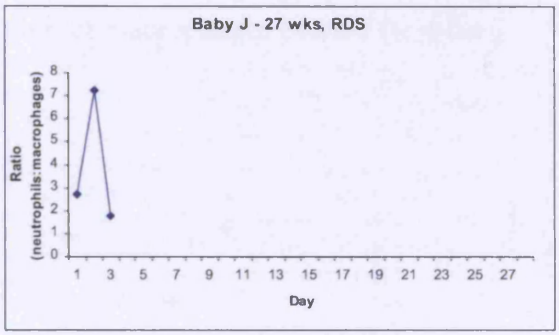

Baby W - 24 wks, CLD
Ratio (neutrophils:macrophages)
Day

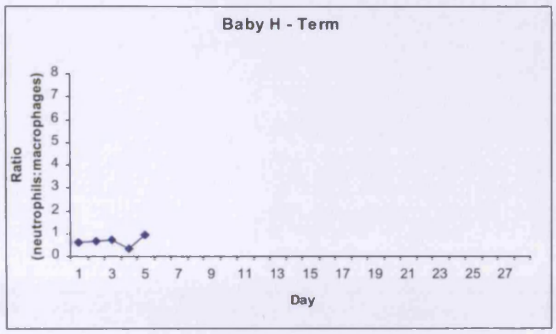

Baby Y - 26 wks, CLD
Ratio (neutrophils:macrophages)
Day

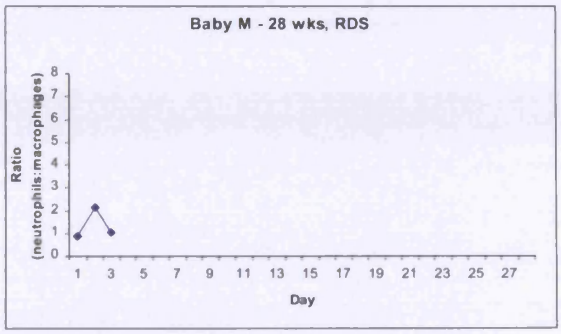

Baby Z - 26 wks, CLD
Ratio (neutrophils:macrophages)
Day

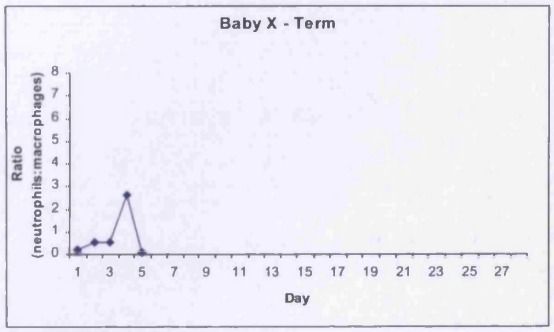












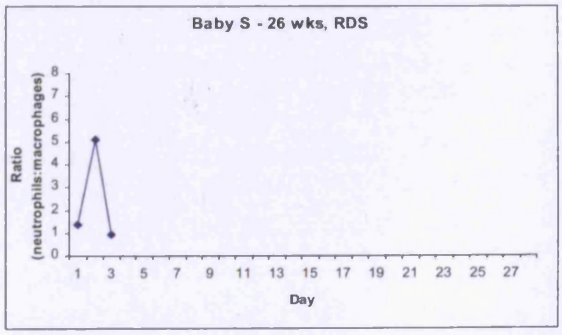
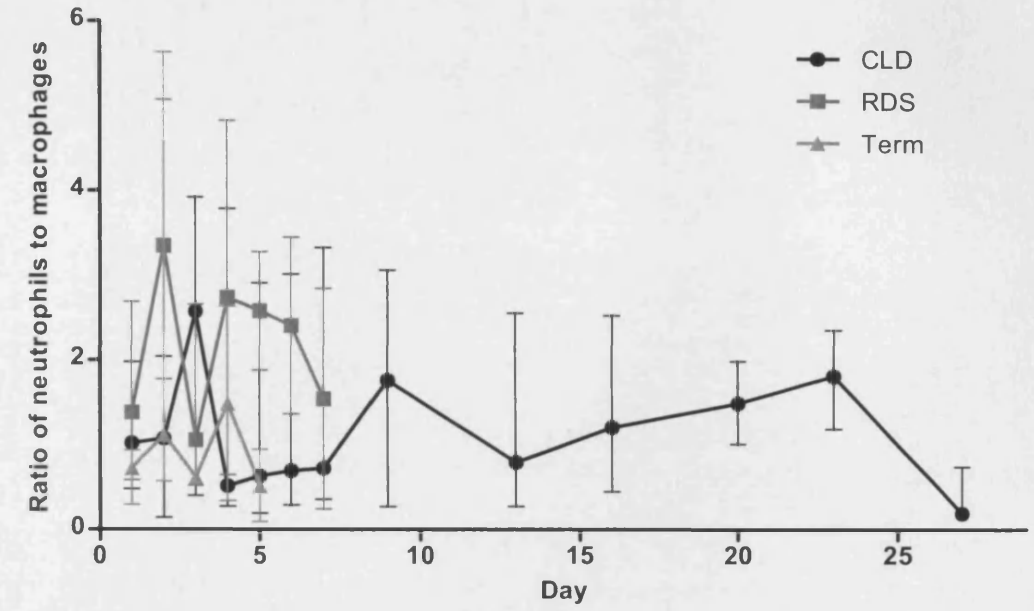
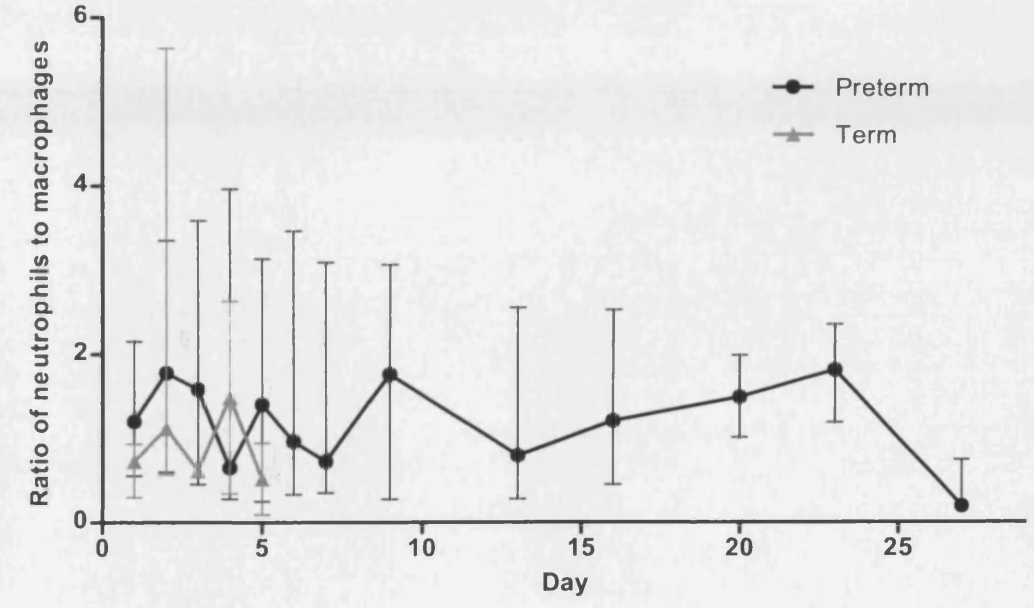


Figure 3.42 Graphs showing ratio of neutrophils to macrophages for individual babies with 3 or more BAL samples.

When median data are viewed longitudinally, patterns for the ratio between neutrophils and macrophages are very similar (Figure 3.43) – all groups showing an early neutrophil spike. There is a second spike which occurs very quickly in RDS and term babies but this second rise in the ratio appears to be delayed in CLD babies. The later samples (beyond day 7) in CLD babies continue to have a ratio of neutrophils: macrophages of >1, indicating an excess of neutrophils until the final sample which may be indicative of ongoing neutrophil recruitment or failure of neutrophil clearance from the lung, both of which indicate an ongoing inflammatory process in infants developing CLD. Term babies seldom seem to have neutrophil numbers in excess of the number of macrophages present (ie ratio ≤ 1).



A



B

Figure 3.43 Graphs showing longitudinal relationship between neutrophils and macrophages (in the form of neutrophil: macrophage ratio) in BAL fluid according to (A) diagnosis and (B) gestation.

3.3.6 Differences between the groups on day 1

There is much debate about the antenatal determinants of CLD. It is ethically and technically almost impossible to measure the inflammatory process within the lung prior to birth. The first available observation of the process was made by us at 12 hours of age in the majority of infants. The first BAL was performed at 12 hours of age in order to have negligible impact on the clinical care of the infant – preterm infants are usually intubated and given a dose of exogenous surfactant via the endotracheal tube within seconds to minutes after delivery (unit policy dictates that surfactant should ideally be given prior to the first breath in extremely preterm infants) – a BAL procedure at this point would prejudice this process, so BAL was performed at 12 hours of age, just prior to the second dose of surfactant being given, when the infant would routinely have endotracheal tube suctioning performed.

I compared results of values obtained for all the aforementioned parameters on the first day of life in order to ascertain whether any differences existed between the groups of infants at this early stage. This may shed light on the in-utero environment of the lungs before birth as well as possibly providing some form of prognostic indicator of the likely progression of lung disease at a very early stage in the clinical course. Such prognostic ability may facilitate specific clinical care interventions to prevent or reduce the severity of CLD.

There were 12 babies in the CLD group, 9 with RDS and all 5 term babies who had information available about cell counts on day 1 of life. When term babies were compared with preterm infants who recovered from RDS and with infants who developed CLD, I found no significant differences between the groups for total cell count on the first day of life (Kruskal-Wallis test, p=0.24; Term mean 1.027 $x10^6$ cells/ml, median 1.04 $x10^6$ cells/ml; RDS mean 3.345 $x10^6$ cells/ml, median 1.95 $x10^6$ cells/ml; CLD mean 1.702 $x10^6$ cells/ml, median 1.336 $x10^6$ cells/ml). As a group, preterm infants had slightly higher cell counts on day 1, but this was not a statistically significant difference (Mann-Whitney U-test, p=0.17; Term mean 1.027 $x10^6$ cells/ml, median 1.040 $x10^6$ cells/ml; Preterm mean 2.406 $x10^6$ cells/ml, median 1.76 $x10^6$ cells/ml).

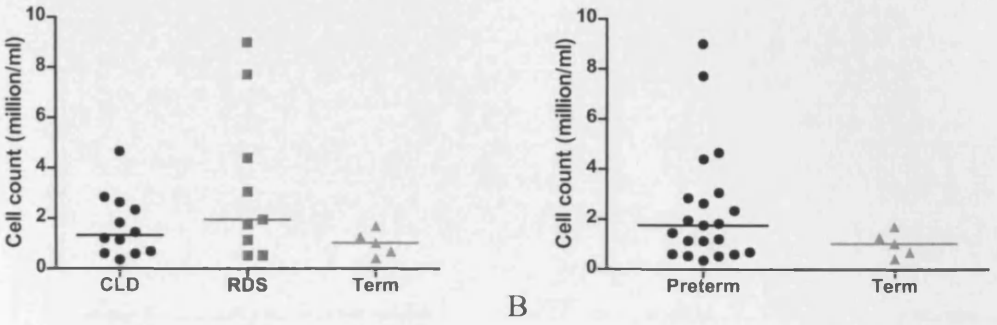


Figure 3.44 Scatterplots showing total cell counts on day 1 for (A) each diagnostic category and (B) the two gestational groups.

Adequate differential cell count data on day 1 were available for 11 babies who developed CLD, 7 of the RDS group and 4 of the term infants. The absolute number of macrophages was similar in all 3 groups (Kruskal-Wallis test, p=0.297; Term mean 0.076 $x10^6$ cells/ml, median 0.077 $x10^6$ cells/ml; RDS mean 0.874 $x10^6$ cells/ml, median 0.025 $x10^6$ cells/ml; CLD mean 0.139 $x10^6$ cells/ml, median 0.023 $x10^6$ cells/ml) (Figure 3.45B and D). The median number of neutrophils present on the first day of life in the lungs of babies whose RDS resolved was skewed mainly by 2 infants who had the highest day 1 neutrophil counts of all the 32 infants recruited. However, there was still no significant difference between the groups (Kruskal-Wallis test, p=0.279; Term mean 0.07 $x10^6$ cells/ml, median 0.067 $x10^6$ cells/ml; RDS mean 1.26 $x10^6$ cells/ml, median 0.77 $x10^6$ cells/ml; CLD mean 0.19 $x10^6$ cells/ml, median 0.04 $x10^6$ cells/ml) (Figure 3.45A and C) when the analysis was performed after excluding these 2 infants.

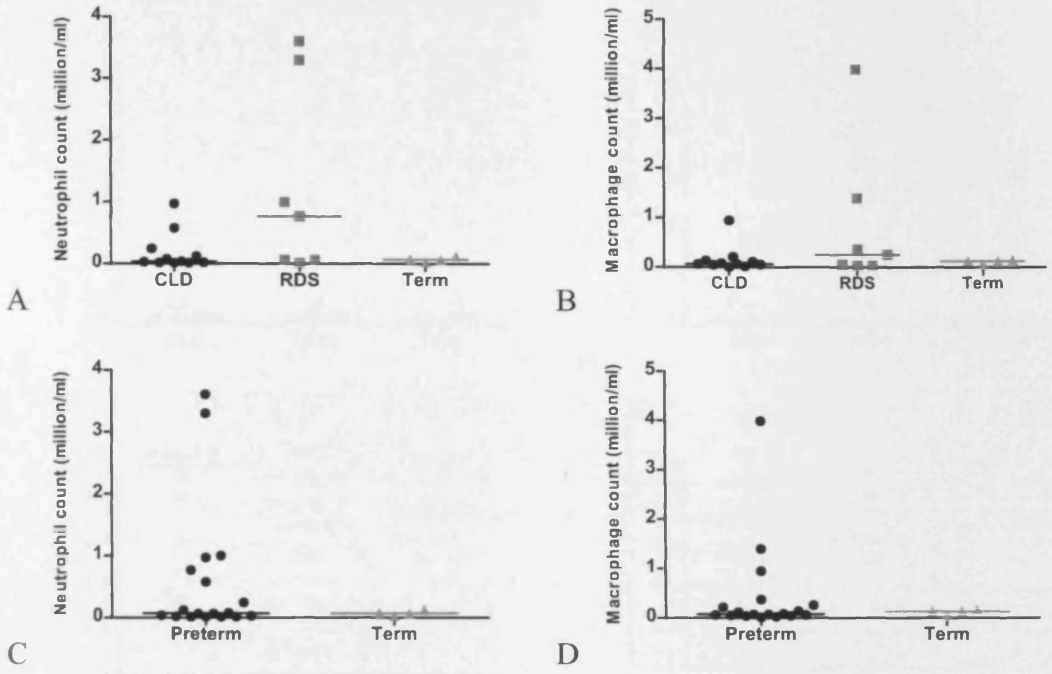


Figure 3.45 Scatterplots showing neutrophil and macrophage counts on the first day of life according to (A and B) diagnostic group and (C and D) gestation.

When macrophage subtypes were assessed, there was no significant difference in the proportion or absolute number of macrophages which expressed CD36 and the proportion which were HLA-DR positive was also not quite significant (p=0.0502) between any of the diagnostic groups. Again the RDS group had two infants with results that were markedly different to the remainder of the group. There were insufficient data on CD14 expression on day 1 to make an appropriate comparison.

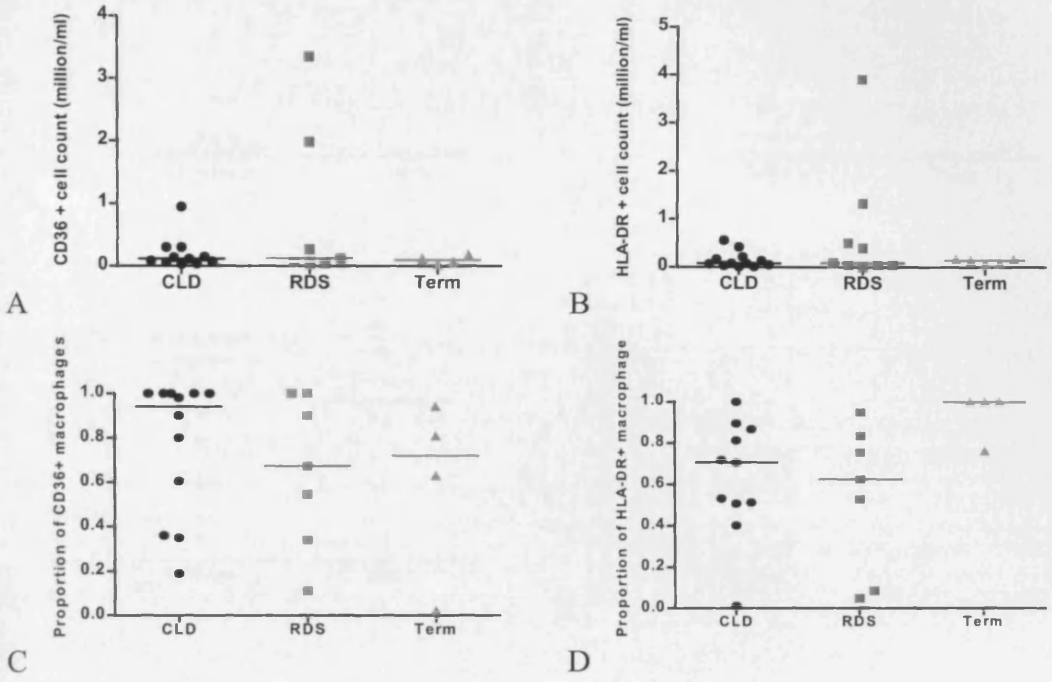


Figure 3.46 Scatterplots showing the number of (A) CD36 positive and (B) HLA-DR positive macrophages in BAL fluid on Day 1 and the proportion of cells which expressed (C) CD36 and (D) HLA-DR.

When term and preterm infants were compared, absolute numbers of HLA-DR+ and CD36+ cells were similar but, when expressed as a proportion of the total macrophage population, term infants had significantly more of their macrophages present in mature form on day 1 (p=0.015, median term 1, median preterm 0.62). This higher proportion of mature (HLA-DR expressing) macrophage from the outset in term infants may represent a significant difference between term and preterm infants, particularly with respect to phagocytosis of invading microbes and apoptotic cells.

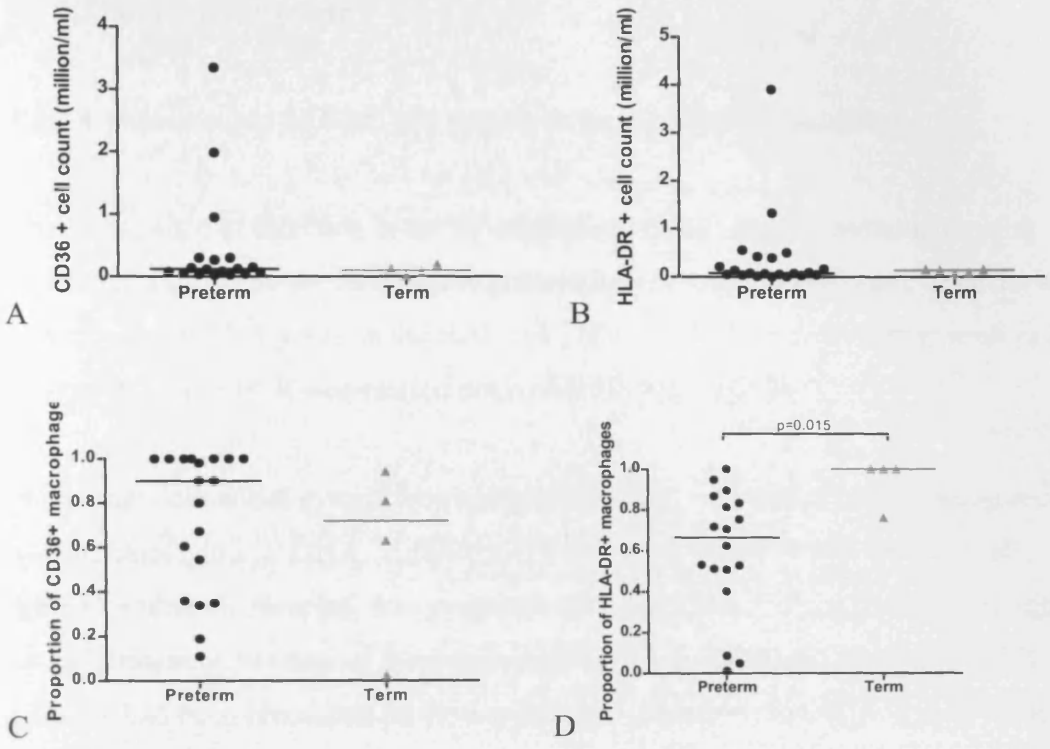


Figure 3.47 Scatterplots showing the absolute numbers (A and B) and relative proportions (C and D) of macrophage subtypes in term and preterm infants on day 1.

3.3.7 The role of infection

3.3.7.1 Relationship of BAL cell counts to the presence of infection

The importance of infection in the development of CLD and the association with neutrophil influx into the lung was examined by detecting the presence of bacterial 16S ribosomal RNA genes in the BAL cell pellet by PCR as described in section 2.1.5 of chapter 2. The PCR was carried out by Mr Michael Beeton.

Human mitochondrial cytochrome oxidase (HMCO) is a marker of the presence of human mitochondrial DNA. Sufficient cell pellet, as judged by the presence of HMCO within the samples, was present in 177/207 samples from 31 of the 32 infants. In the remaining 30 samples there was insufficient cell pellet remaining for PCR after material had been processed for flow cytometry. Genes for 16S rRNA were detected in 35/177 (19.8%) of the tested lavage samples and at some stage in 14/31 infants (45.2%).

Significantly more infants who went on to develop CLD had 16S rRNA genes detected in their BAL fluid at some point (11/15 or 73.3%) compared to infants whose RDS resolved (2/10 or 20%), Chi square test, p=0.0089. The number of preterm babies who had 16S rRNA detected (11 CLD + 2 RDS = 13/25 or 52%, 95% CI 33.50% - 69.97%) was not statistically greater than that of term control infants (1/5 or 20%, 95% CI 2.03% - 64.04%) p=0.1904, probably partly as a result of the small number of term infants involved (Table 3.3).

	Babies with 16s rRNA detected (%)	Babies with 16s rRNA not detected (%)	TOTALS
CLD	11 (73.3)	4 (26.7)	**15**
RDS	2 (20)	8 (80)	**10**
Term	1 (20)	4 (80)	**5**
Died	0 (0)	1 (100)	**1**
TOTALS	**14 (45.2)**	**17 (54.8)**	**31**

Table 3.3 Table showing number (and percentage) of infants in each diagnostic category with and without 16S rRNA detected at any point during their period of ventilation.

Both antenatal and postnatal infection have been implicated in the pathogenesis of CLD. In order to further define the possible roles for each of these infections, infants with 16S rRNA genes detectable in the first 3 days of life were assumed to have acquired these in the antenatal period. Beyond the first 3 days of life, it is more likely that micro-organisms in the lung may have been acquired postnatally, while the infant was ventilated on the neonatal unit.

There was no significant difference between CLD and RDS babies when compared for the presence of 16S rRNA genes in the first 3 days of life (3/15 or 20% in CLD vs 0/10 or 0% in RDS) (Chi square test, p=0.471) (Table 3.4), nor was there a difference between term (1/5 or 20%) and preterm infants (3 CLD + 0 RDS = 3/25 or 12%) (p=0.823) in the first 3 days. This may indicate that the hospital-acquired infections associated with prolonged ventilation are important in the pathogenesis of CLD, rather than antenatal bacterial infection as is commonly thought. However, numbers in each group were small.

	Babies with 16s rRNA detected by day 3 (%)	Babies with 16s rRNA not detected by day 3 (%)	TOTALS
CLD	3 (20)	12 (80)	**15**
RDS	0 (0)	10 (100)	**10**
Term	1 (20)	4 (80)	**5**
Died	0 (0)	1 (100)	**1**
TOTALS	**14 (45.2)**	**17 (54.8)**	**31**

Table 3.4 Table showing number (and percentage) of infants in each diagnostic category with and without 16S rRNA detected in BAL samples during the first 3 days of life.

Of the 129 samples from infants developing CLD which were judged to be adequate by the presence of HMCO, 28 (21.7%) had 16S rRNA genes detected, which was not significantly more than the 6/30 (20%) samples from infants whose RDS resolved, p=0.8374. There was also no significant difference in the number of 16S rRNA positive samples from preterm infants (34/159, 21.4%) and the 1/14 (7.1%) from term control infants (p=0.2035) (Table 3.5). The very small number of term controls makes interpretation of this result difficult.

	Samples with 16s rRNA detected (%)	Samples with 16s rRNA not detected (%)	TOTALS
CLD	28 (21.7)	101 (78.3)	**129**
RDS	6 (20)	24 (80)	**30**
Term	1 (7.1)	13 (92.9)	**14**
Died	0 (0)	4 (100)	**4**
TOTALS	**35 (19.8)**	**142 (80.2)**	**177**

Table 3.5 Table showing number (and percentage) of BAL samples from infants in each diagnostic category with and without 16S rRNA detected.

While more infants who developed CLD had 16S rRNA detectable in their BAL samples, a similar proportion of individual BAL samples from CLD and RDS babies had 16S rRNA genes present. Overall there were 11 babies with a total of 28 positive samples who developed CLD and 2 babies whose RDS resolved who supplied a total of 6 positive samples.

This information is difficult to interpret but may indicate that babies who develop CLD have more than one episode of infection with each being of short duration and babies whose RDS resolves tend to have only a single episode prior to being extubated. This pattern can be seen from the episodes of 16S rRNA positive BAL samples marked on individual babies' data (arrows on Figures 3.13, 3.18, 3.26, 3.31 – particularly babies CC, G and O), but may be more evident from a larger sample. Other possible explanations for the differences observed between the frequency of detection of 16S rRNA genes between CLD and RDS babies could be that RDS infants are only colonised with microbes and CLD babies develop a true infection or that the immune response to the presence of a microbe differs between the two groups – neither of these conclusions can be drawn from the data I collected.

Data were, however, collected on the type of organism from which the 16S rRNA genes originated by sequencing of the 16S rRNA PCR product. It can be seen in Figure 3.49 that the two infants whose RDS resolved had *S. epidermidis* identified on sequencing of the 16S rRNA genes detected in their BAL samples, an organism not generally thought to be a pathogen in the neonatal lung; whereas babies who developed CLD had a variety of other organisms identified (but including *S. epidermidis*), which are generally considered to be pathogenic, particularly in the neonatal lung.

3.3.7.2 Total cell counts in infection

Samples in which 16S rRNA was detected had significantly higher total cell counts than samples which were negative for 16S rRNA (Mann-Whitney U-test, p=0.0001) (16S negative median 1.18 $x10^6$ cells/ml, mean 1.74 $x10^6$ cells/ml; 16S positive median 2.20 $x10^6$ cells/ml, mean 2.90 $x10^6$ cells/ml). There was no significant difference in the peak cell counts (16S positive median 4.21 $x10^6$ cells/ml, mean 5.11 $x10^6$ cells/ml; 16S negative median 1.95 $x10^6$ cells/ml, mean 3.33 $x10^6$ cells/ml) (Mann-Whitney U-test, p=0.118)

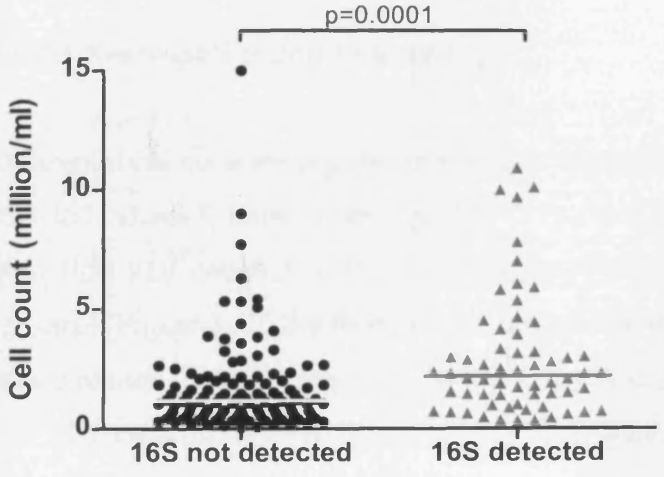


Figure 3.48 Scatterplot showing total cell count in BAL according to presence or abscence of 16S rRNA genes in the same sample. Horizontal lines represent medians.

When the 16S rRNA genes were sequenced, the highest cell counts were associated with organisms considered to be pathogens in neonatal practise and lower cell counts associated with the presence of *S. epidermidis* (Figure 3.49).

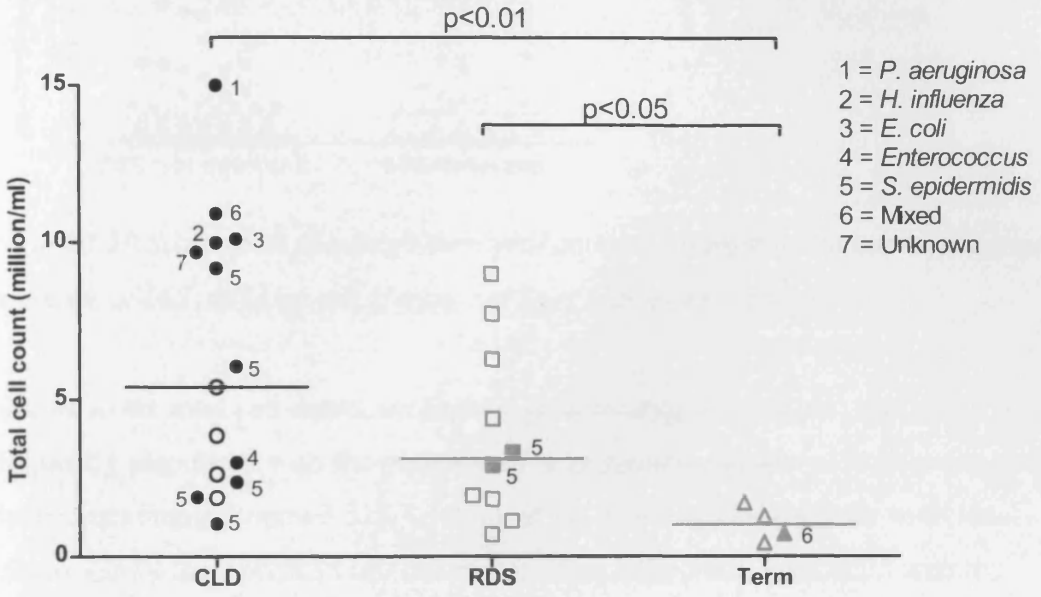


Figure 3.49 Scatterplot of peak cell count for each diagnostic group. The filled boxes were 16S rRNA gene positive and the empty boxes were negative. Sequencing data for 16S rRNA gene positive organisms is also shown.

3.3.7.3 Neutrophil counts in infection

Neutrophil counts were significantly higher in samples where 16S rRNA genes were detected (Mann-Whitney U-test, p=0.0003) (16S negative median 0.12 $x10^6$ cells/ml, mean 0.53 $x10^6$ cells/ml; 16S positive median 0.67 $x10^6$ cells/ml, mean 1.22 $x10^6$ cells/ml) (Figure 3.50) but there was no significant difference in the peak neutrophil counts related to the presence of infection (Positive mean 3.65 $x10^6$ cells/ml, median 2.04 $x10^6$ cells/ml; negative mean 1.37 $x10^6$ cells/ml, median 0.91 $x10^6$ cells/ml) (Mann-Whitney U-test, p=0.105).

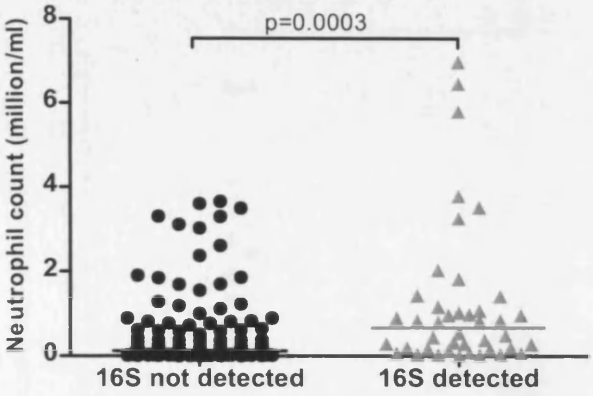


Figure 3.50 Scatterplot showing neutrophil counts in samples with and without the presence of 16S rRNA genes. Horizontal lines represent medians.

Similar to the total cell count, the highest peak neutrophil counts in each baby were frequently associated with the presence of an organism considered to be pathogenic in the neonatal lung (Figure 3.51). Looking at the data longitudinally for individual infants shows that spikes in cell counts are often temporally associated with the detection of 16S rRNA genes in the cell pellet. (See individual baby data in Figure 3.13 previously.)

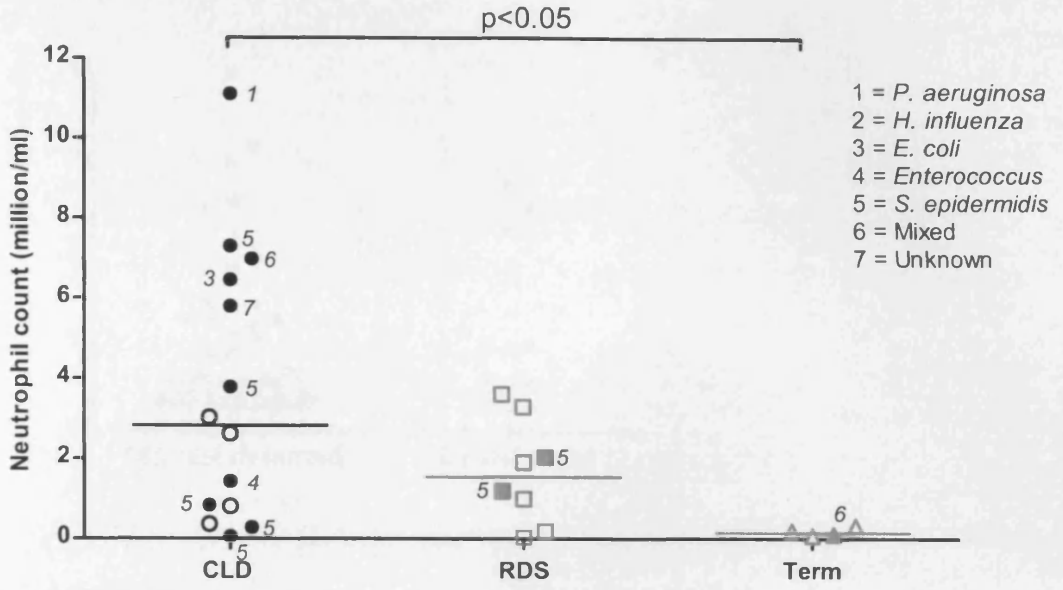


Figure 3.51 Scatterplot of peak neutrophil count for each diagnostic group. The filled boxes were 16S rRNA gene positive and the empty boxes were negative. Sequencing data for 16S rRNA gene positive organisms is also shown. One sample (from baby BB) was identified as 16S rRNA gene positive but no organism could be sequenced. There was no differential cell count for baby A (term) or B (CLD- H. influenza detected).

3.3.7.4 Macrophage counts in infection

The macrophage count is also significantly higher in infected babies (Mann-Whitney U-test, p=0.0003; 16S negative median 0.17 $x10^6$ cells/ml, mean 0.40 $x10^6$ cells/ml; Positive median 0.44 $x10^6$ cells/ml, mean 0.72 $x10^6$ cells/ml). This suggests that the overall total cell count increase is contributed to by both neutrophils and macrophages. Once again, there is no significant difference in the peak macrophage counts (positive mean 1.07 $x10^6$ cells/ml, median 0.74 $x10^6$ cells/ml; negative mean 1.23 $x10^6$ cells/ml, median 0.51 $x10^6$ cells/ml) (Mann-Whitney U-test, p=0.786). The individual baby data shown in Figure 3.26 previously also confirm a temporal association between the detection of 16S rRNA and an increase in the macrophage count.

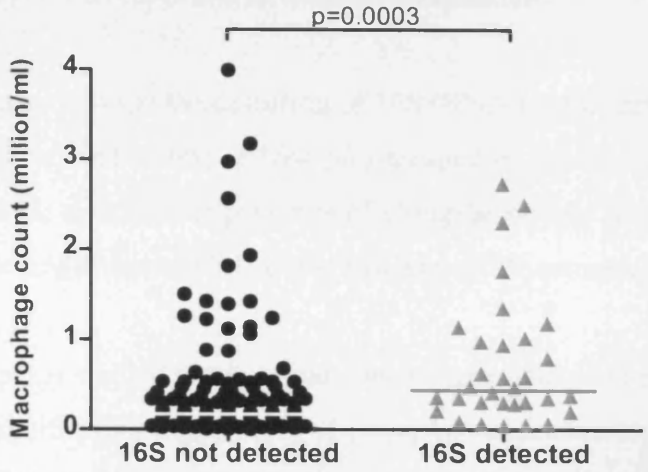


Figure 3.52 Scatterplot showing macrophage count in samples with and without 16S rRNA detected.

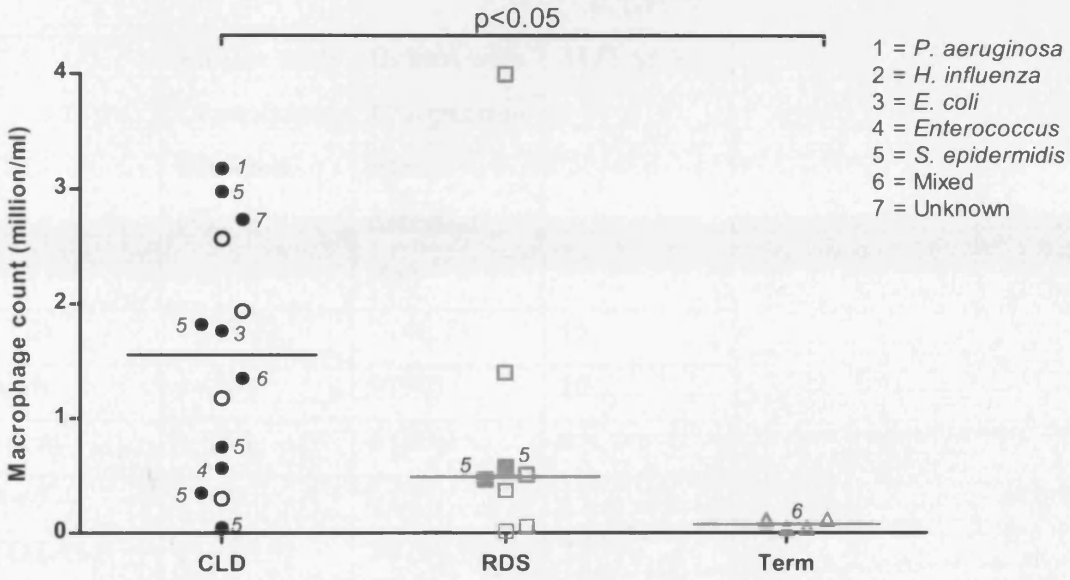


Figure 3.53 Scatterplot of peak macrophage count for each diagnostic group. The filled boxes were 16S rRNA gene positive and the empty boxes were negative. Sequencing data for 16S rRNA gene positive organisms is also shown. One sample (from baby BB) was identified as 16S rRNA gene positive but no organism was able to be sequenced. There was no differential cell count for baby A (term) or B (CLD- H. influenza detected).

3.3.7.5 *Ureaplasma* infection/colonisation

In addition to the detection of 16S rRNA for bacterial infection, BAL samples also underwent culture in *Ureaplasma* specific medium (as described in 2.1.6 above) in order to detect the presence of *Ureaplasma spp.* which have been implicated in the pathogenesis of CLD but whose exact role remains highly controversial.

In this studied group of patients, infants who went on to develop CLD had significantly higher rates of *Ureaplasma* detected in their BAL fluid at some point (8/15 or 53.3%) compared to infants whose RDS resolved (1/10 or 10%) (Chi square test, p=0.027). The number of preterm babies who had *Ureaplasma* detected (9/25 or 36%) was not statistically greater than that of term control infants (1/5 or 20%) (p=0.488).

	Babies with *Ureaplasma* detected (%)	**Babies with *Ureaplasma* not detected (%)**	**TOTALS**
CLD	8 (53.3)	7 (46.7)	**15**
RDS	1 (10)	9 (90)	**10**
Term	1 (20)	4 (80)	**5**
Died	1 (100)	0 (0)	**1**
TOTALS	**11 (35.5)**	**20 (64.5)**	**31**

Table 3.6 Table showing the prevalence of Ureaplasma spp. *in BAL samples.*

In line with current practice and protocols on the neonatal unit, the majority (7/11) of infants in whom *Ureaplasma* was detected did not receive any specific treatment for this. Four babies were treated with macrolide antibiotics which resulted in the organism being cleared from 2 infants, a highly resistant organism persisting in one baby and the organism briefly being cleared before returning after antibiotic treatment was discontinued in the fourth infant. All 4 of these babies went on to develop CLD.

3.3.7.6 Differential cell counts in babies with *Ureaplasma*

3.3.7.6.1 Neutrophils

There was no significant difference in either percentage (Mann-Whitney U-test, p=0.5839) or absolute neutrophil count (Mann-Whitney U-test, p=0.5388) in babies in whom *Ureaplasma* was detected.

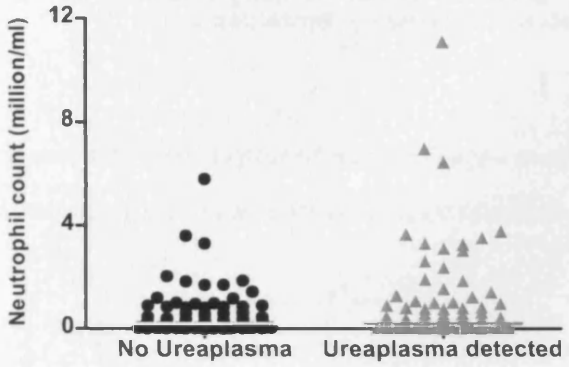


Figure 3.54 Scatterplot of neutrophil counts in babies with or without Ureaplasma *detected. Horizontal lines represent medians.*

3.3.7.6.2 Macrophages

Both the percentage macrophages and absolute macrophage counts (Figure 3.55) were significantly higher in babies in whom *Ureaplasma* was detected (Percentages: Mann-Whitney U-test, p=0.0045; Negative median 14.2%, mean 18.8%; Positive median 21.2%, mean 24.5%) (Counts: Mann-Whitney U-test, p=0.038; Negative median 0.21 $x10^6$ cells/ml, mean 0.43 $x10^6$ cells/ml; Positive median 0.31 $x10^6$ cells/ml, mean 0.53 $x10^6$ cells/ml). Peak macrophage counts were also significantly higher in babies with positive cultures for *Ureaplasma* (Mann-Whitney U-test, p=0.006; Negative median 0.40 $x10^6$ cells/ml, mean 0.77 $x10^6$ cells/ml; Positive median 1.76 $x10^6$ cells/ml, mean 1.74 $x10^6$ cells/ml).

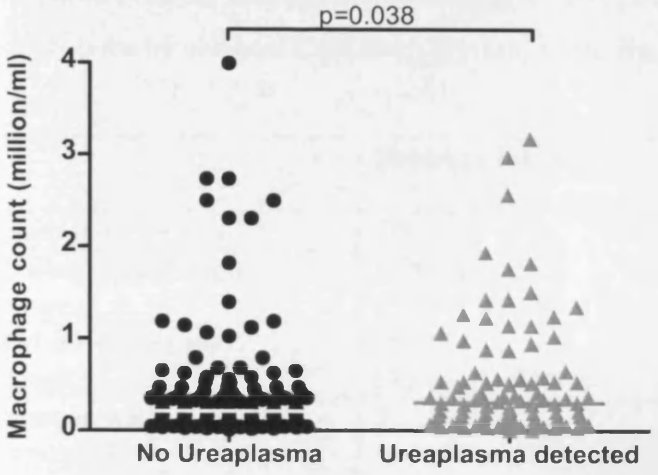


Figure 3.55 Scatterplot of macrophage counts in babies with or without Ureaplasma *detected. Horizontal lines represent medians.*

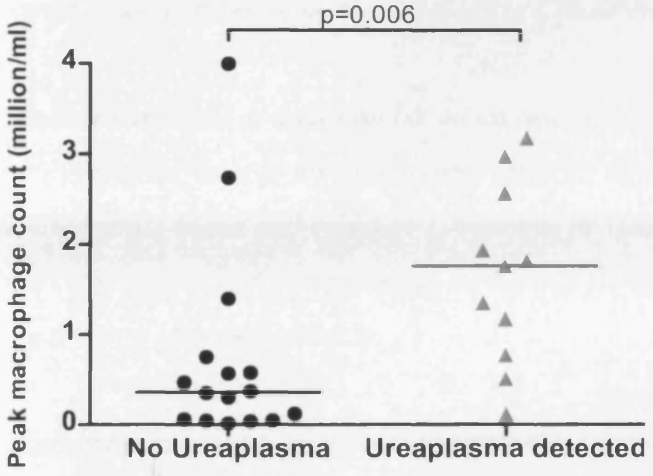


Figure 3.56 Scatterplot of peak macrophage counts of babies with and without Ureaplasma. *Horizontal lines represent medians.*

3.3.7.7 Antenatal infection

Once again, I sought to understand if any differences between antenatally infected and uninfected infants could be found, by using data from the first day's BAL sample as there is evidence to suggest that the majority of cases of spontaneous preterm labour occur as the result of an infective process (Gomez et al., 1997), even if a specific organism cannot be isolated or identitifed (Klein and Gibbs, 2004, Sanchez, 1993). I

compared preterm infants born following spontaneous preterm labour against preterm infants born by elective Caesarean section, when the mother was not in labour.

	Preterm labour	**Elective Caesarean section (no labour)**
Number of babies	20	6
Median birthweight (range)	0.905 kg (0.56-1.92 kg)	0.965 kg (0.7-1.18 kg)
Median gestation (range)	26^{+2} ($23^{+4} - 31^{+2}$)	28^{+1} ($27^{+0} - 29^{+3}$)
Number with *Ureaplasma* diagnosed	9	0
Number with PROM	5	1
Number with clinical suspicion of infection*	11	3

Table 3.7 Table showing characteristics of preterm infants born following spontaneous preterm labour or by elective Caesarean section.

**Clinical suspicion of infection based on one or more of the following being recorded in the maternal notes prior to delivery: pyrexia, offensive smelling vaginal discharge, elevated white blood cell count or C-reactive protein, result of high vaginal swab shows organism/s not considered to be normal flora, uterine tenderness or irritability suggestive of chorioamnionitis.*

Infants born following spontaneous preterm labour were not significantly different in weight (p=0.784) from their electively delivered counterparts. The preterm labour group had a median gestation which was statistically significantly lower than the elective Caesarean group (p=0.0224), possibly related to intrauterine infection precipitating the earlier onset of labour in these pregnancies.

It is immediately apparent that all the babies in whom *Ureaplasma* was detected were in the preterm labour group (Chi square test, p=0.042). *Ureaplasma* is well recognised as a cause of preterm labour (Embree and Embil, 1980, Kataoka et al., 2006) and the transmission from mother to infant has been shown to be highest in the most preterm infants (Alfa et al., 1995). The number of infants in each category with PROM was

not significantly different (Chi square test, p=0.67), nor was the number with suspected infection (Chi square test, p=0.82).

In the preterm labour group, 11/20 infants developed CLD compared to 4/6 of the electively delivered infants (Chi square test, p=0.612). Larger numbers of patients would be required to make an adequate statistical comparison between the two groups.

3.3.7.7.1 Cell counts on day 1 in relation to mode of delivery

The total cell count just failed to reach significance in infants born following spontaneous preterm labour compared to those born by elective Caesarean section (p=0.051), however a larger sample may have produced a significant result.

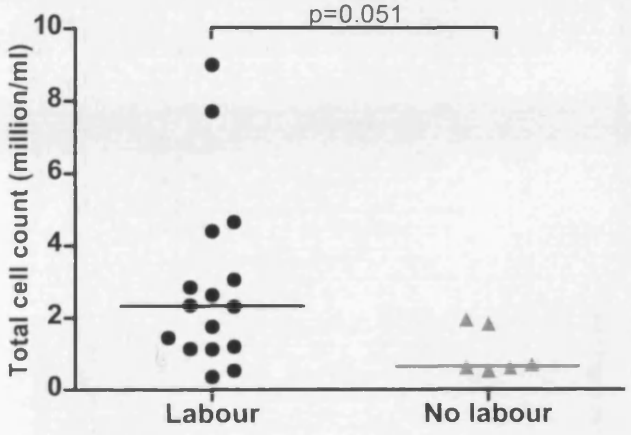


Figure 3.57 Scatterplot showing total cell count on the first day of life for infants born following spontaneous onset of labour against infants born by elective Caesarean section without labour. Horizontal lines represent medians.

There were no significant differences between the two groups with respect to differential counts or macrophage subtypes.

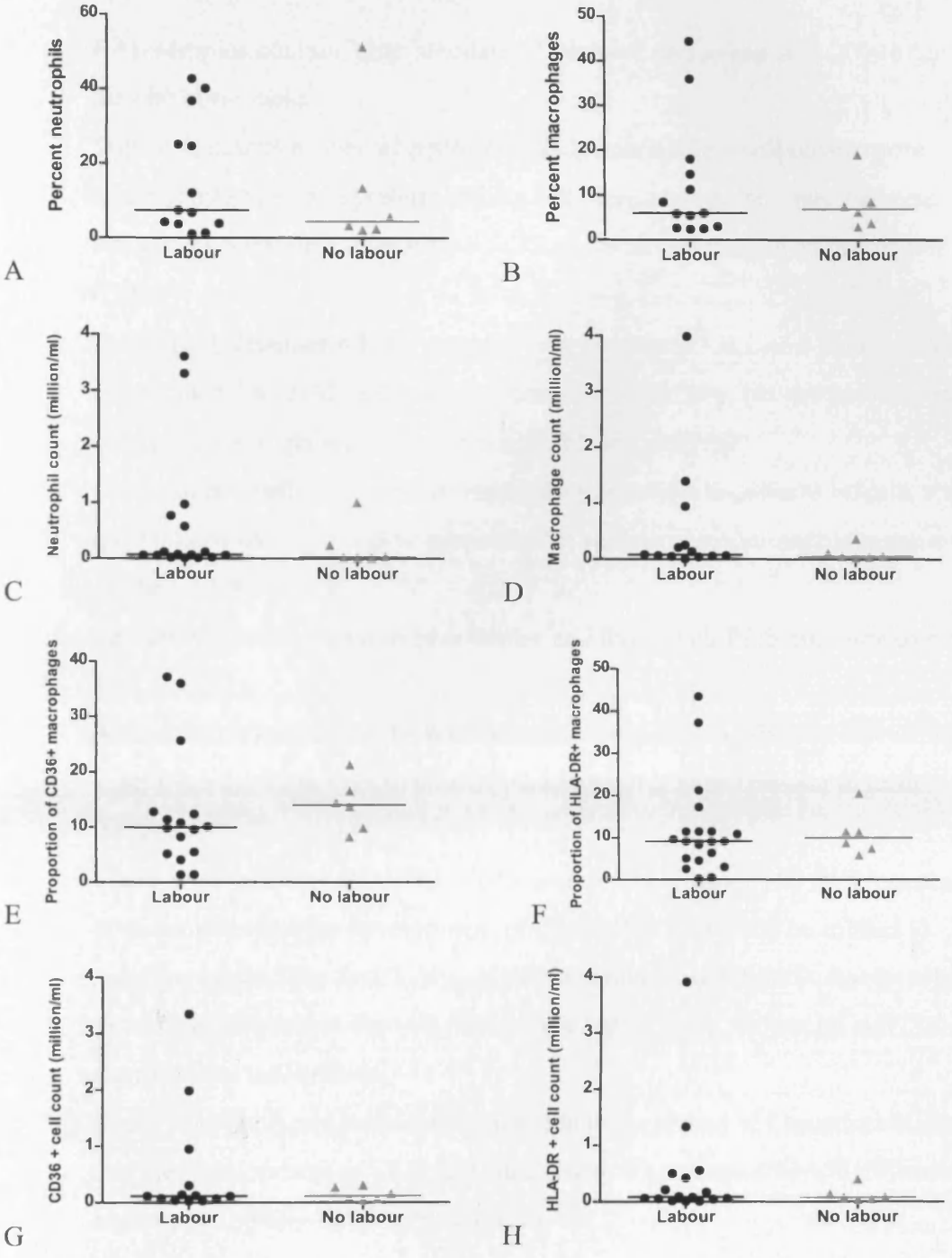


Figure 3.58 Scatterplots comparing infants following spontaneous labour compared with those born by elective Caesarean section before the onset of labour. There were no significant differences in (A) proportion of the sample made up of neutrophils, (B) proportion of the sample made up of macrophages, (C) neutrophil count, (D) macrophage count, (E & G) proportion or absolute numbers of macrophages expressing CD36 or HLA-DR (F & H).

3.4 Summary of key results

1. BAL samples contain large amounts of "debris" and as much as 20% of cells may be non-viable.
2. Total cell counts as well as neutrophil and mononuclear cell counts were significantly higher in preterm infants but there were no obvious differences in cell counts between babies whose RDS resolved and those who progressed to CLD.
3. Neutrophils from term BAL samples express more TLR 2 and TLR 4 on the cell surface than BAL neutrophils from preterm infants, but the amounts of surface CD14 expression were not significantly different.
4. Mononuclear cells have a more immature phenotype in preterm infants, with term infants having a higher proportion of mature alveolar macrophages even on the first day of life.
5. Cell counts peak earliest in term babies and those with RDS and later in babies who get CLD.
6. Almost no difference can be seen between the groups on the first day of life, apart from the higher proportion of mature macrophages present in term lavages.
7. There is a significant association between the presence of 16S rRNA genes in BAL samples and the development of CLD. This appears to be related to infection beyond the first 3 days of life in babies who get CLD. Babies who have samples positive for 16S rRNA have higher total, neutrophil and mononuclear cell counts.
8. There is a significant relationship between the presence of *Ureaplasma spp.* and the development of CLD and babies with *Ureaplasma* have significantly higher macrophage counts in BAL samples.
9. There were no appreciable differences between infants delivered by elective Caesarean section and those born following spontaneous preterm labour, apart from the prevalence of *Ureaplasma* being significantly higher in the preterm labour group.

3.5 Discussion

3.5.1 Overview

This portion of the study provides a detailed analysis of the inflammatory cell infiltrate in the lungs of a group of ventilated term and preterm infants using flow cytometry. The study includes 32 infants, of which 27 were less than 32 weeks' gestational age. It is the only study, to my knowledge, that examines in such detail, by flow cytometry the cellular constituents of BAL samples in newborn term and preterm babies.

The median gestational age for the infants who developed CLD was 26 +0 weeks and for those whose RDS resolved it was 27 +1 weeks. This was statistically significant and it is to be expected that those infants who did not develop CLD were more mature. Despite this difference in gestational age, there was no statistically significant difference in birthweight between the groups, although babies whose RDS resolved had a median birthweight 150 g more than the median for the CLD group. This is probably in keeping with a widely held view among neonatologists that infants who are "stressed" in-utero (reflected by reduced growth) experience less severe postnatal lung disease.

In the majority of the preterm cases, at least one dose of antenatal steroids had been administered to the baby's mother prior to delivery. Less than half of the mothers received a second dose of antenatal steroids because, as a result of preterm labour, delivery of the infant occurred prior to the second dose being due. This may be a source of bias in this study because of the small sample size. Statistics for the neonatal unit reveal this cohort of infants to be slightly unusual, in that unit statistics reveal that 88.5% of infants born at less than 32 weeks' gestation in 2007 received a full course of antenatal steroids (Cherian et al., 2007). All the preterm infants were intubated at birth and received exogenous surfactant therapy. All infants born with respiratory distress received postnatal antibiotics for at least 48 hours, until blood cultures taken at birth could be confirmed as negative. Antibiotics were continued for 5 or 7 days if infection was proven or clinically suspected.

The presence of a PDA which required medical or surgical intervention was a significant feature of the CLD group. PDA has not been shown to be an independent risk factor for CLD (Arnon et al., 1993, Fraser et al., 2004). It may be that in this relatively small sample, the presence of a PDA in association with lung disease which showed signs of clinical progression towards CLD, prompted the attending clinician to choose medical or surgical PDA treatment. PDA closure may not have been undertaken in the RDS group as these babies may have been felt to be improving without the need for PDA closure. Additionally, the signs or symptoms of PDA may result in a longer duration of ventilation and the use of higher pressures and inspired oxygen levels, each increasing the likelihood of an "at-risk" infant developing CLD.

Our study benefits from the presence of a group of term control infants, who were ventilated for non-respiratory reasons. Although there were only five infants recruited to this arm of the study, I was able to observe the responses of largely "normal" term infant lungs to low pressure, low volume ventilation in air or <28% oxygen and compare these with the findings in preterm infants. Recruitment of patients into the term control group was not easy due to the stringent entry criteria used in order to try to ensure the "normality" of the lungs of the term group. Clearly, healthy term infants are not usually ventilated. The main criteria for ventilation of term infants tend to be respiratory distress (e.g. secondary to infection or transient tachypnoea of the newborn), which would frequently require oxygen concentrations >28%, a key exclusion criterion of our study, as hyperoxia has documented pro-inflammatory effects on lung (Davis et al., 1991). Alternatively, term infants may be ventilated for neurological problems, such as hypoxic ischaemic encephalopathy (HIE). Such infants were also excluded from the term control group as the period of hypoxia responsible for producing HIE, would also be very likely to trigger inflammatory changes within the lung. All of our control group were infants who underwent surgery for gastroschisis on the first day of life and required ventilation post-operatively. Gastroschisis usually occurs as an isolated anomaly with no associated lung pathology, so that at birth the lungs should be healthy. I recognise that gastroschisis may be a cause of systemic illness and that even healthy lungs may not remain "normal" after a period of ventilation, however, it remains the best control group available and a reasonable comparison between ventilated term and preterm lungs. Gastroschisis is not a common condition (4.6/10 000 births (Drewett et al., 2006) and

not every child born with gastroschisis requires post-operative ventilation for more than a few hours, which explains why only five term control infants were recruited.

Many studies which have examined lung lavage samples from newborn infants have used tracheal aspirate fluid (TAF), however, these samples largely reflect upper airway secretions. By using BAL fluid this study analyses inflammatory infiltrates from the lower airways, which may be particularly important in CLD where alveolar changes (larger, simplified alveoli), rather than more proximal airway abnormalities, are seen.

3.5.2 Development of flow cytometry analysis of BAL samples

Flow cytometry has not previously been published as a method for analysis of the celllar constituents of neonatal BAL samples. It has, however been used fairly extensively in studies of adult lung disease, including COPD and ARDS (Matute-Bello et al., 1997, Rosseau et al., 2000a, Pletz et al., 2004). Using the published information in the adult literature as a guide, I developed a method for the processing and analysis of neonatal BAL samples by flow cytometry, showing that the use of DTT as a mucolytic had no effect on any of the antigens which I studied.

Flow cytometry is a potentially more accurate method for identification of cell types and surface antigens than previously published techniques for analysis of neonatal BAL samples, which include immunocytochemistry and light microscopy and which rely on human observation for counting and recording cell types (Grigg et al., 1991, Kotecha et al., 2003, Oei et al., 2003). I found significant differences between total cell counts and differential counts based on the method used to analyse the cells, namely microscopy or flow cytometry.

There are a number of possible reasons for this:-

- cytospins varied in quality, dependent on the amount of mucus and/or cellular debris present which made counting more difficult and complex.

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There are a number of possible reasons for this:-

- cytospins varied in quality, dependent on the amount of mucus and/or cellular debris present which made counting more difficult and complex.

- when very little BAL material was available, there were very few cells on the cytospin slide and counted cytospin may not have been representative of the whole sample.
- when cells on cytospin slides were counted, at least 300 cells were counted for each sample in duplicate cytospins. Flow cytometry parameters were set up to count at least 10 000 events (with debris gated out). Thus flow cytometry differentials are more likely to be accurately representative of the sample. (The more events counted on a cytospins, the closer the result was to FACS results.)
- the flow cytometer requires a single cell suspension in order to accurately count and identify cells. At times there was clumping of BAL cells due to cell activation and/or the presence of mucus. These cell clumps would not be accurately counted by the flow cytometer but could be observed and counted more accurately on a cytospin slide.
- clusters of apoptotic neutrophils on a cytospin slide may have been within a macrophage but the macrophage may not have been clearly identified and thus its contents would have been individually counted and the macrophage not counted. The flow cytometer would simply count the macrophage with its ingested contents as 1 large macrophage, not several neutrophils.
- there was some inter-observer difference noted when I re-counted (blinded) a proportion of the cytospins
- inflammatory or immature macrophages (more monocyte-like phenotype) may not have been accurately counted as macrophages because of their small size and altered staining on cytospins when compared to more mature macrophages.
- diff-quik staining of cytospins produced results which were very variable in quality and therefore ease of counting. This may have contributed to difficulty in correct identification of some cells.

The use of flow cytometry, although potentially more accurate than counted cytospins, for the detailed analysis of neonatal BAL samples is a very time consuming process. For optimal results in this study, BAL cells were stained with antibodies and analysed on the flow cytometer immediately following the lavage procedure to avoid any deterioration in the sample quality or any ongoing necrosis or apoptosis of cells. The process of obtaining the BAL, separating and staining the cells and FACS

analysis took at least 4 hours per sample. By virtue of the study design, in which the first lavage was done when the infant was 12 hours old to minimise the impact of the procedure on clinical care, then repeated daily for 7 days, a large number of the BAL samples were taken at night and out of normal working hours, making the acquisition of data for the study relatively arduous. Fixation of stained BAL samples using paraformaldehyde to allow flow cytometry analysis to be delayed until "office hours" was considered but rejected for routine use as fixation changes the forward and side scatter patterns of cells on flow cytometry and would only have saved around 30 minutes per sample. Cytospins, on the other hand, can be made relatively quickly after the BAL has been performed and then either fixed or stained for analysis at a more convenient time and may thus be more suited for routine clinical applications.

3.5.3 Debris and non-viable cells

A proportion of each BAL sample contained viable cells which could be identified as neutrophils or macrophages by either flow cytometry or cytospin slides but there was a large amount of non-viable debris in many of the BAL samples. The amount of viable material increased with increasing duration of extra-uterine life.

In this study, I think it is likely that the debris is composed mainly of cell fragments as shown by CD59 and To-Pro 3 staining, revealing the debris to originate from human cells and/or contain DNA. Some of the debris may be artefact, created by the processing of the BAL sample. Although every care was taken to be gentle in any manipulation of the samples, it is probable that some damage to the cells may be caused by the pipetting and centrifugation required to study them. Debris may also originate from dead or damaged cells sloughed in-utero during lung growth and development – this may account for the larger amount of debris in initial samples. The initiation of an inflammatory process in the lungs may generate more debris in the early stages which then reduces as the process becomes established, but this is merely speculative as the debris component of BAL samples was not studied in detail.

No other study has reported the amount of non-viable material in infant BAL samples. Although there is no significant difference in the amount of debris between groups, the composition of the debris may in fact be variable and have an impact on the

outcome for the infant. The disposal of debris and the pro- or anti-inflammatory effects of this may also be of significance in the resolution of RDS or the development of CLD.

3.5.4 Cell counts

As with previous studies, this study demonstrates increasing cell counts, particularly neutrophils over the first few days of life in preterm infants. In published cohorts, cell counts were significantly greater in lavage fluid from infants who went on to develop CLD compared to infants whose RDS resolved (Merritt et al., 1983, Ogden et al., 1984, Watterberg et al., 1994), however this was not found in our group of babies, probably as a result of the relatively small number of infants studied. These papers also describe a pattern of elevated cell counts which remain high in infants who develop CLD compared to counts that rise over the first few days of life but then fall in infants whose RDS resolves. It important to note that most such studies have grouped data, which will have the effect of smoothing out an individual infant's fluctuations in cell counts. By analysing individual infants as well as grouped data, our study demonstrates that whilst overall (median) cell counts increase and remain elevated in CLD, individual babies tend to have cell counts which increase in an episodic manner and then return to lower levels. Most babies show a cell count rise in the first few days of life and then babies who remain intubated have further sporadic rises in cell count. Nevertheless, increases in cell counts, and particularly that of neutrophils, in BAL fluid demonstrate that inflammatory changes occur.

a) Neutrophil cell surface markers

CD14 expression on blood neutrophils has been reported to be lower in preterm infants than in adults or term babies (Henneke et al., 2003), however I found the expression of CD14 appeared similar between neutrophils from BAL samples in term and preterm infants. Individual infants in the preterm group had a large proportion of the neutrophil population expressing high levels of CD14, particularly on the first 2 days of life. This may reflect antenatal exposure to infection as CD14 is important in the recognition of pathogens by the cells and may be upregulated in the presence of LPS (Coimbra et al., 2004).

Overall the percentage of neutrophils showing detectable TLR 2 and TLR 4 expression in term infants was significantly more than in preterm infants even in this small sample. TLR 2 expression on leucocytes has been found to be slightly reduced in term infants compared to adults in one study of cord and adult blood (Viemann et al., 2005) but very similar in another (Sadeghi et al., 2007), however in the second study, response to TLR 2 signalling via MyD88 was reduced in newborns. Our finding of a significant reduction in the proportion of lung neutrophils expressing TLR 2 may indicate a further impairment in the ability of the preterm newborn to respond to pulmonary infection.

TLR 4 expression has been reported to increase with gestational age (Sadeghi et al., 2007) on blood cells and this may hold true for cells which have migrated into the airways. My finding of a reduced percentage of lung neutrophils expressing TLR 4 in preterm infants is supportive of this. It has been noted that while TLR 2 is upregulated markedly in neonatal sepsis, no such alterations are seen in TLR 4 (Viemann et al., 2005).

b) Macrophages

Macrophages show similar patterns of increase to the total cell count and neutrophil count, also showing sporadic peaks in association with the detection of infection in the BAL sample. Flow cytometry enables a detailed look at the different sub-types of macrophage present in BAL samples and this has not previously been undertaken in neonatal BAL fluid. Alveolar monocyte and macrophage phenotypes have been studied by flow cytometry in adults with both healthy and diseased lungs (Taylor et al., 2000, Umino et al., 1999, Ward et al., 2001). All agree that the study of alveolar macrophages by this method is useful but complicated by the high levels of autofluorescence of the macrophages and all the studies use different markers to classify and differentiate the cells. The only study of alveolar macrophage markers in the paediatric population (Grigg et al., 1999) studied BAL fluid from children from 7 days to 17 years of age using immunocytochemistry and found that relative immaturity of alveolar macrophages may be a contributor to increased severity of pulmonary infection in young children. BAL samples from preterm infants in our

study had higher numbers of monocyte-like (CD14 high) macrophages than term babies. Preterm infants also had a higher proportion of the total macrophage count in the form of monocyte-like (CD14 high) macrophages than term babies. Term infants had almost all their macrophages displaying high levels of HLA-DR, a marker of a mature alveolar macrophage, whereas in preterm infants, this proportion was lower. If day 1 alone is reviewed, term babies have a median of around 90% of their macrophages expressing high levels of HLA-DR, compared to just over 60% in the preterm group.

Together, this implies that the macrophage population in preterm infants is altogether less mature at delivery than in term infants. This immature macrophage population may be present as a result of rapid and ongoing recruitment of monocytes to the lung in the face of continuing inflammation. Immaturity may render the macrophage less able to cope with phagocytosis of pathogens, possibly leading to infection becoming established rather than being cleared. Immature (monocyte-like) macrophages have been shown to have augemented release of pro-inflammatory mediators in adults (Rosseau et al., 2000a, Maus et al., 2002c, Maus et al., 2001) and, although this has not yet been proven in infants, it is likely that the higher proportion of immature macrophages in preterm babies could also alter the ability of the macrophage population to modulate cytokine production and lead to further, excess neutrophil recruitment, which may overwhelm the macrophage capacity to clear the effete neutrophils. In addition, immature macrophages may be less able to phagocytose apoptotic neutrophils and this will be discussed in chapter 4. As a result, a large number of neutrophils is able to accumulate in the ventilated preterm infant lung.

c) Patterns of influx

The timing of neutrophil versus macrophage influx is of particular note. Previous studies (Kotecha et al., 2003) showed a total cell count which rose from birth and peaked on about day 4 of life in infants with RDS and on day 10 in infants who developed CLD. Our cohort showed peak total cell counts on day 2 in RDS and day 7 in CLD compared to day 1 in term infants. The Kotecha *et al* paper only compared samples from preterm infants taken twice weekly and not daily as in our project, thus possibly missing important peaks in cell counts. Additionally, cell counting by

cytospins and haemocytometer preparations may have contributed to the differences between our project and previously published work as these are to some extent operator dependent.

The macrophage count in this study reached its peak on day 10/11. Our data from the current cohort show that neutrophil and macrophage counts appear to increase almost simultaneously. This perceived difference may be related to the method of cell identification (cytospins in Kotecha *et al* compared to FACS in this study) and also to the small sample sizes available in studies of this nature. The Kotecha *et al* paper also did not perform daily samples during the first week of life which may imply that the peak cell counts which I observed early in the course of ventilation (days 3 - 4) were simply missed due to the sampling schedule used.

Not only is the timing of influx of different cell types of interest, but the timing of cell influx in the different diagnostic categories is also interesting. Term babies experience their peak total cell counts within the first 24 hours, followed by RDS babies and then CLD babies have highest cell counts at almost 3 days of age (when looking at first 5 days alone) or day 7 (for entire ventilated period, but this is influenced by cell count fluctuations related to possible infective episodes later in the period of intubation). In order to try to elucidate factors which may differentiate between the groups very early in their ventilatory course and to shed some light on possible antenatal predeterminants of the course of pulmonary inflammation, various parameters were analysed on day 1 of life alone. No significant differences in total or differential cell counts were observed on the first day of life and for this reason it is unlikely that a cell count or differential count from a first day BAL sample would be clinically useful in the early identification of infants at highest risk of CLD.

d) Infection

A striking feature of all the longitudinal data for cell counts in individual babies is the sudden episodic increases that were observed. The most likely explanation for these sudden increases is the innate immune response to pulmonary infection. Ventilated preterm infants are at high risk of developing infections and much of neonatal care is devoted to preventing or treating these. All infants born with respiratory distress on

our unit are started on benzylpenillicin and gentamicin to treat potential infection and stringent precautions are in place to minimise nosocomial infection. Despite this, episodes of proven or suspected sepsis are common.

Identification of infection in neonates is difficult. Clinically, a variety of signs including temperature instability, poor perfusion, increased ventilatory requirements, changes in the infant's usual behaviour or activity levels or poor feed tolerance may be noticed. Neonates are frequently treated for "presumed" or "suspected" sepsis based on clinical suspicion as all of these signs are non-specific and may not be present in the early stages of infection. Laboratory findings such as raised (or reduced) white cell counts, reduced platelet counts or elevated C-reactive protein may guide clinical judgement and cultures of blood, urine, airway secretions or cerebrospinal fluid may or may not identify pathogenic organisms. Special investigations such as X-rays may further assist in diagnosis of infection but X-ray findings, particularly chest X-ray features associated with infection, may lag behind clinical findings both in onset and resolution of changes.

Culture of BAL fluid is the best clinical method to identify a chest infection and identify an organism, however growth of a specific organism is frequently difficult to obtain given the small volumes of BAL fluid obtained and the widespread and early use of antibiotics when infection is suspected. The use of PCR to detect genes coding for 16S rRNA has been shown to increase sensitivity of bacterial detection in a number of clinical settings compared to standard cultures, such as in amniotic fluid (Markenson et al., 1997, Oyarzun et al., 1998) and in cystic fibrosis (Rogers et al., 2006) and it is now widely used in a research setting. One study has used this technique in neonatal BAL fluid (Miralles et al., 2005), however, this only sought the presence of 16S rRNA genes on the first day of life and focused mainly on the role of antenatal infection on preterm labour. One previous study has used 16S rRNA genes to specifically examine the link between infection and pulmonary inflammation in the development of CLD (Miralles et al., 2005).

Although infection has been implicated in the development of CLD, there are surprisingly relatively few studies to support this. Antenatal infection, particularly with *Ureaplasma urealyticum* has been implicated in the onset of preterm labour

(Goldenberg et al., 2000) and may be an independent risk factor for the development of CLD (Schelonka et al., 2005). Postnatal infection is less well examined. Several studies (Rojas et al., 1995, Van Marter et al., 2002, Liljedahl et al., 2004) found an association with systemic infection and the development of CLD. Cordero *et al* (Cordero et al., 1997) found that colonisation of the airways with Gram negative bacteria was associated with the development of CLD and Groneck *et al* (Groneck et al., 2001) demonstrated increased perinatal infections in tracheal aspirate fluid of infants who develop CLD.

Using PCR techniques to identify 16S rRNA genes, I demonstrated the presence of bacterial DNA in one fifth (35/177) of BAL samples. This may reflect true lower airway bacterial infection, although it is also possible that contamination could occur from the endotracheal tube through which the suction catheter must pass to perform the BAL. Among babies who developed CLD 11/15 had microbial DNA detected in their BAL sample at some stage, compared to 2/10 of the RDS group. The use of these PCR techniques would support the hypothesis that development of CLD is associated with the presence of bacteria. When the data are reviewed for only the first 3 days of life, in an attempt to discriminate between antenatally and postnatally acquired organisms, the relationship between the presence of 16S rRNA genes and the development of CLD is not significant. Although the numbers are small, as this study was attempting to develop a methodology for neonatal BAL analysis by flow cytometry and not powered to detect differences in the development of CLD related to infection, this may suggest that it is infection in those babies who remain intubated which may be the cause of the lung injury that leads to the development of CLD, rather than antenatally acquired organisms.

The predominant organism detected in this cohort of infants was *S. epidermidis*. It was the only organism identified in the group of babies whose RDS resolved, while infants in the CLD group had a variety of organisms identified, including *S. epidermidis* but also a larger number of organisms which are widely recognised as pathogenic. The role of *S. epidermidis* in the neonatal lung is unclear. It is an organism which is frequently associated with indwelling venous or arterial catheters and *S. epidermidis* bacteraemia is always promptly treated with antibiotics and removal of the catheter, however *S. epidermidis* may equally colonise the

endotracheal tube and be found in lung lavage samples. Many neonatal clinicians regard this as a contaminant, introduced during endotracheal suction, and will not routinely treat an infant for isolation of *S. epidermidis* from endotracheal secretions. Recently, more attention has been paid to the role of *S. epidermidis*, in particular its interaction with the neonatal immune system (Hartel et al., 2008). *S. epidermidis* colonisation of the endotracheal tube may be truly innocuous, however infection of the lung itself might be more harmful and the difference between endotracheal tube colonisation and true lung infection may help to explain the presence of *S. epidermidis* in both RDS and CLD groups. However, with respect to *S. epidermidis,* it has been previously shown that *S. epidermidis* is a weaker inducer of the neutrophil inflammatory response than an organism like *S. aureus* (Nilsdotter-Augustinsson et al., 2004), which may also in part account for our findings that *S. epidermidis* detection may be less frequently associated with the development of CLD than detection of other organisms. Further knowledge of the role of this organism may be a useful addition to neonatal care but this would require differentiation of colonisation of the endotracheal tube from infection in the lung itself and may thus entail the use of an animal model.

Ureaplasma was considered separately from other types of infection as its role in the development of CLD is far more controversial among neonatologists, possibly because its role in precipitating preterm labour and delivery is difficult to separate from the role of prematurity in the development of CLD (Embree and Embil, 1980, Schelonka and Waites, 2007, Kataoka et al., 2006). Also, treatment of *Ureaplasma* infection or colonisation has not been shown to reduce the development of CLD, although there is very little evidence for this conclusion (Mabanta et al., 2003).

This study supports a significant relationship between the development of CLD and the presence of *Ureaplasma spp.* in the preterm lung.

Overall, our data suggest that the presence of bacteria in the lung is associated with increased inflammation within the airways, as evidenced by elevated cell counts. As neutrophils enter the lungs they may release their proteases such as elastase and MMP-9 as well as reactive oxygen species in response to pathogens. These proteases and reactive oxygen species may be responsible for the tissue injury and abberant

lung growth that is central to the development of CLD. Levels of human neutrophil elastase were measured in our samples and its role will be explored further in chapter 5.

3.5.5 Conclusion

In this chapter I have presented flow cytometry data on the cellular component of BAL fluid from a group of 32 ventilated term and preterm infants. Detailed flow cytometry analysis of BAL cells in this population of patients had not been previously published.

I have tried to describe the nature and progress of the inflammatory response in these infants and attempted to relate the number, type and sub-type of cells present to the development of CLD. Infants who develop CLD have higher cell counts and a larger proportion of the pulmonary mononuclear cell population are present in a more immature form, which may signify that immaturity of the innate immune system predisposes these preterm infants to a more uncontrolled or dysregulated inflammatory response and the development of CLD. Longitudinal data for individual infants over their entire ventilation course may be more informative regarding the development of CLD than looking at groups of babies due to the rapid and variable fluctuations in cell counts in individual babies, particularly in response to infection. Neither total nor differential cell counts on the first day of life were able to detect which infants might go on to develop CLD.

I also related the flow cytometry findings to the presence of bacterial 16S rRNA genes and *Ureaplasma spp.* in the lavage samples to show that infants in whom either organism is detected are a greater risk of developing CLD.

Chapter 4

Apoptosis

Chapter 4
Apoptosis

4.1 Introduction

In chapter 3 I described the cellular component of the inflammatory response in the newborn lung, however once inflammatory cells have arrived in the airways, it is necessary for them to be removed again in order for the inflammatory process to be resolved. A lack of adequate or appropriate resolution of the inflammatory response has been linked with the development of CLD (Kotecha et al., 2003).

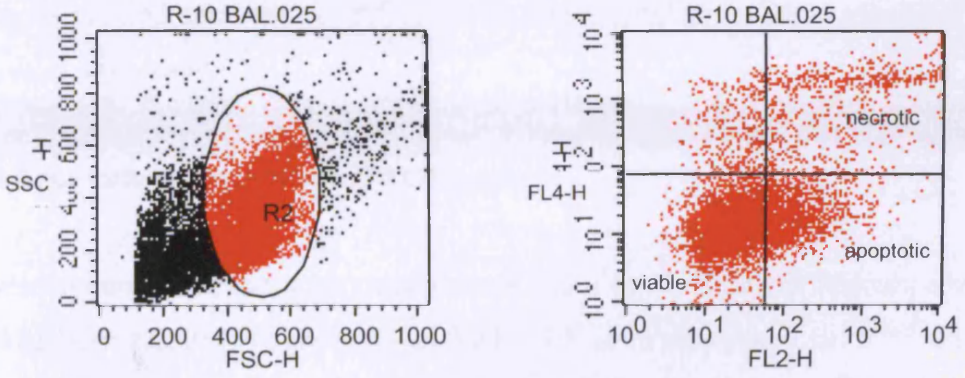
Cells, particularly neutrophils, are removed from the lung in a number of ways. In particular, neutrophils may undergo apoptosis and ingestion by macrophages, which helps to resolve inflammation, or they may undergo necrosis, resulting in a magnification of the inflammatory cascade. I hypothesised that the development of CLD may be linked to a reduction in neutrophil apoptosis, leading to a greater number of viable neutrophils remaining in the lung causing tissue injury and ongoing inflammation. Additionally, the immature macrophage phenotype demonstrated in chapter 3 may have an impact on phagocytosis of neutrophils that have become apoptotic, resulting in apoptotic neutrophils undergoing secondary necrosis, further increasing inflammation and tissue injury.

In this chapter I shall examine neutrophil apoptosis in BAL samples and endeavour to understand the relationships between neutrophil apoptosis and the development of CLD. Firstly, I shall attempt to further describe the BAL neutrophil population in terms of cell viability, apoptosis or necrosis, hypothesising that babies who go on to develop CLD will have fewer apoptotic and more viable and necrotic neutrophils present in the lung, contributing to tissue injury. I will also look at the impact of infection on neutrophil apoptosis, necrosis and viability in BAL samples. I will review data for the first few days of life in an attempt to determine if there is a parameter that may be predictive for the development of CLD. Additionally, I shall attempt to understand the relationship between the presence of macrophages in BAL samples and the apoptotic neutrophil population.

4.2 Detection of apoptotic cells by cytospins vs FACS

The externalisation of phosphatidylserine (PS) on the cell membrane is one of the early signs of apoptosis. Annexin-V is a protein of the annexin family which preferentially binds to negatively charged phospholipids like PS in the presence of Ca^{2+}. Changes in PS asymmetry can be detected before morphological changes associated with apoptosis have occurred and before the integrity of the cell membrane has been lost. The use of a DNA stain (To-Pro 3) aids the differentiation of necrotic (Annexin V +, To-Pro 3 +) from apoptotic (Annexin V +, To-Pro 3 -) cells.

Neutrophils were gated on forward/side scatter plots, based on their typical size and granularity. This gating was confirmed to be around neutrophils by assessing CD15 positivity. The Annexin V and To-Pro 3 characteristics of these cells were then assessed.



A B

Figure 4.1 Flow cytometry plots showing (A) gating on neutrophils (R2) and (B) Annexin-V (FL2) and To-Pro 3 (FL4) staining for the gated population.

Using flow cytometry it is possible to detect apoptosis before the morphological changes that can be seen on cytospins become apparent, therefore I expected a higher percentage of apoptotic cells to be detected by FACS and this was seen in our results, where the median percentage of the neutrophil population identified as apoptotic was 12.38% by FACS and only 1.52% on counted cytospins (Mann-Whitney U-test, $p<0.0001$; Cytospin mean 3.51%, median 1.52%; FACS mean 13.05%, median

12.38%) (Figure 4.2). Data that follow in this chapter are those obtained by flow cytometry, rather than on counted cytospins.

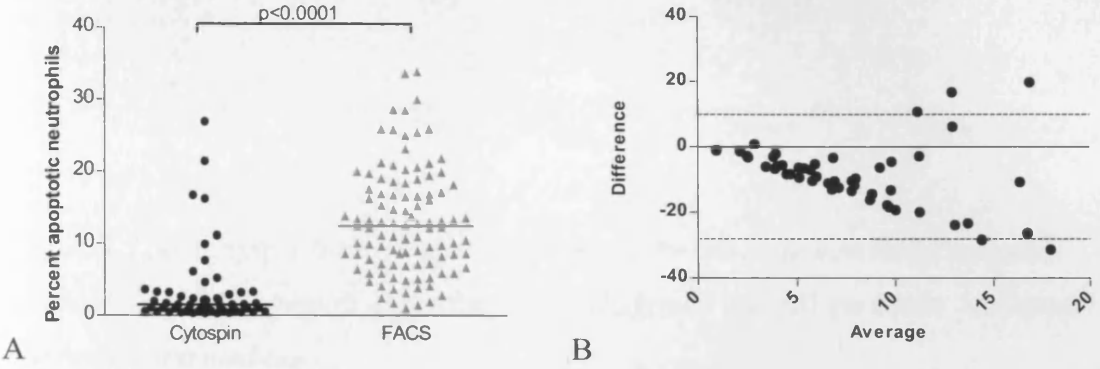


Figure 4.2 (A) Scatterplot showing the percentage of apoptotic cells in each BAL according to two different methods, namely counted on cytospin preparations and detected by FACS analysis. Horizontal lines represent medians.
(B) Bland-Altman plot showing comparison between cytospins and flow cytometry for detection of apoptotic cells in BAL samples. Horizontal dotted lines represent 95% levels of agreement.

4.3 Apoptotic neutrophils in BAL samples

The total number of apoptotic cells was not significantly different between either term and preterm infants (Preterm median 0.026 $x10^6$ cells/ml, mean 0.105 $x10^6$ cells/ml; Term median 0.011 $x10^6$ cells/ml, mean 0.018 $x10^6$ cells/ml; Mann-Whitney U-test, p=0.122), or between any of the diagnostic groups (CLD median 0.024 $x10^6$ cells/ml, mean 0.103 $x10^6$ cells/ml; RDS median 0.029 $x10^6$ cells/ml, mean 0.115 $x10^6$ cells/ml; Term median 0.011 $x10^6$ cells/ml, mean 0.018 $x10^6$ cells/ml; Kruskal-Wallis test, p=0.111), although it can be seen that preterm infants, regardless of diagnosis, had a tendency to have higher numbers of apoptotic neutrophils present in BAL samples (Figure 4.3). This is not surprising in view of the higher absolute number of neutrophils present in preterm BAL samples.

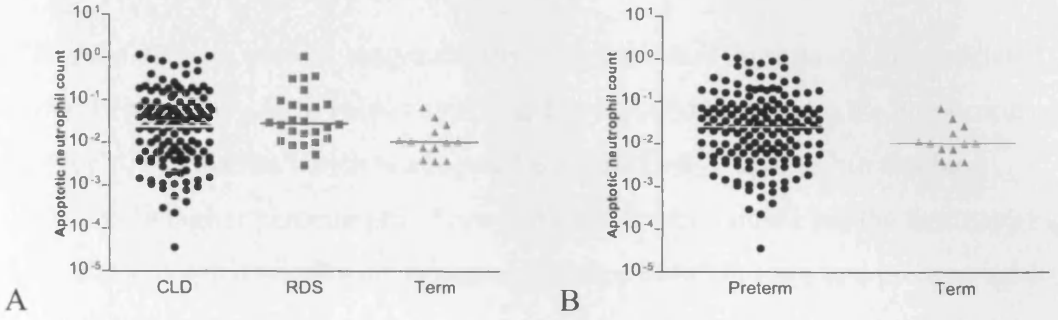


Figure 4.3 Scatterplots (using log scale) showing the absolute number of apoptotic neutrophils in BAL samples according to (A) diagnosis and (B) gestation. Horizontal lines represent medians.

However, when the percentage of the neutrophil population which was apoptotic was analysed, a significantly higher percentage of the neutrophil population was apoptotic in term infants when compared to preterm infants (Preterm median 12.38%, mean 13.83%; Term median 21.62%, mean 25.68%; Mann-Whitney U-test, p=0.0009) (Figure 4.4B). Babies with RDS and those with CLD had very similar proportions of the neutrophil population in apoptosis (CLD median 12.52%, mean 13.71%; RDS median 10.46%, mean 14.48%; Term median 21.62%, mean 25.68%; Kruskal-Wallis test, p=0.0032; CLD vs term <0.01, RDS vs term <0.01) (Figure 4.4A). In other words, although preterm infants had higher numbers of neutrophils in BAL samples than their term counterparts, the proportion of the neutrophils which were apoptotic was significantly smaller in the preterm group.

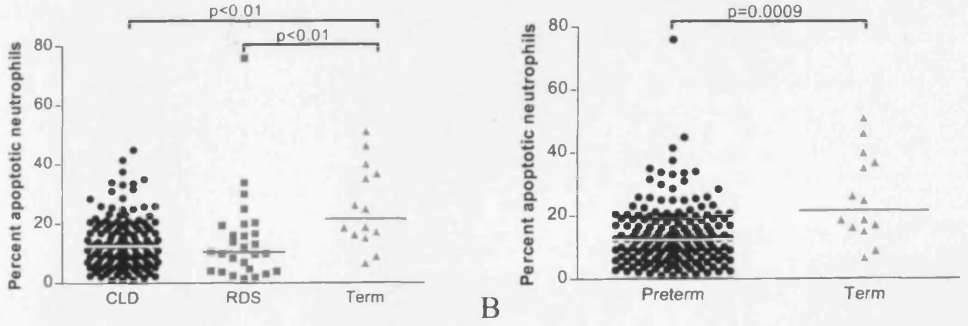
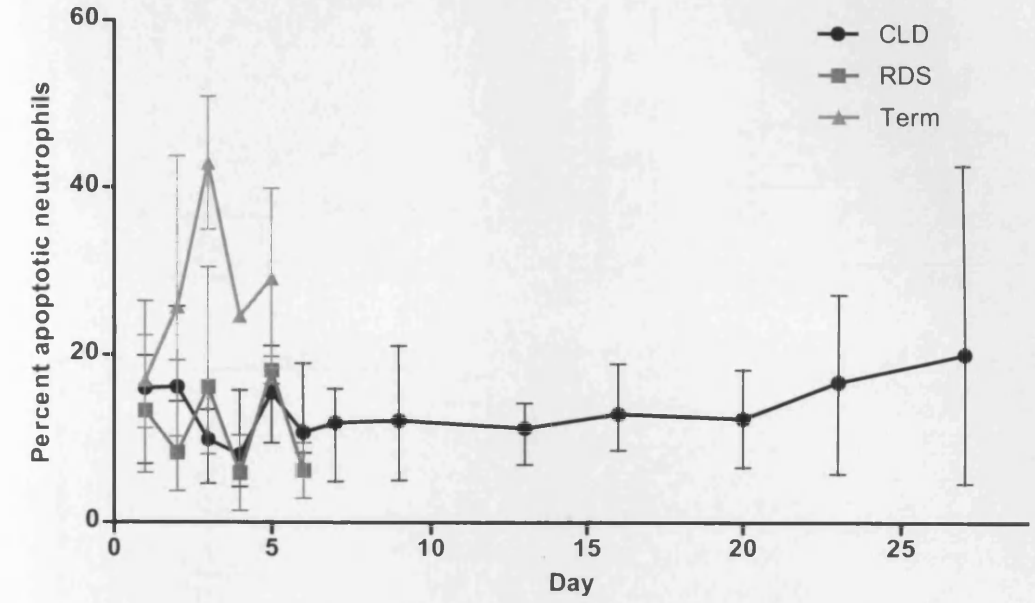
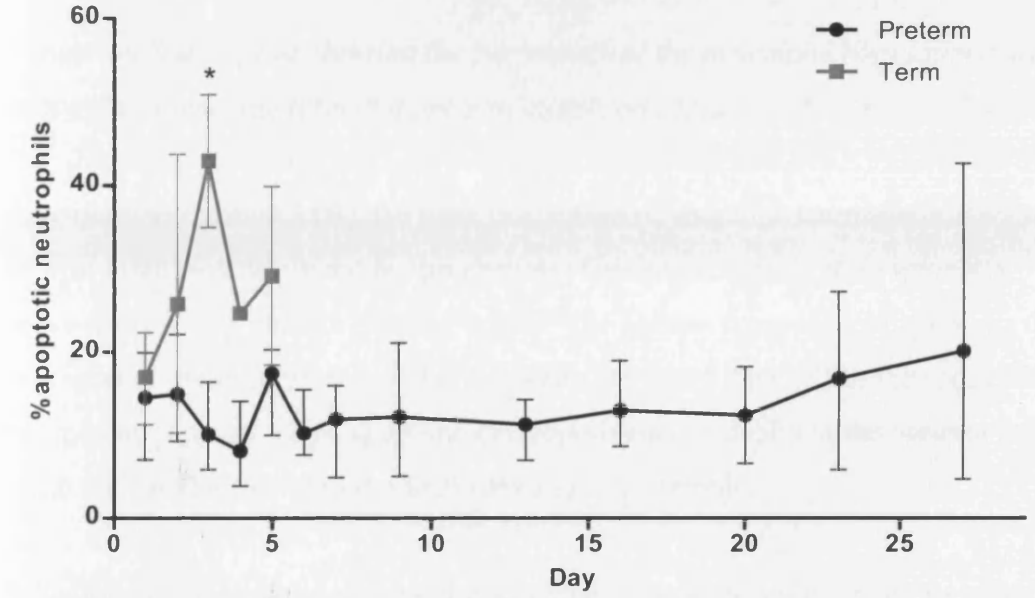


Figure 4.4 Scatterplots showing the percentage of the neutrophil population which was apoptotic in BAL samples, according to (A) diagnosis and (B) gestation. Horizontal lines represent medians.

When the data are viewed longitudinally over the entire duration of the ventilated period (Figure 4.5), term babies appear to have a sudden surge in the proportion of the neutrophil population which is apoptotic over the first 3 days of life and have consistently higher percentages of apoptotic neutrophils on all but the first day (Figure 4.6). There is a statistically significant difference between term and preterm infants on day 3 (Figure 4.6). The median percentage of the neutrophil population which is apoptotic is very similar between all the groups of babies on day 1 and there is no statistically significant difference between the groups on day 2 either (Term mean 26.61%, median 25.79%; preterm mean 16.04%, median 14.94%; Mann-Whitney U-test, p=0.342). However, there is a significant difference between term and preterm infants on day 3 (Term mean 42.96%, median 42.96%; preterm mean 13.23%, median 10.06%; Mann-Whitney U-test, p=0.019). After this point, there are too few data in the term group to allow for meaningful comparisons to be made as all but 2 of the babies had no usable data for days 4 and 5 due to most having been extubated and others having very low total cell counts which did not allow sufficient cells to be analysed by flow cytometry for apoptosis. No significant difference in the percentage of neutrophils which were apoptotic was found between preterm babies who developed CLD and those whose RDS resolved on days 1-7.



A



B

Figure 4.5 Graphs showing changes in percentage of apoptotic neutrophils over the course of the ventilation period according to (A) diagnosis and (B) gestation. It can be seen that there is little difference between the RDS and CLD sub-groups of preterm infants. (p=0.019)*

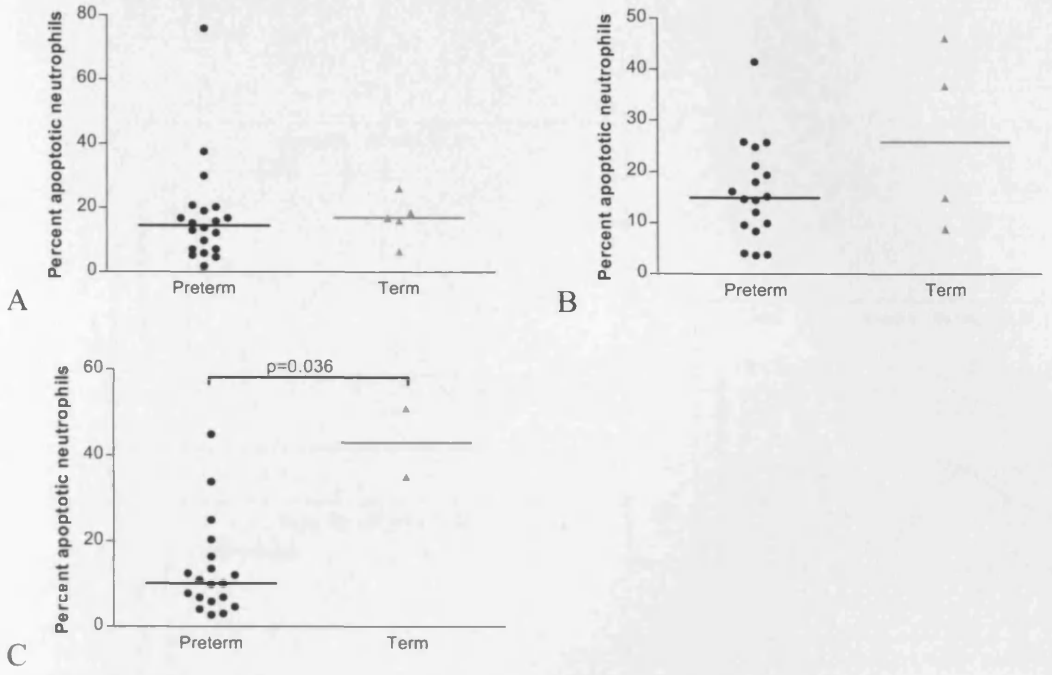
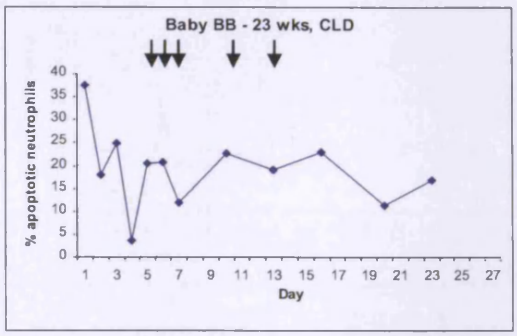
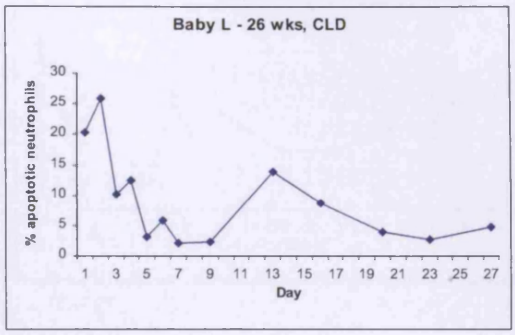


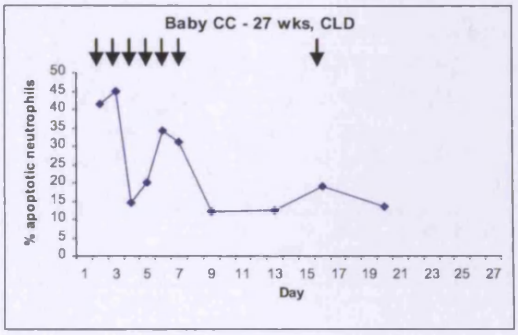
Figure 4.6 Scatterplots showing the percentage of the neutrophil population which is apoptotic, comparing term and preterm infants on (A) day 1, (B) day 2 and (C) day 3.

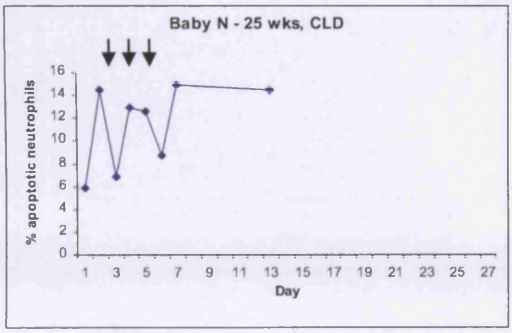
According to figure 4.5 (B), the peak percentage of apoptotic neutrophils is achieved in term infants on day 3 and the proportion of apoptotic cells in term infants is consistently higher than in preterm infants. The highest proportion of apoptotic cells is present in preterm infants on day 5 (if only the first 5 days of life are considered) but median proportions of apoptotic neutrophils rise gradually in the preterm group to reach their highest level in the final (day 27) BAL sample.

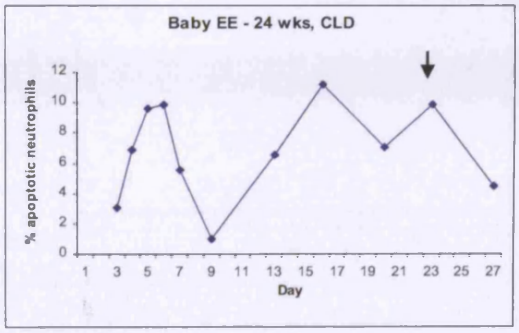
When looking longitudinally at either numbers or proportions of apoptotic cells, grouped data for preterm infants show some initial day-to-day variations in apoptotic cells followed by a very slowly increasing proportion of apoptotic cells as the CLD babies approach the 27th day of life. However, when data from individual babies are reviewed, the slow increase in median apoptotic cells can be seen to be contributed to by short duration, episodic increases and decreases in the proportion of apoptotic cells. This can be seen in figure 4.7.

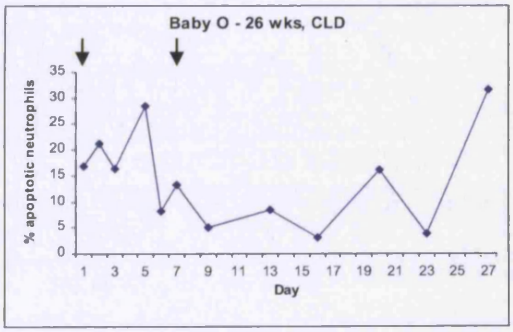

Baby BB - 23 wks, CLD
% apoptotic neutrophils
Day

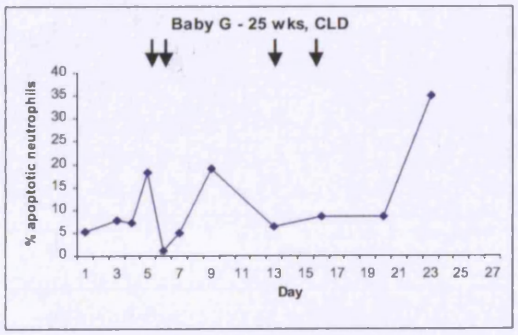

Baby L - 26 wks, CLD
% apoptotic neutrophils
Day

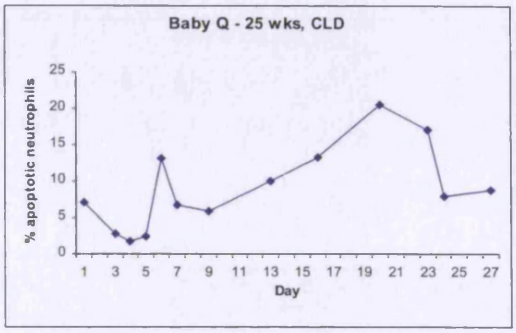

Baby CC - 27 wks, CLD
% apoptotic neutrophils
Day

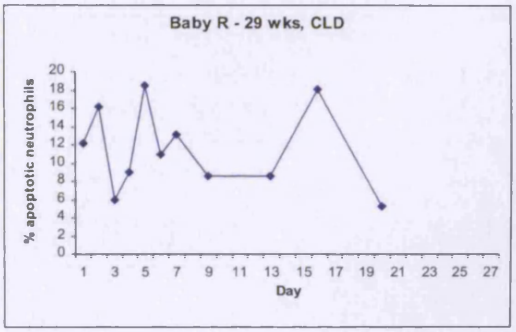

Baby N - 25 wks, CLD
% apoptotic neutrophils
Day

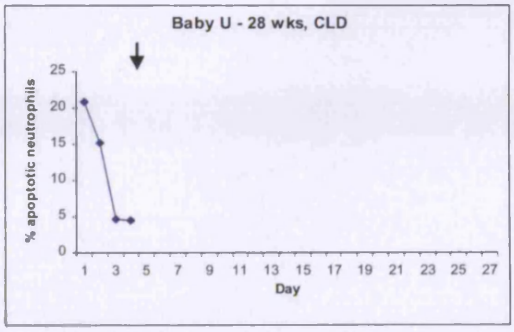

Baby EE - 24 wks, CLD
% apoptotic neutrophils
Day

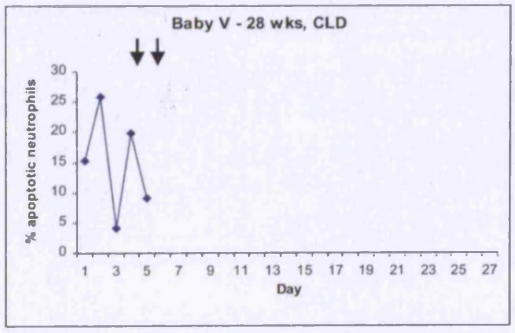

Baby O - 26 wks, CLD
% apoptotic neutrophils
Day

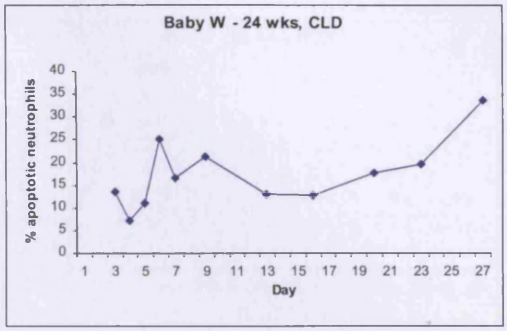

Baby G - 25 wks, CLD
% apoptotic neutrophils
Day

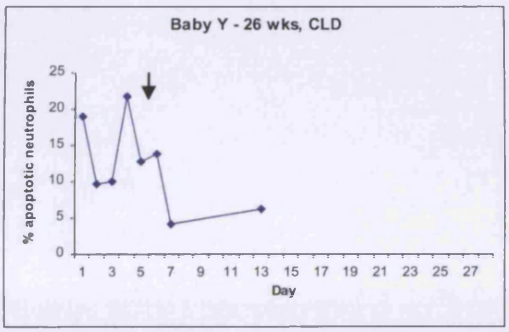

Baby Q - 25 wks, CLD
% apoptotic neutrophils
Day

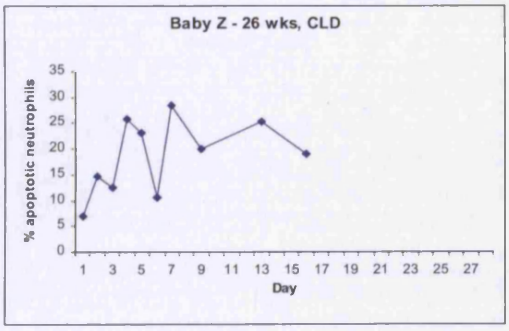

Baby R - 29 wks, CLD
% apoptotic neutrophils
Day

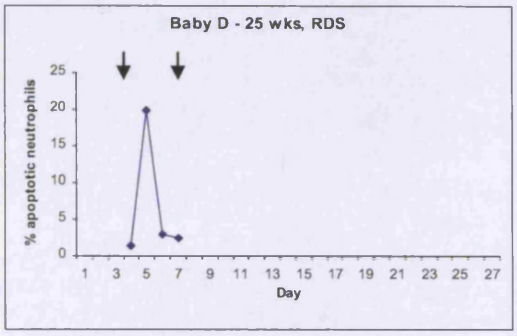

Baby U - 28 wks, CLD
% apoptotic neutrophils
Day

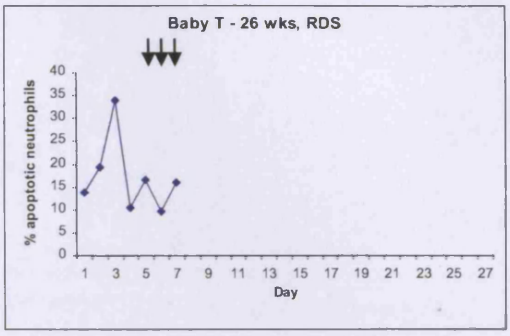

Baby V - 28 wks, CLD
% apoptotic neutrophils
Day

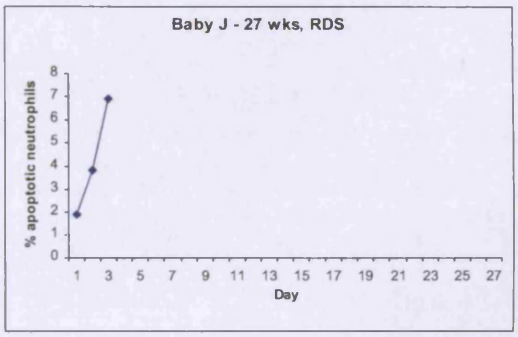

Baby W - 24 wks, CLD
% apoptotic neutrophils
Day

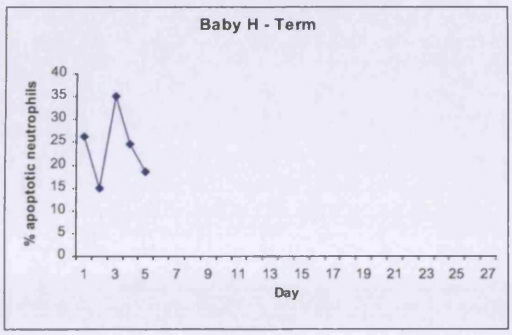

Baby Y - 26 wks, CLD
% apoptotic neutrophils
Day

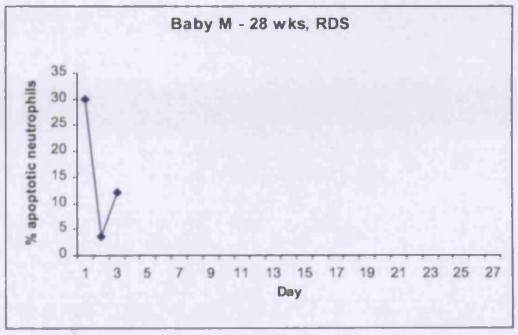

Baby Z - 26 wks, CLD
% apoptotic neutrophils
Day

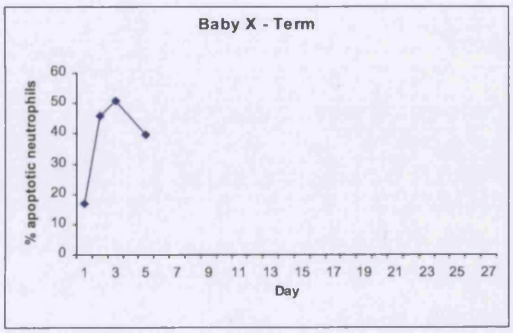












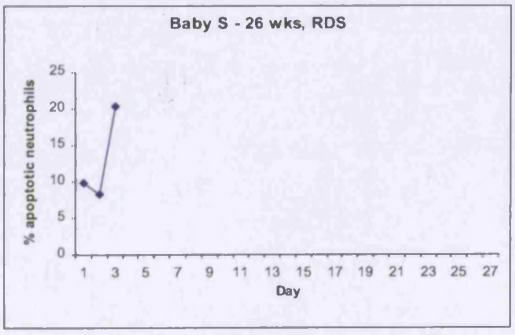


Figure 4.7 Graphs showing percentage of neutrophil population which is apoptotic for all babies with 3 or more BAL samples. Vertical arrows represent samples in which 16S rRNA was detected.

There is also a significant difference in the peak percentage of apoptotic neutrophils among the 3 groups (CLD mean 20.24%, median 15.52%; RDS mean 10.64%, median 7.12%; Term mean 29.53%, median 32.02%; Kruskal-Wallis test, p=0.0395) (Figure 4.8). Despite the apparent trend, seen in Figure 4.7, among individual infants developing CLD to have higher percentages of apoptotic neutrophils in later BAL samples, very little difference can be seen in the median percentage of peak apoptotic neutrophils between the first 5 days of life (12.5%) and the whole ventilated period (15.5%).

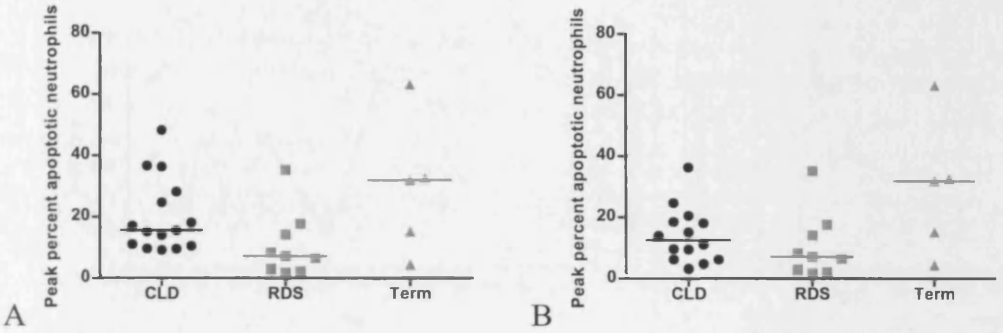
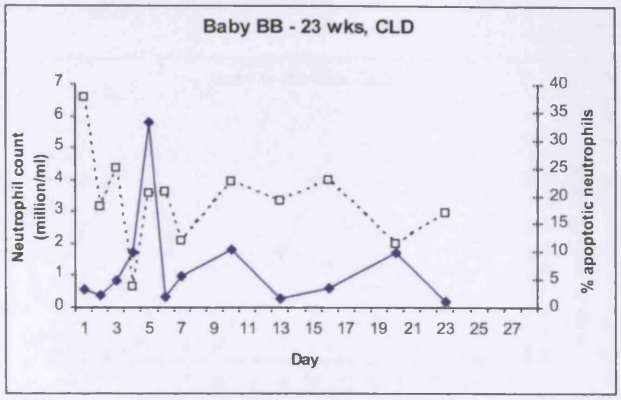
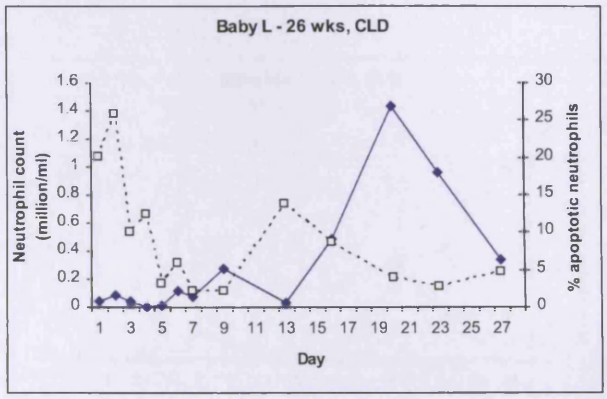
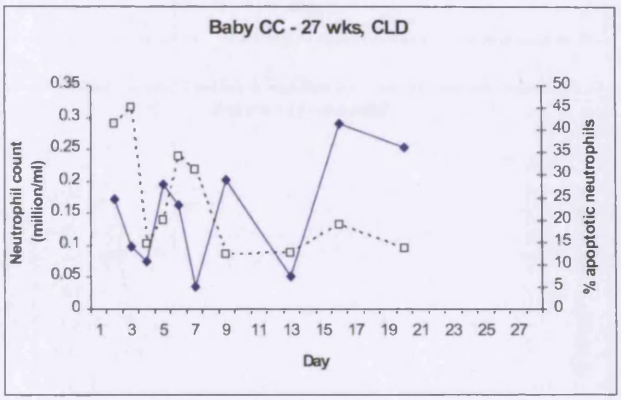


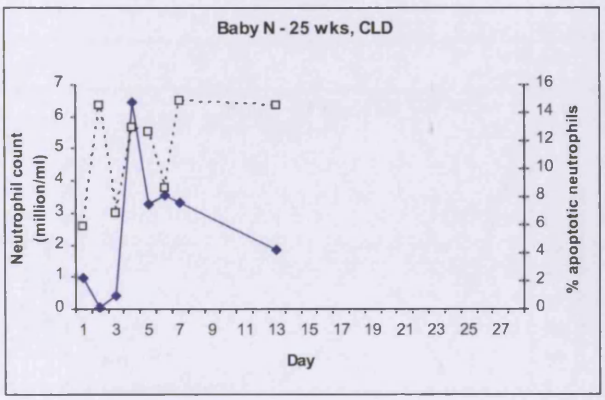
Figure 4.8 Scatterplots showing the highest percentage of apoptotic cells reached in each individual infant in (A) the entire ventilated period and (B) the first 5 days of life. Horizontal lines represent medians in each group.

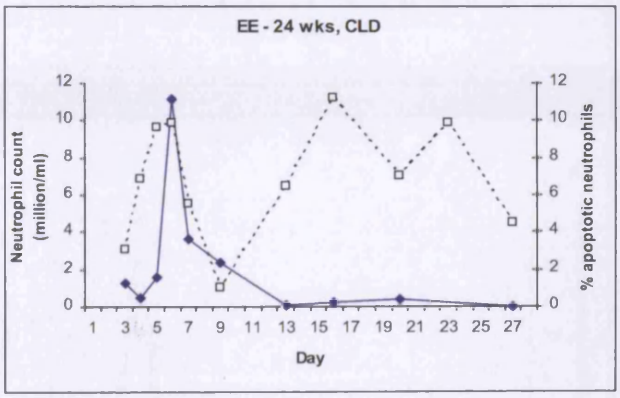
It might be expected that the proportion of apoptotic neutrophils would fluctuate in parallel with the total neutrophil count or with the proportion of the total cell count made up of neutrophils. In figure 4.9 it can be seen that, in many babies, while neutrophil counts are relatively low, the percentage of apoptotic neutrophils are relatively high but there are also periods when the neutrophil count and percentage apoptotic cells appear to rise simultaneously. Figure 4.10 shows a significant linear relationship between the number of neutrophils present and the number of apoptotic neutrophils. This may indicate that, in addition to a reduction in the percentage of apoptotic neutrophils in preterm infants, lung injury also occurs as a result of an inability to clear away any apoptotic neutrophils, which go on to become necrotic.

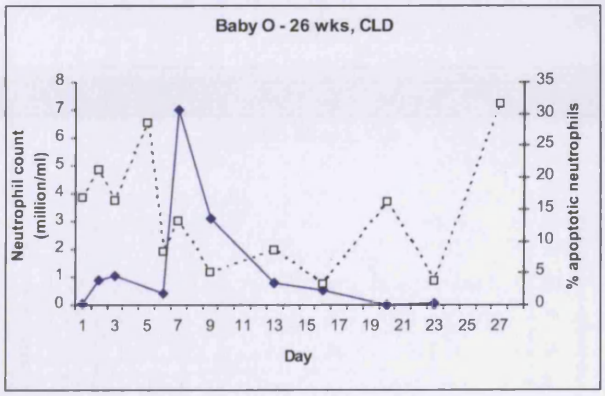

Baby BB - 23 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day

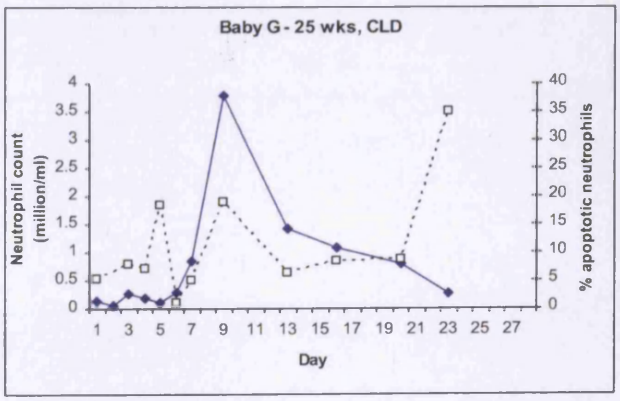

Baby L - 26 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day

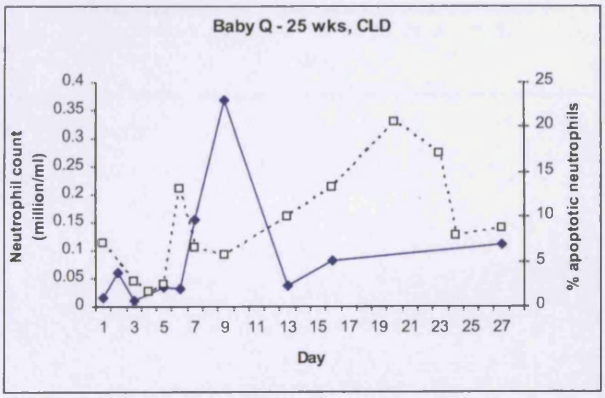

Baby CC - 27 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day

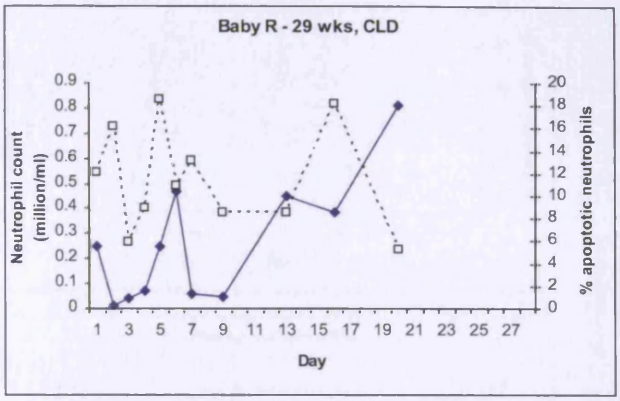

Baby N - 25 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day

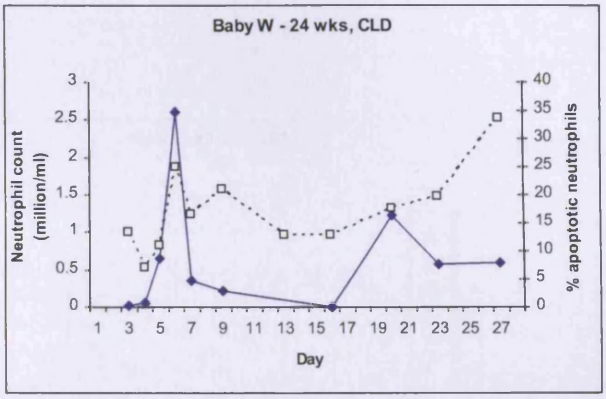

EE - 24 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day

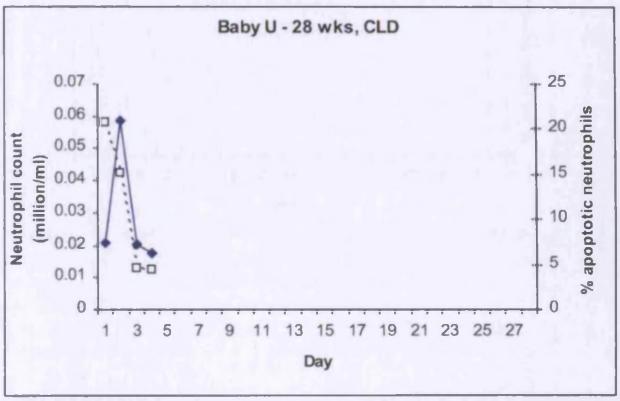

Baby O - 26 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day

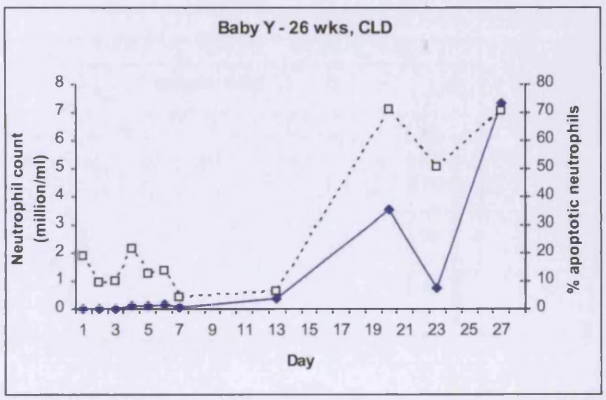

Baby G - 25 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day

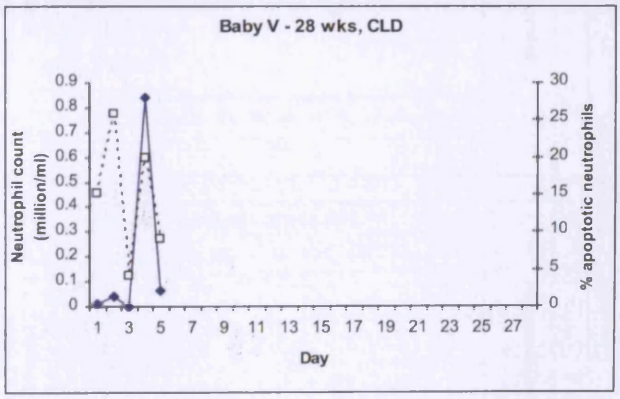

Baby Q - 25 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day

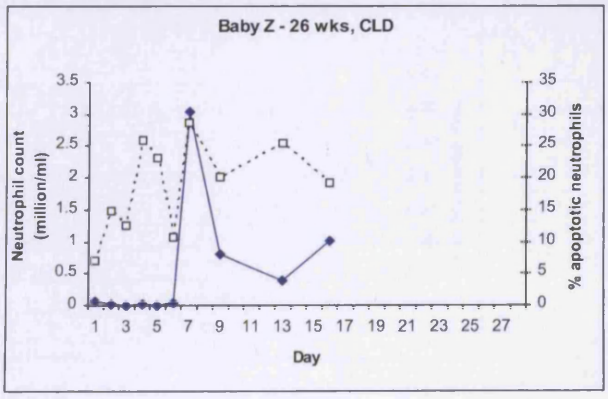

Baby R - 29 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day


Baby W - 24 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day


Baby U - 28 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day


Baby Y - 26 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day


Baby V - 28 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day


Baby Z - 26 wks, CLD
Neutrophil count (million/ml)
% apoptotic neutrophils
Day

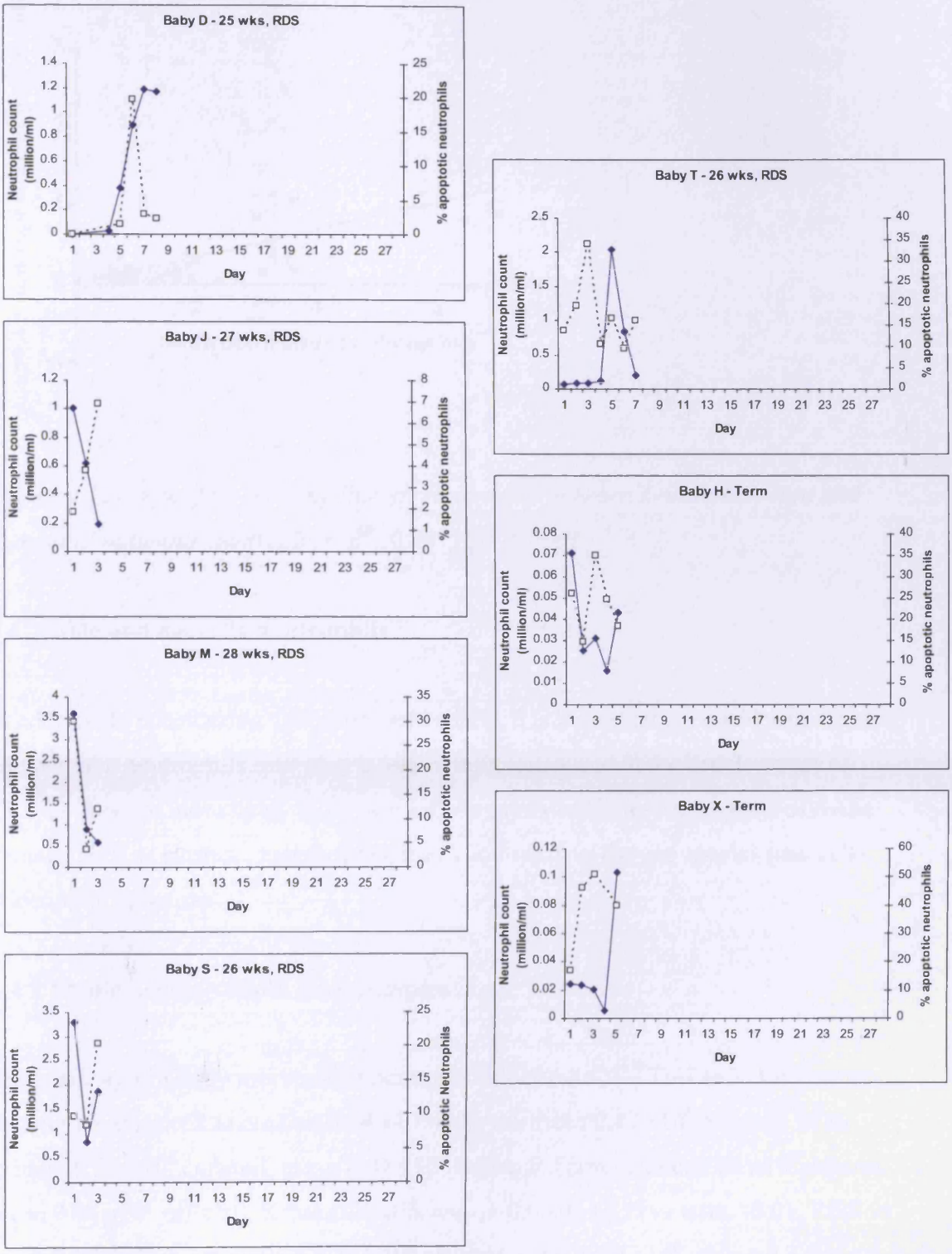


Figure 4.9 Graphs showing relationship between the proportion of apoptotic neutrophils and the total neutrophil count in individual infants with 3 or more BAL samples. (Solid line indicates absolute neutrophil count, broken line indicates percentage of apoptotic neutrophils.)

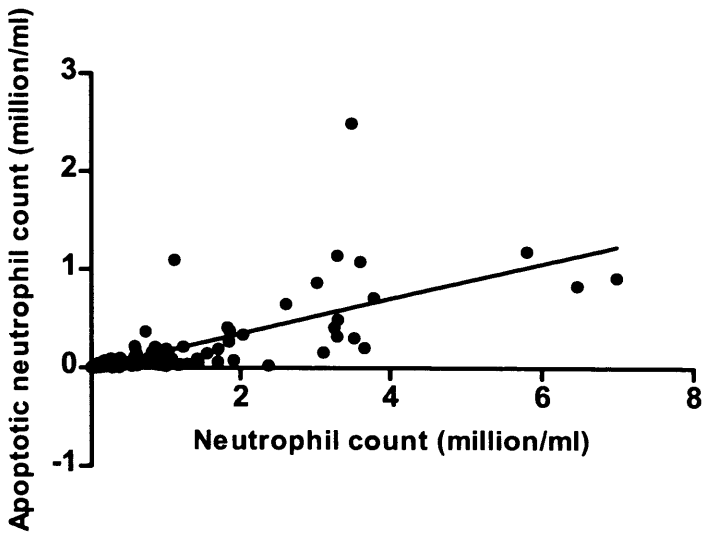


Figure 4.10 Scatterplot showing linear relationship between neutrophil count and number of apoptotic neutrophils. $R^2=0.5371$, $p<0.0001$

4.4 Viable and necrotic neutrophils

In addition to considering apoptotic neutrophils, it is important to consider that viable and necrotic neutrophils may play a role in lung injury and in the development of CLD as they are more likely to release inflammatory mediators and agents of tissue damage such as elastase, metalloproteinases and reactive oxygen species than cells undergoing apoptosis.

4.4.1 Viable neutrophils in BAL samples

There are significantly more viable neutrophils (Annexin V -, To-Pro 3 -) in preterm than term babies (CLD median 0.14 $x10^6$ cells/ml, mean 0.47 $x10^6$ cells/ml; RDS median 0.37 $x10^6$ cells/ml, mean 0.47 $x10^6$ cells/ml; Term median 0.03 $x10^6$ cells/ml, mean 0.04 $x10^6$ cells/ml; Kruskal-Wallis test, p=0.0006, CLD vs term <0.01, RDS vs term<0.001) (Preterm median 0.15 $x10^6$ cells/ml, mean 0.47 $x10^6$ cells/ml; Term median 0.03 $x10^6$ cells/ml, mean 0.04 $x10^6$ cells/ml; Mann-Whitney U-test, p=0.0008). This was not unexpected due to the higher total number of neutrophils in preterm BAL samples.

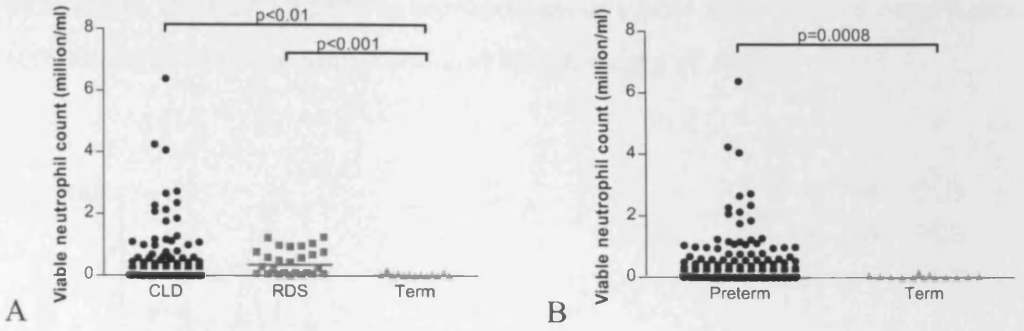


Figure 4.11 Scatterplots showing the viable neutrophil count in BAL samples according to (A) diagnosis and (B) gestation. Horizontal lines represent medians.

When the percentage of viable neutrophils is considered as a proportion of the total neutrophil population, no significant difference is observed among term, RDS and CLD babies (CLD median 62.46%, mean 59.97%; RDS median 59.84%, mean 57.77%; Term median 50.95%, mean 49.72%; Kruskal-Wallis test, p=0.076), however, there is a significantly higher proportion of viable neutrophils in preterm infants when considered as a group (Preterm median 61.87, mean 59.62; Term median 50.95, mean 49.72; Mann-Whitney U-test, p=0.026) .

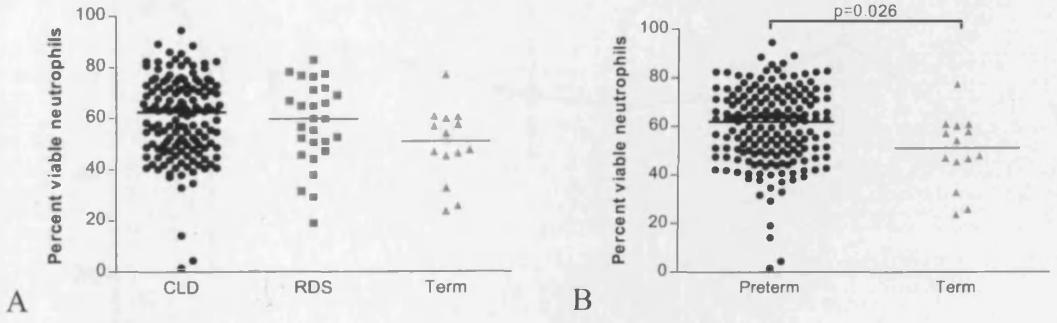
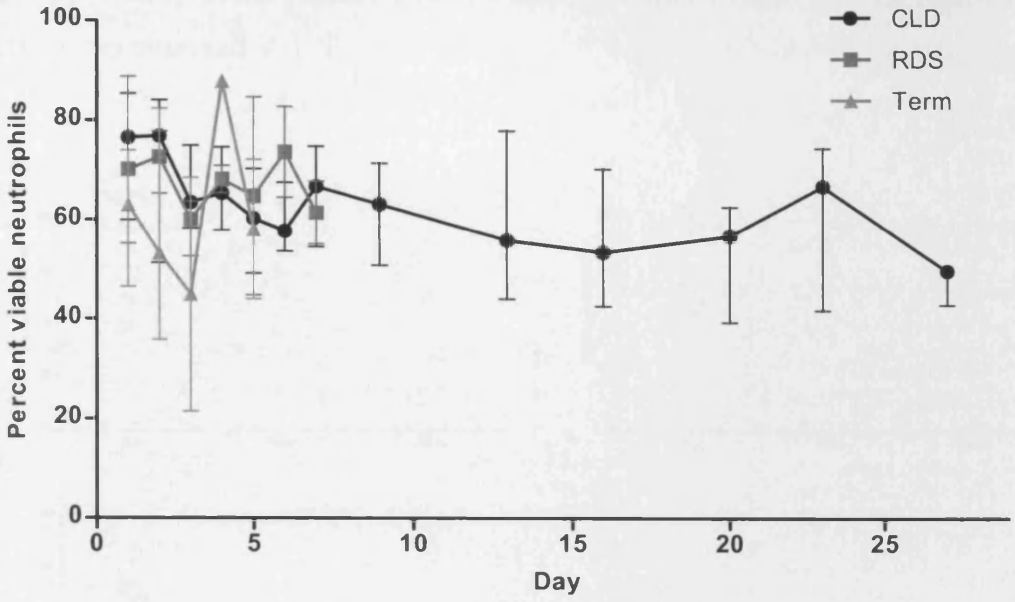


Figure 4.12 Scatterplots showing the percentage of the BAL neutrophils which were viable according to (A) diagnosis and (B) gestation. Horizontal lines represent medians.

When data for the percentage of viable neutrophils are viewed longitudinally over the entire length of the ventilation period, it can be seen that all 3 groups of infants have similar proportions of their neutrophil population as viable cells initially and that

infants who develop CLD have a proportion of viable neutrophils around a median of 60% for most of the second, third and fourth weeks of life.



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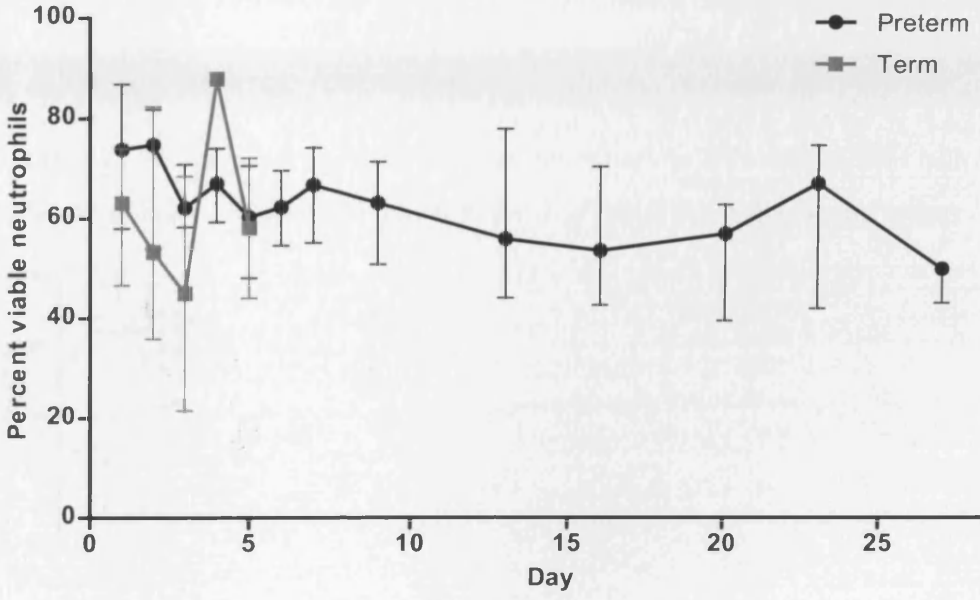


Figure 4.13 Graphs showing changes in median percentage of viable neutrophils over the course of the ventilation period according to (A) diagnosis and (B) gestation. Error bars represent interquartile ranges.

Once again, with the objective of identifying a possible prognostic indicator for the development of CLD, the first 3 days of life were reviewed in detail with respect to the percentage of viable neutrophils present, and despite the term group tending to have persistently lower percentages of viable neutrophils, there were no significant differences observed.

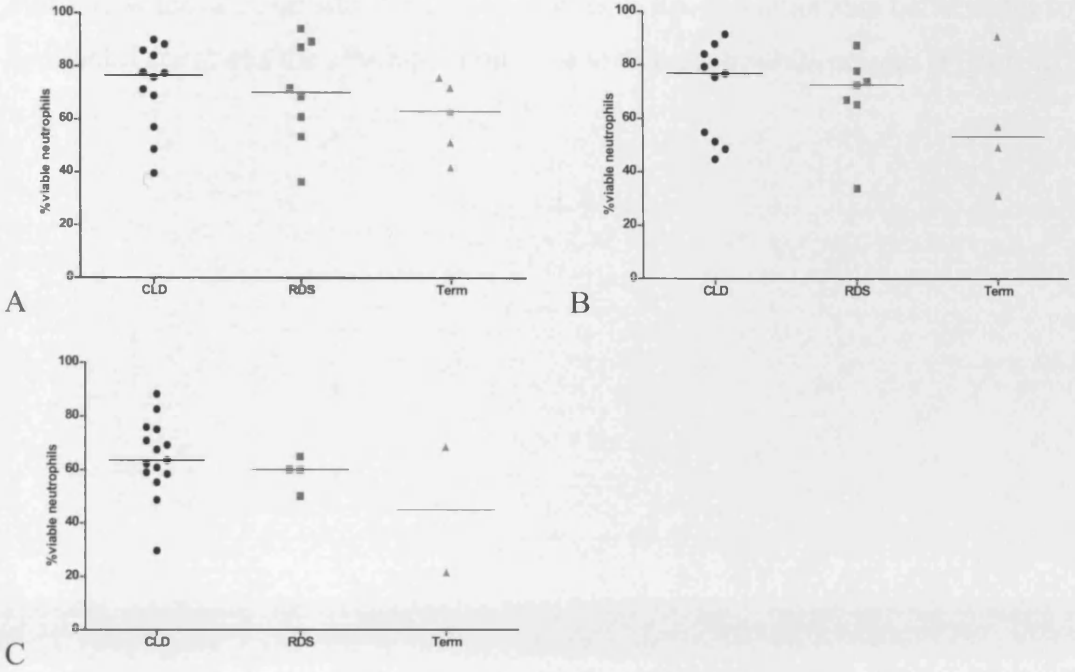


Figure 4.14 Scatterplots showing the percentage of the BAL neutrophils which were viable on (A) day 1, (B) day 2 and (C) day 3 of life in each diagnostic group.

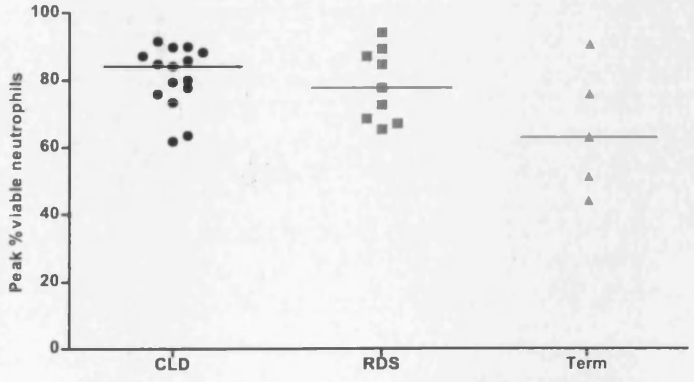
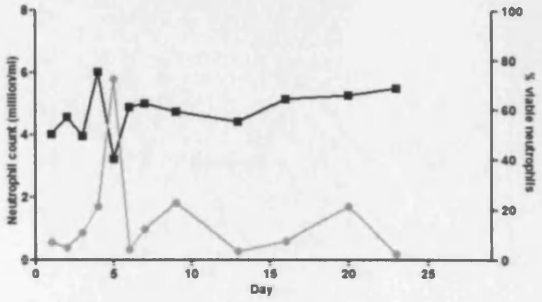


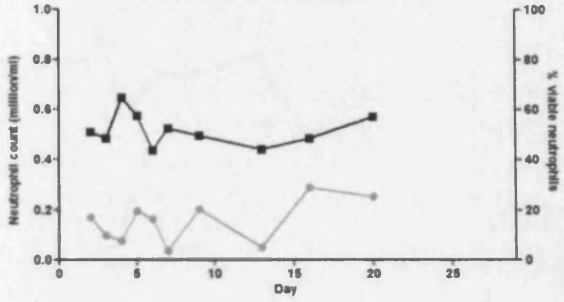
Figure 4.15 Scatterplot showing the median peak percentage of viable neutrophils in each infant according to diagnostic group.

When data for individual infants are reviewed, it can be seen that the proportion of viable neutrophils in each infant is fairly constant but often falls around the time of a total neutrophil count increase (Figure 4.16). This may be related to large numbers of neutrophils dying by necrosis causing more neutrophils to be recruited to the site of inflammation or possibly the neutrophil count peak coincides with an increase in apoptosis of the neutrophils that are present in an attempt to control neutrophil numbers at the affected site. There is, however, a linear relationship between the total neutrophil count and the absolute number of viable neutrophils present (Figure 4.17).

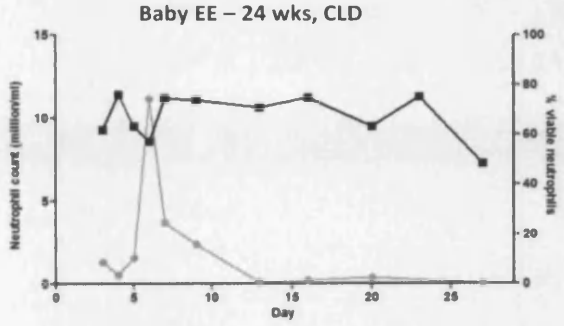
Baby BB – 23 wks, CLD



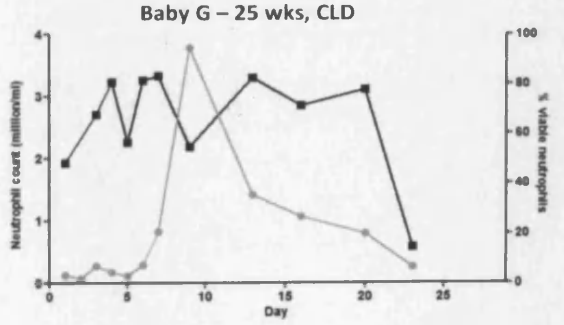
Baby CC – 27 wks, CLD



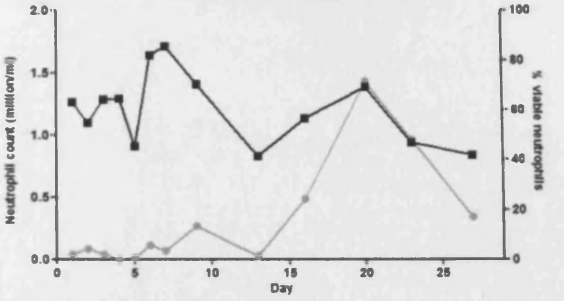
Baby EE – 24 wks, CLD



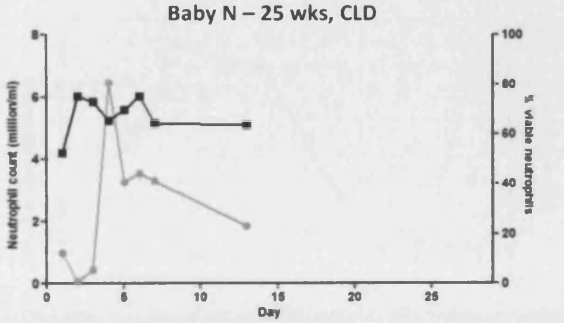
Baby G – 25 wks, CLD



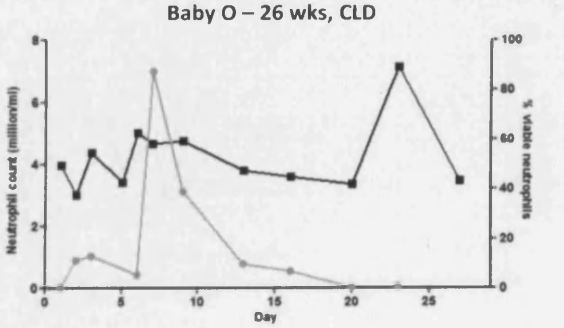
Baby L – 26 wks, CLD



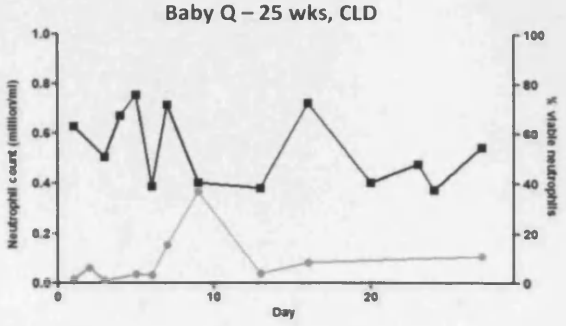
Baby N – 25 wks, CLD



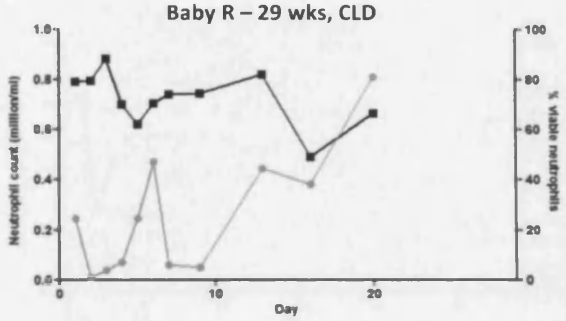
Baby O – 26 wks, CLD



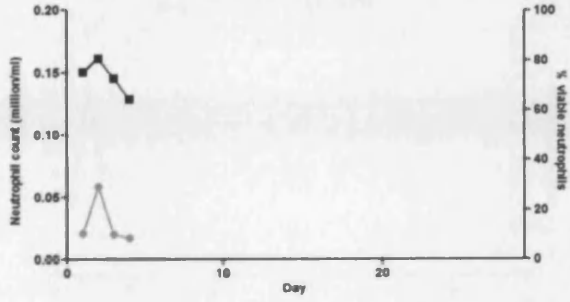
Baby Q – 25 wks, CLD



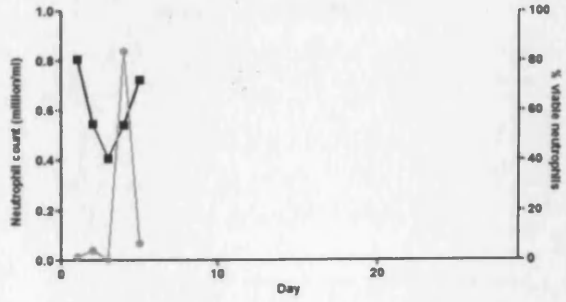
Baby R – 29 wks, CLD



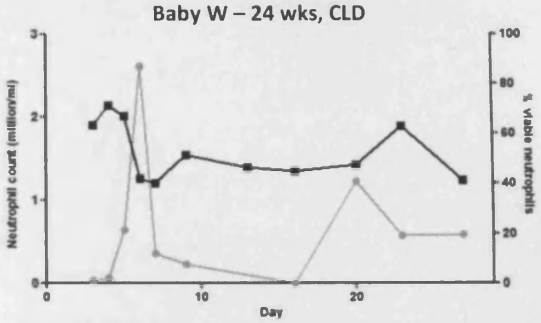
Baby U – 28 wks, CLD



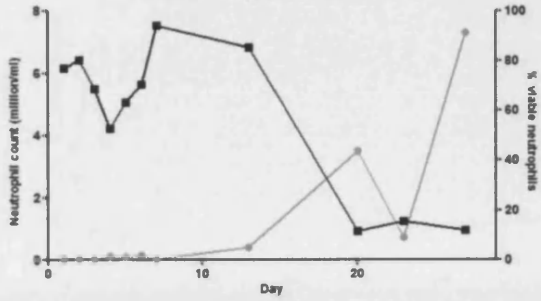
Baby V – 28 wks, CLD



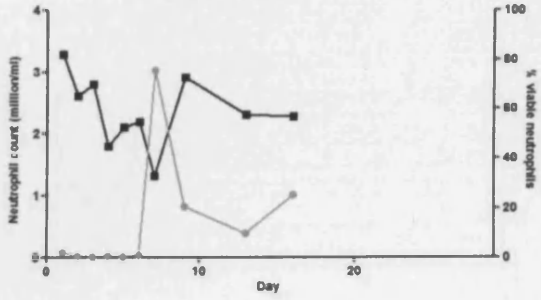
Baby W – 24 wks, CLD



Baby Y – 26 wks, CLD



Baby Z – 26 wks, CLD



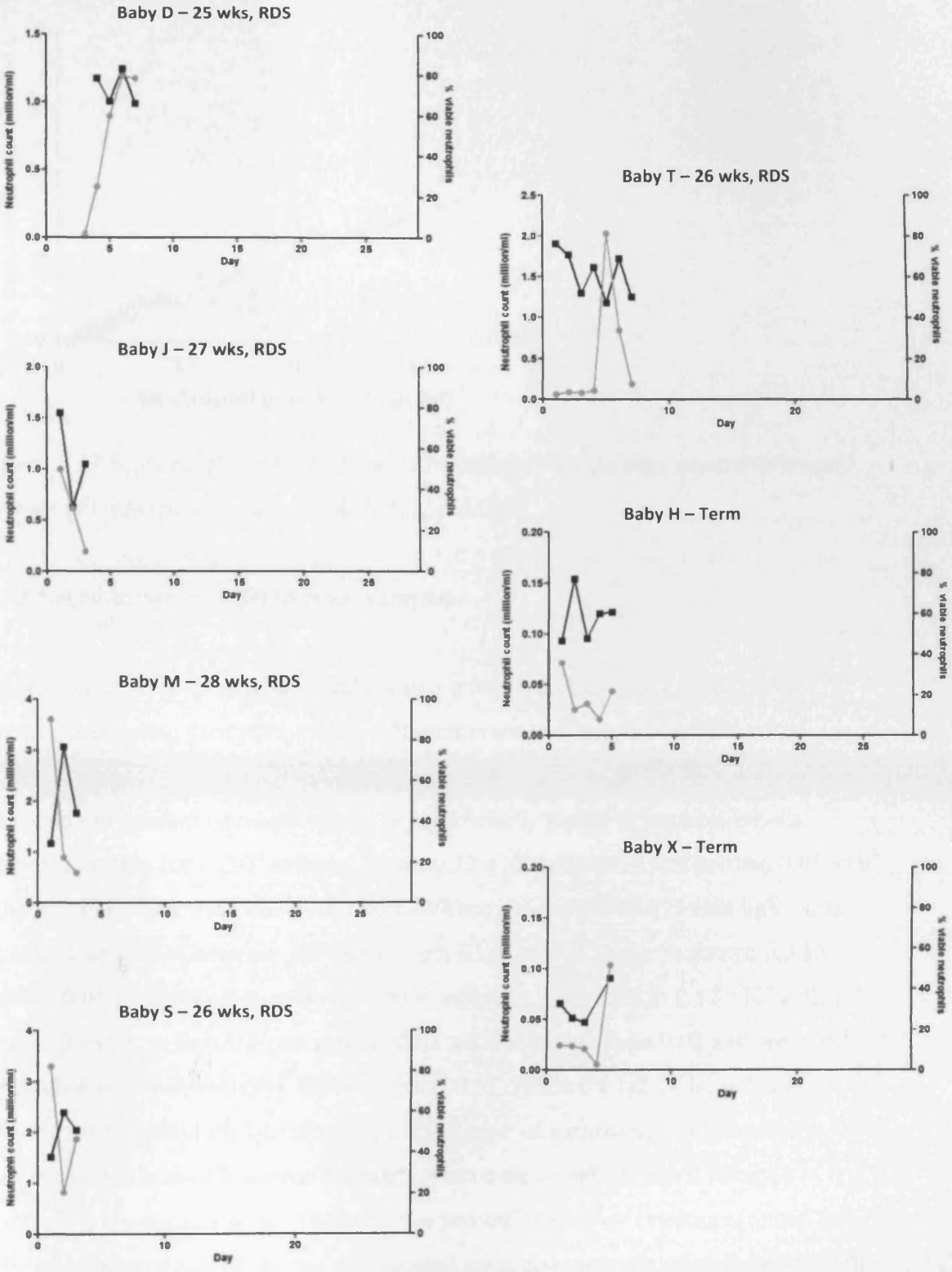


Figure 4.16 Graphs showing relationship between total neutrophil count (grey line) and the proportion of the neutrophil population that was viable (black line) for all infants with 3 or more BAL samples.

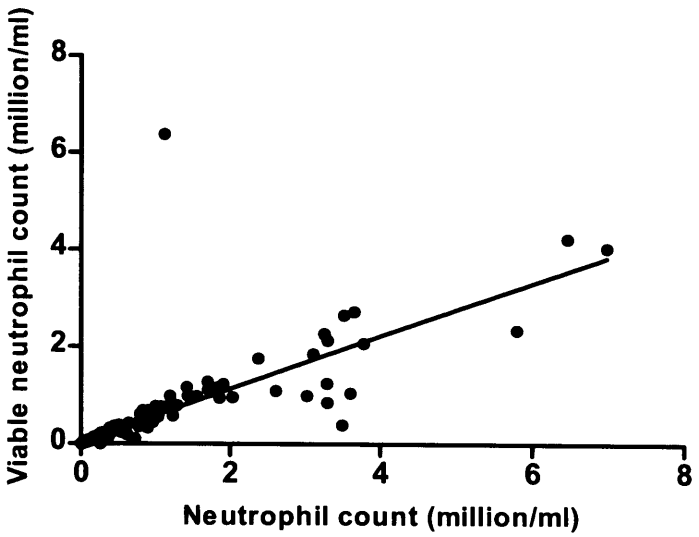


Figure 4.17 Scatterplot showing linear relationship between total neutrophils and number of viable neutrophils. $R^2=0.6285$, $p<0.0001$

4.4.2 Necrotic neutrophils in BAL samples

Necrotic cells were those neutrophils which were both Annexin V and To-Pro 3 positive, indicating disruption of the cell membrane and exposure of DNA.

The absolute number of necrotic cells is significantly higher in preterm infants (Preterm median 0.07 x10^6 cells/ml, mean 0.22 x10^6 cells/ml; Term median 0.01 x10^6 cells/ml, mean 0.02 x10^6 cells/ml; Mann-Whitney U-test, p=0.009) although, once again, no difference between the babies with RDS and CLD can be shown (CLD median 0.05 x10^6 cells/ml, mean 0.21 x10^6 cells/ml; RDS median 0.17 x10^6 cells/ml, mean 0.30 x10^6 cells/ml; Term median 0.01 x10^6 cells/ml, mean 0.02 x10^6 cells/ml; Kruskal-Wallis test, p=0.004, RDS vs term <0.01) (Figure 4.18). A large load of necrotic neutrophils may be responsible for release of various pro-inflammatory factors into the peri-cellular environment. Macrophages which ingest necrotic neutrophils are stimulated to release further pro-inflammatory cytokines, rather than anti-inflammatory mediators that are released upon phagocytosis of an apoptotic cell. Thus, this high load of necrotic neutrophils may help to potentiate the inflammatory response, both through the direct consequence of necrosis and the release of the neutrophil's toxic contents and through the phagocytosis of necrotic neutrophils causing release of further pro-inflammatory mediators from macrophages.

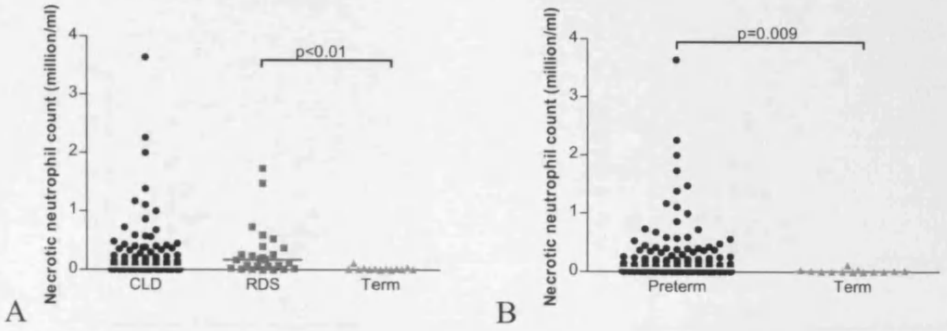


Figure 4.18 Scatterplots showing the necrotic neutrophil count in BAL samples according to (A) diagnosis and (B) gestation.

The proportion of the neutrophil population that was necrotic was not significantly different between the groups (CLD median 24.50%, mean 26.30%; RDS median 30.43%, mean 27.76%; Term median 24.98%, mean 24.61%; Kruskal-Wallis test, p=0.74); (Preterm median 24.91%, mean 26.53%; Term median 24.98%, mean 24.61%; Mann-Whitney U-test, p=0.706) (Figure 4.18).

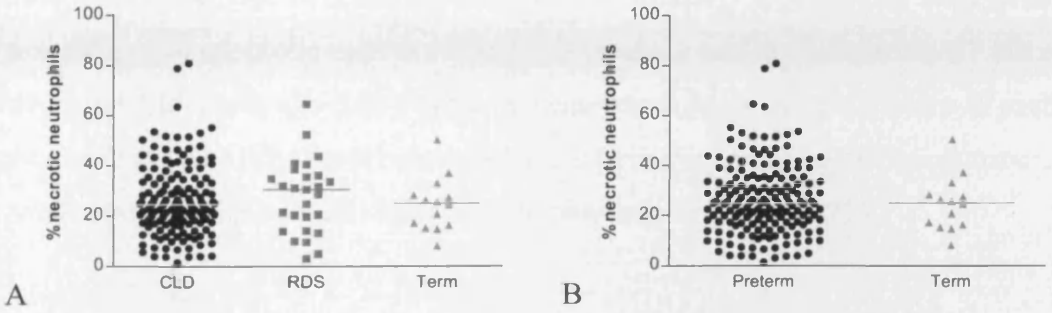
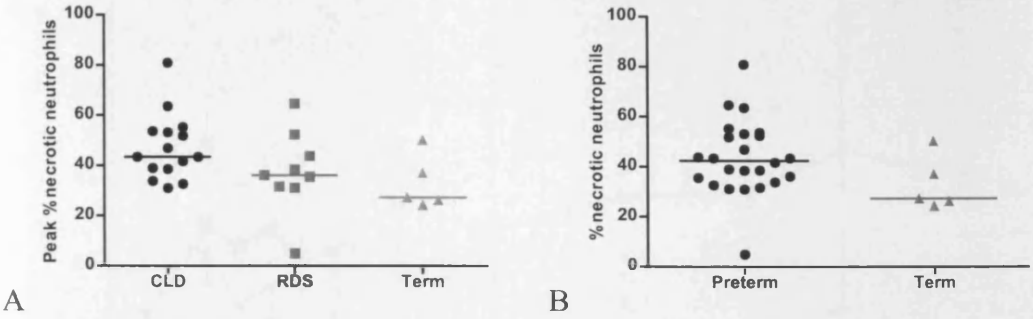


Figure 4.19 Scatterplots showing the percentage of necrotic neutrophils in BAL samples according to (A) diagnosis and (B) gestation.

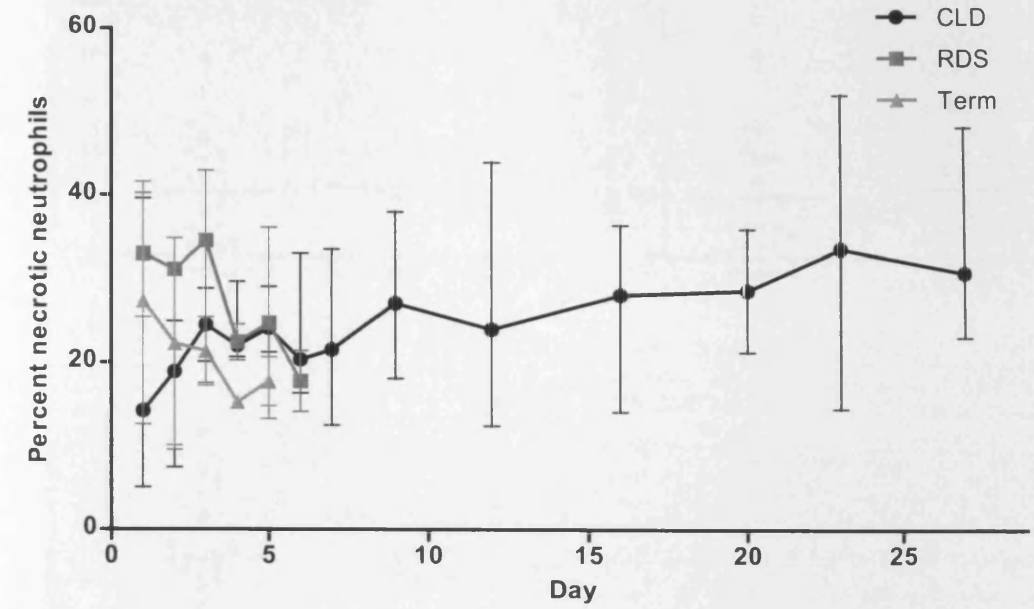
Although the overall number of necrotic neutrophils was significantly higher in BAL from preterm infants (Figure 4.18, above), analysis of the peak percentages of necrotic neutrophils showed no significant difference in the peak percentages of necrotic neutrophils in preterm infants (CLD mean 47.21%, median 43.37%; RDS mean 37.62%, median 36.14%; Term mean 33.11%, median 27.36%; Kruskal-Wallis test, p=0.0565)(Preterm mean 43.61%, median 42.28%; Term mean 33.11%, median 27.36%; Mann-Whitney U-test, p=0.606) (Figure 4.20).



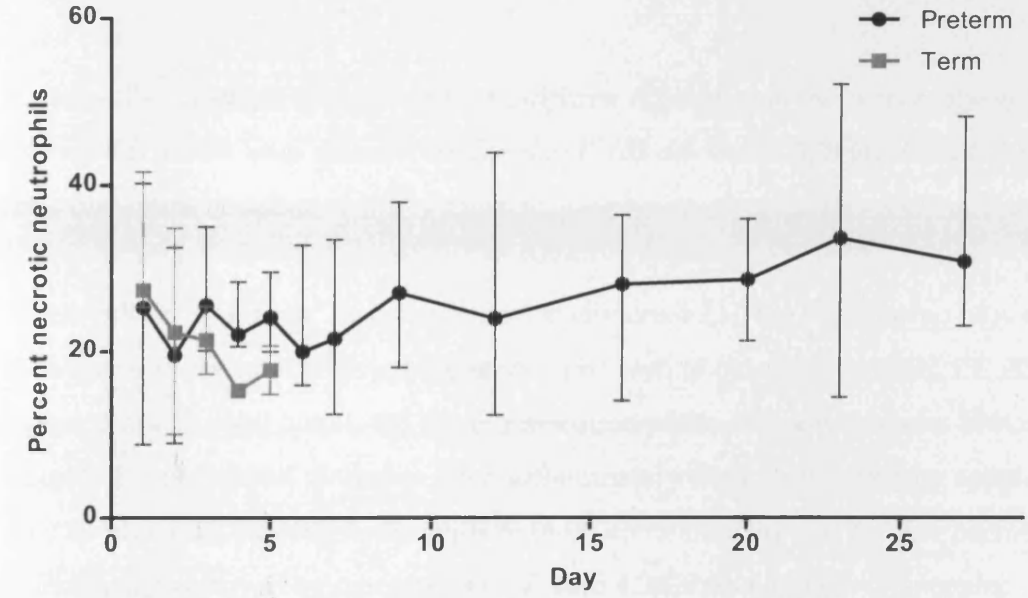
A B

Figure 4.20 Scatterplot showing peak percentage of necrotic neutrophils in BAL samples, indicating a trend towards a higher percentage of necrotic neutrophils in BAL fluid from babies who developed CLD.

In figure 4.21B (below) it can be seen that the highest proportion of necrotic neutrophils occurs on day 1 in term infants; whereas preterm infants have the largest percentage of necrotic cells on day 27. If only the first 5 days of life are compared, preterm infants, both those developing CLD and those whose RDS is resolving, have their peak percentage in necrotic cells at day 3. Similarly for apoptotic cells, peak percentages of apoptotic cells occur in RDS on day 3 and in CLD on day 27 but on day 3 in CLD if only the first 5 days are considered. More detailed review of each of the first 3 days of life shows no significant differences in percentage of necrotic neutrophils present between term and preterm infants (Figure 4.22).



A



B

Figure 4.21 Graphs showing the median percentage of the BAL neutrophil population which is necrotic according to (A) diagnosis and (B) gestation.

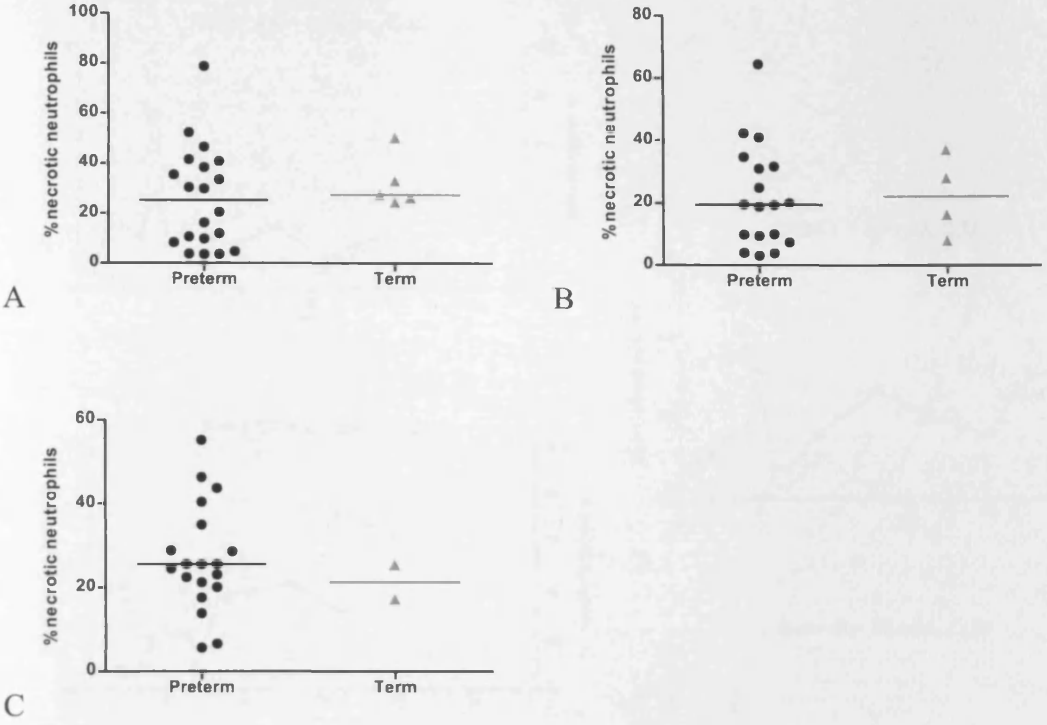
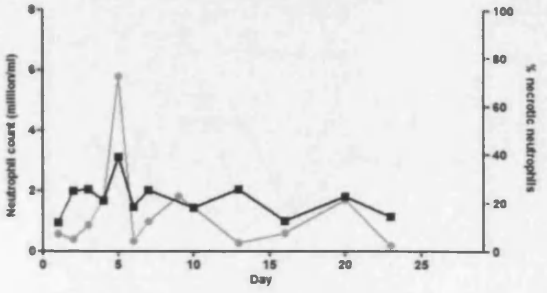


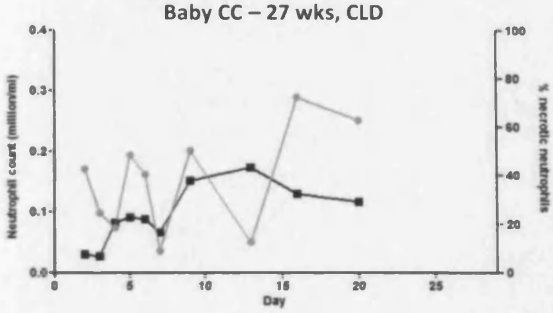
Figure 4.22 Scatterplots showing no significant difference in the percentage of neutrophils which were necrotic on (A) day 1, (B) day 2 and (C) day 3 of life between term and preterm infants.

When individual infants' data are reviewed (Figure 4.23), the relationship of necrotic neutrophils to the total neutrophil count can be seen to change in parallel, i.e. the higher the neutrophil count, the larger proportion of the cells are necrotic. This may suggest that neutrophil clearance from inflammatory sites, particularly by apoptosis, may be impaired, causing neutrophils to build up in numbers and become necrotic before being removed by macrophages. Figure 4.24 shows a linear relationship between neutrophil count and number of necrotic neutrophils.

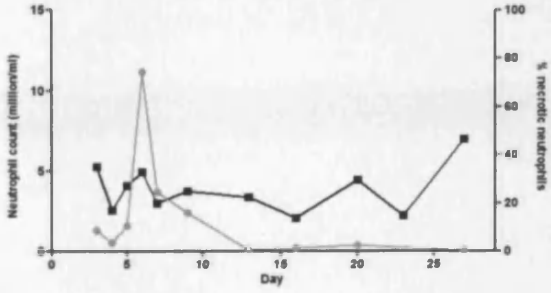
Baby BB – 23 wks, CLD



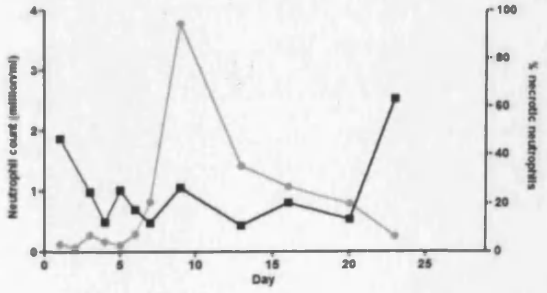
Baby CC – 27 wks, CLD



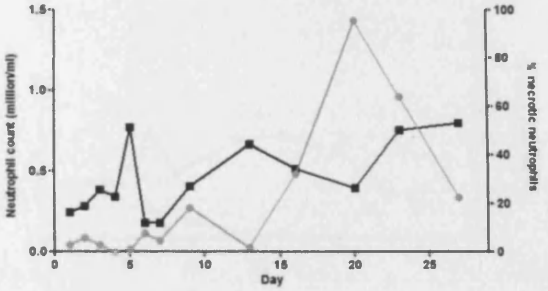
Baby EE – 24 wks, CLD



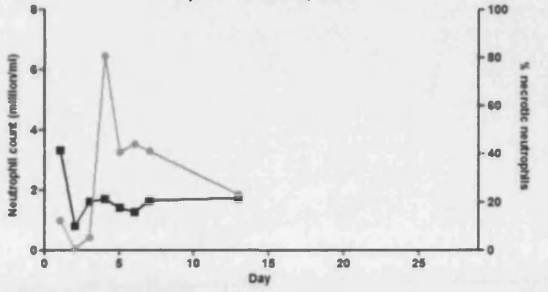
Baby G – 25 wks, CLD



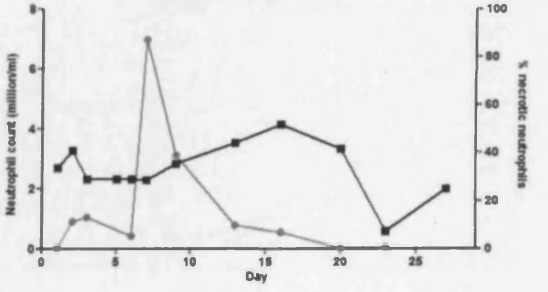
Baby L – 26 wks, CLD



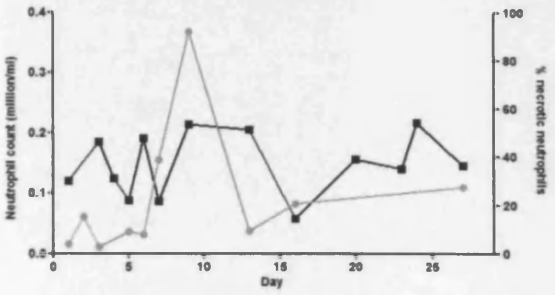
Baby N – 25 wks, CLD



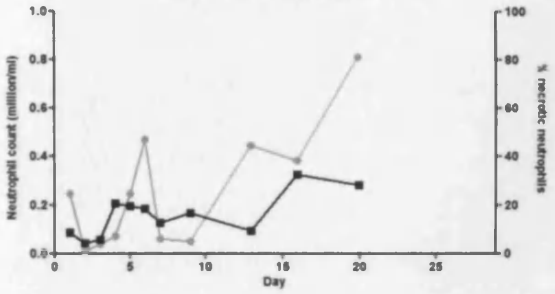
Baby O – 26 wks, CLD



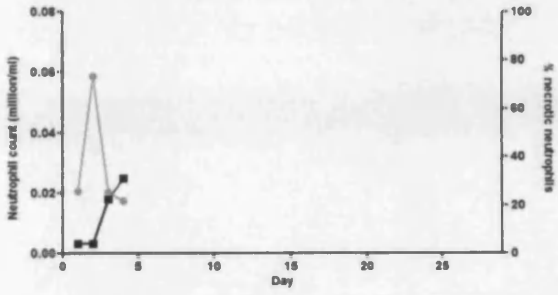
Baby Q – 25 wks, CLD



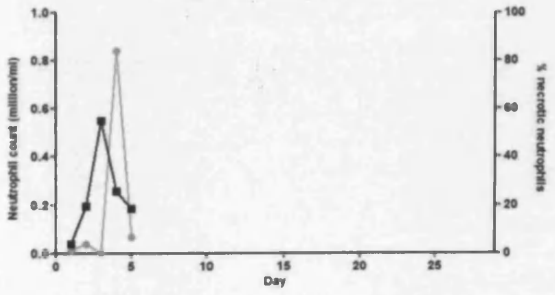
Baby R – 29 wks, CLD



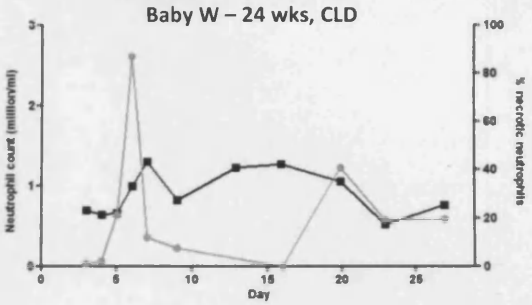
Baby U – 28 wks, CLD



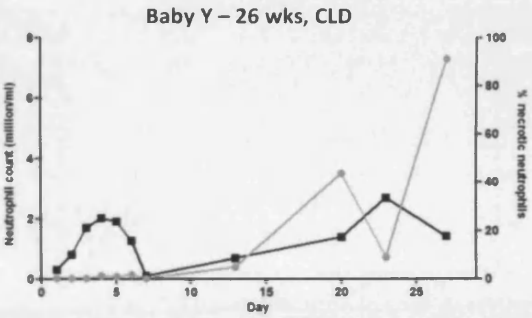
Baby V – 28 wks, CLD



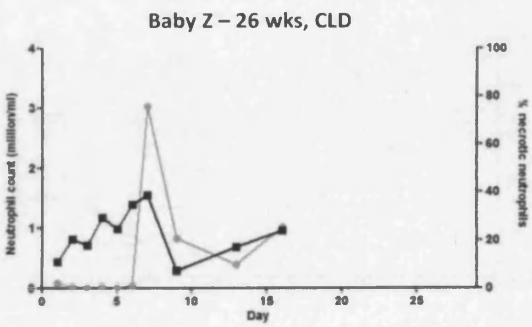
Baby W – 24 wks, CLD



Baby Y – 26 wks, CLD



Baby Z – 26 wks, CLD



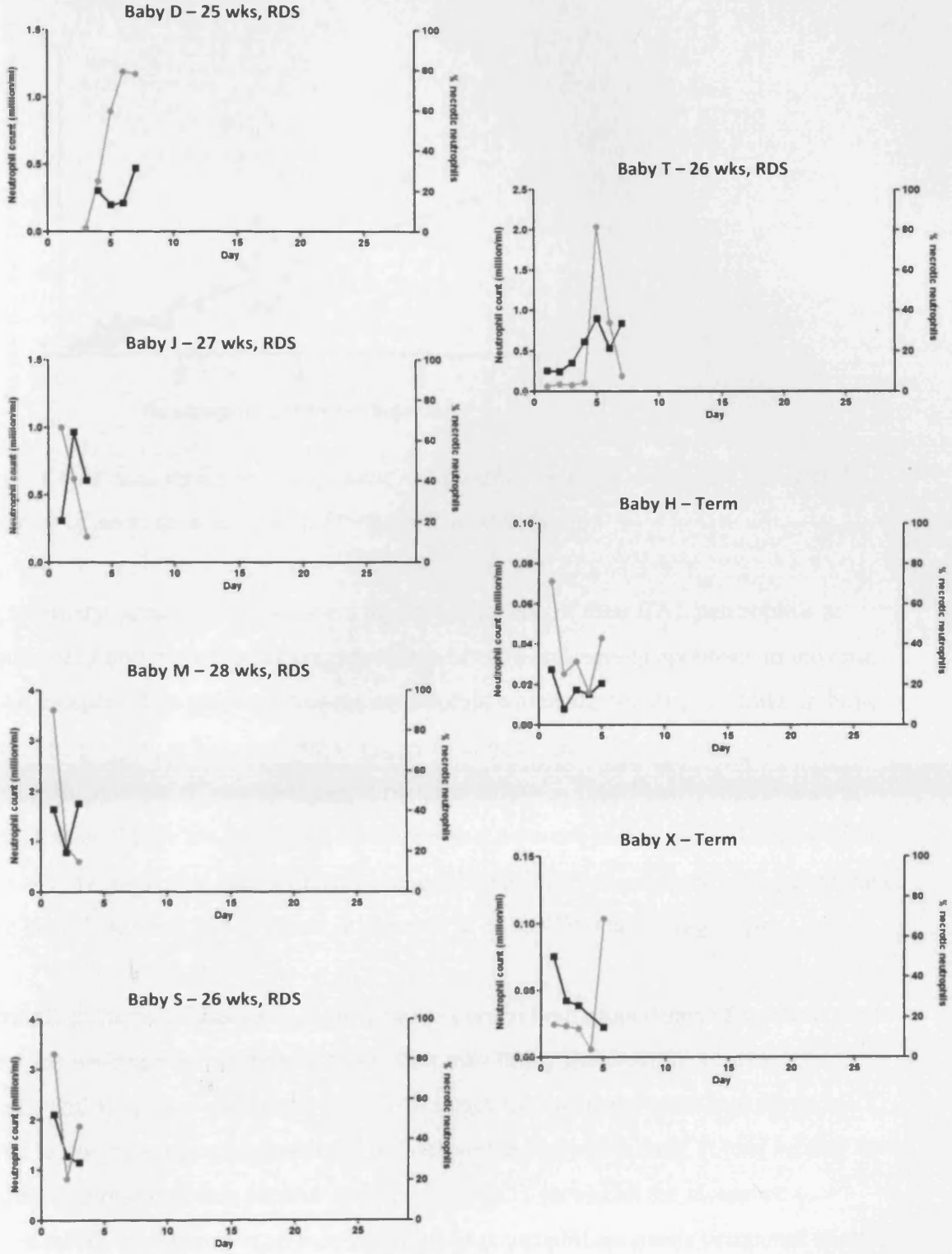


Figure 4.23 Graphs showing neutrophil count (grey line) and percentage necrotic neutrophils (black line) for individual infants with at least 3 BAL samples.

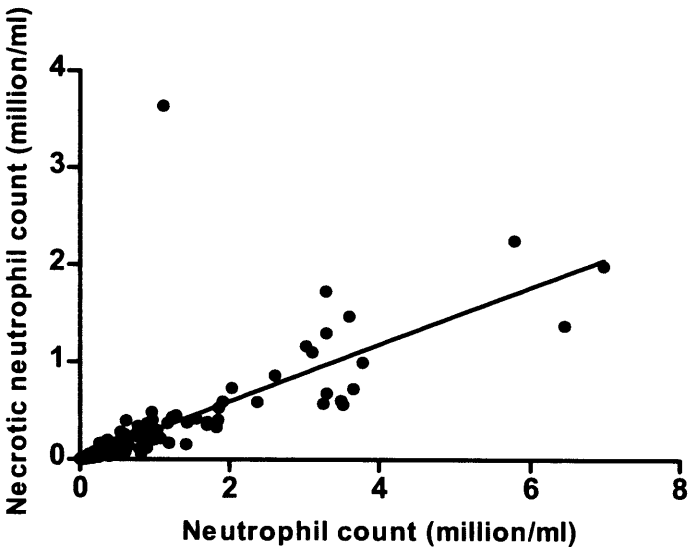


Figure 4.24 Scatterplot showing linear relationship between neutrophil count and number of necrotic neutrophils. $R^2=0.5960$, $p<0.0001$

In summary, preterm infants have a higher proportion of their BAL neutrophils as viable cells and there is a higher percentage of cells undergoing apoptosis in the term BAL samples. The proportion of the neutrophils which are necrotic is similar in both term and preterm infants but due to higher total neutrophil counts in the preterm group, the number of necrotic cells in preterm infants is significantly higher than in term babies. There are no significant differences between babies with RDS and CLD, which may imply that rates of neutrophil apoptosis may be associated with gestational age or with another factor which is common to both RDS and CLD groups.

Overall, preterm infants have slightly later peaks in both proportions of apoptotic and necrotic neutrophils than term infants. This may imply that both the process of neutrophil apoptosis and apoptotic cell clearance (allowing the uncleared apoptotic cells to undergo secondary necrosis) are delayed in preterm infants. It may be that the higher neutrophil counts present in preterm infants overwhelm the clearance mechanisms in addition to an inherent delay in neutrophil apoptosis compared to term infants.

4.5 Relationship between neutrophil apoptosis and the presence of infection

The presence of 16S rRNA genes has already been temporally related to spikes in the total cell count and neutrophil count (Section 3.3.7 and Figures 3.15 and 3.17). I tried to clarify further the relationship between the presence of microbial genes in BAL samples and the proportion of apoptotic neutrophils present. It is likely that the pro-inflammatory factors associated with infection and which promote neutrophil recruitment also have an anti-apoptotic effect on the neutrophils e.g. TNFα and IL-1β (Colotta et al., 1992). This would have the effect of further increasing neutrophil counts.

No statistically significant differences were found in the percentage of the neutrophil population that was apoptotic, necrotic or viable between samples where 16S rRNA genes were detected and samples where the 16S rRNA gene was not found (Figure 4.25).

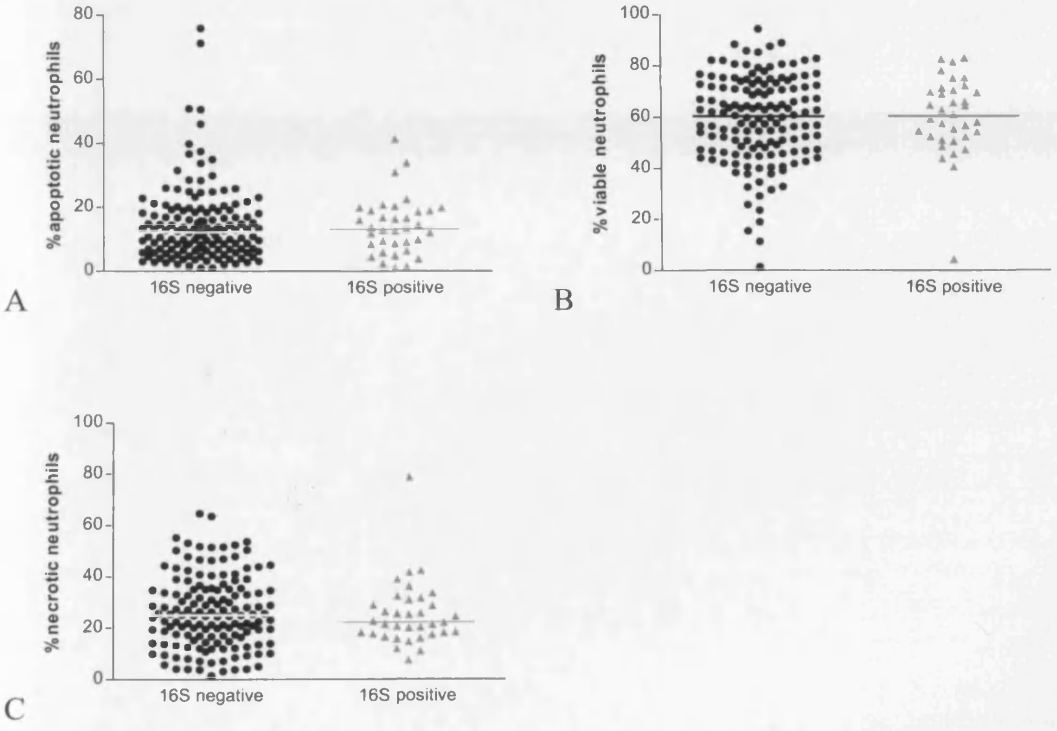
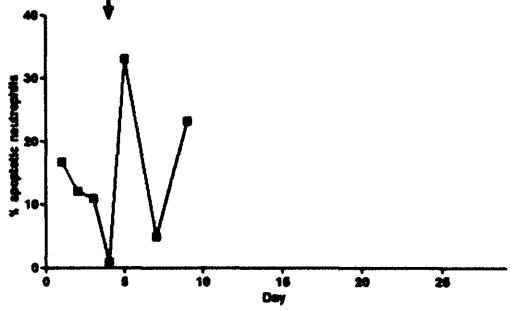


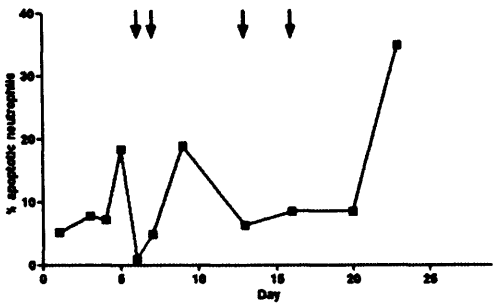
Figure 4.25 Scatterplots showing percentage of (A) apoptotic, (B) viable and (C) necrotic neutrophils in BAL samples of babies in whom 16S rRNA was detected compared to those without.

When looking at longitudinal data for individual babies (Figure 4.26), the percentage of apoptotic neutrophils is often low relative to the peak for that baby when the BAL sample is positive for 16S rRNA genes or that the percentage of apoptotic cells begins to fall once the 16S rRNA gene is detected. However similar variations in the number or percentage of apoptotic cells can also be observed in infants in whom infection was not detected so this is unlikely to be a significant observation.

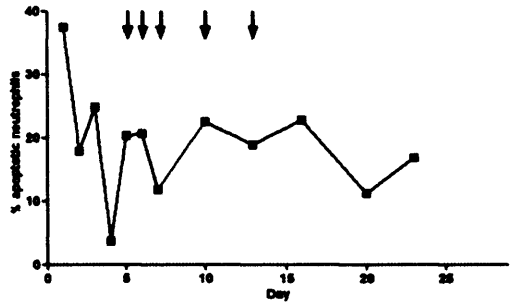
Baby B – 25 wks, CLD



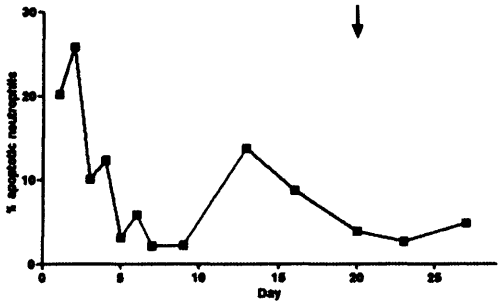
Baby G – 25 wks, CLD



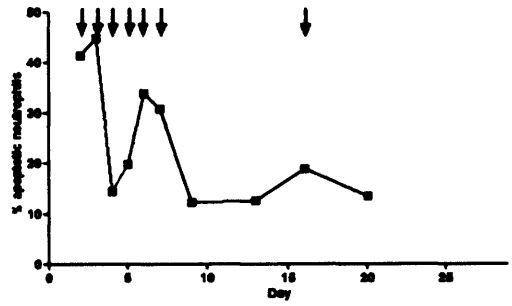
Baby BB – 23 wks, CLD



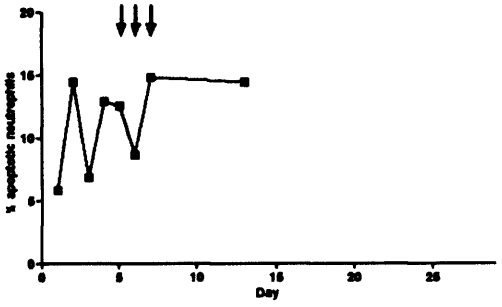
Baby L – 26 wks, CLD



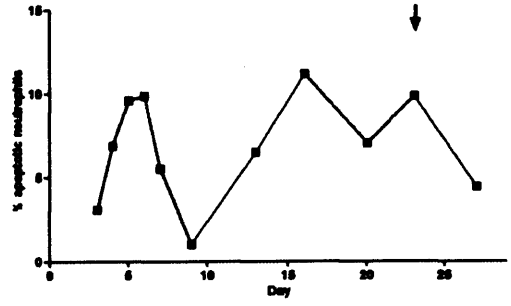
Baby CC – 27 wks, CLD



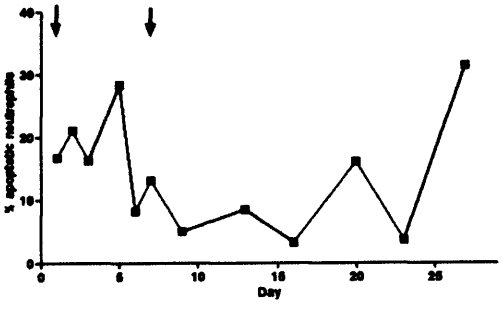
Baby N – 25 wks, CLD



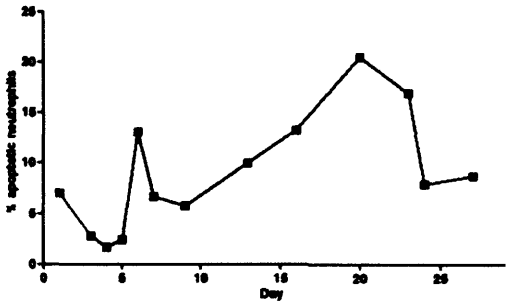
Baby EE – 24 wks, CLD



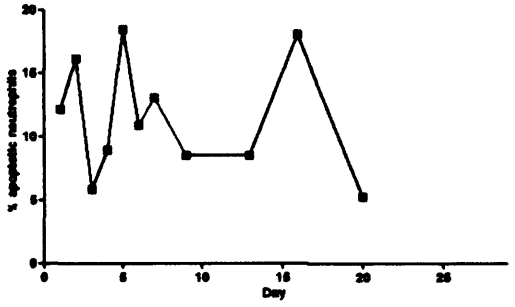
Baby O – 26 wks, CLD



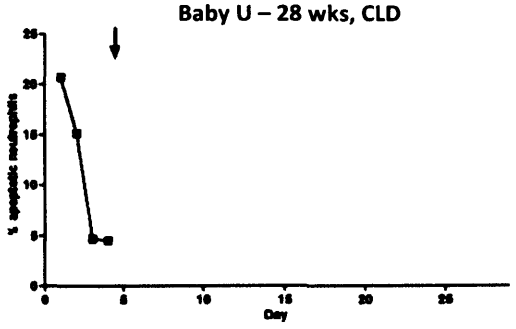
Baby Q – 25 wks, CLD



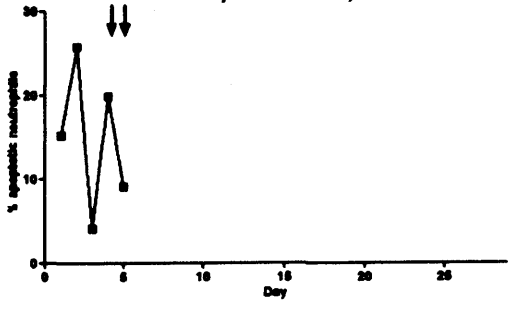
Baby R – 29 wks, CLD



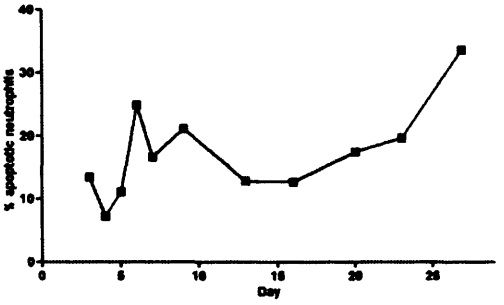
Baby U – 28 wks, CLD



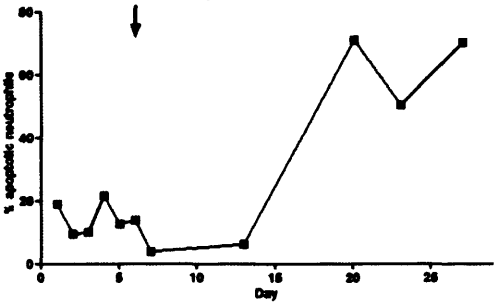
Baby V – 28 wks, CLD



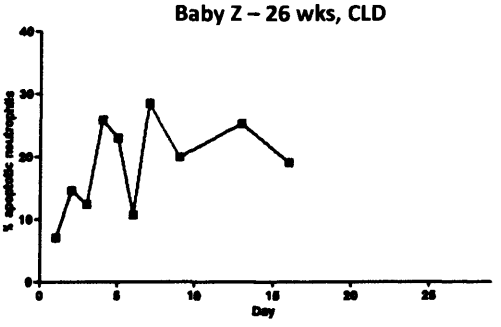
Baby W – 24 wks, CLD



Baby Y – 26 wks, CLD



Baby Z – 26 wks, CLD



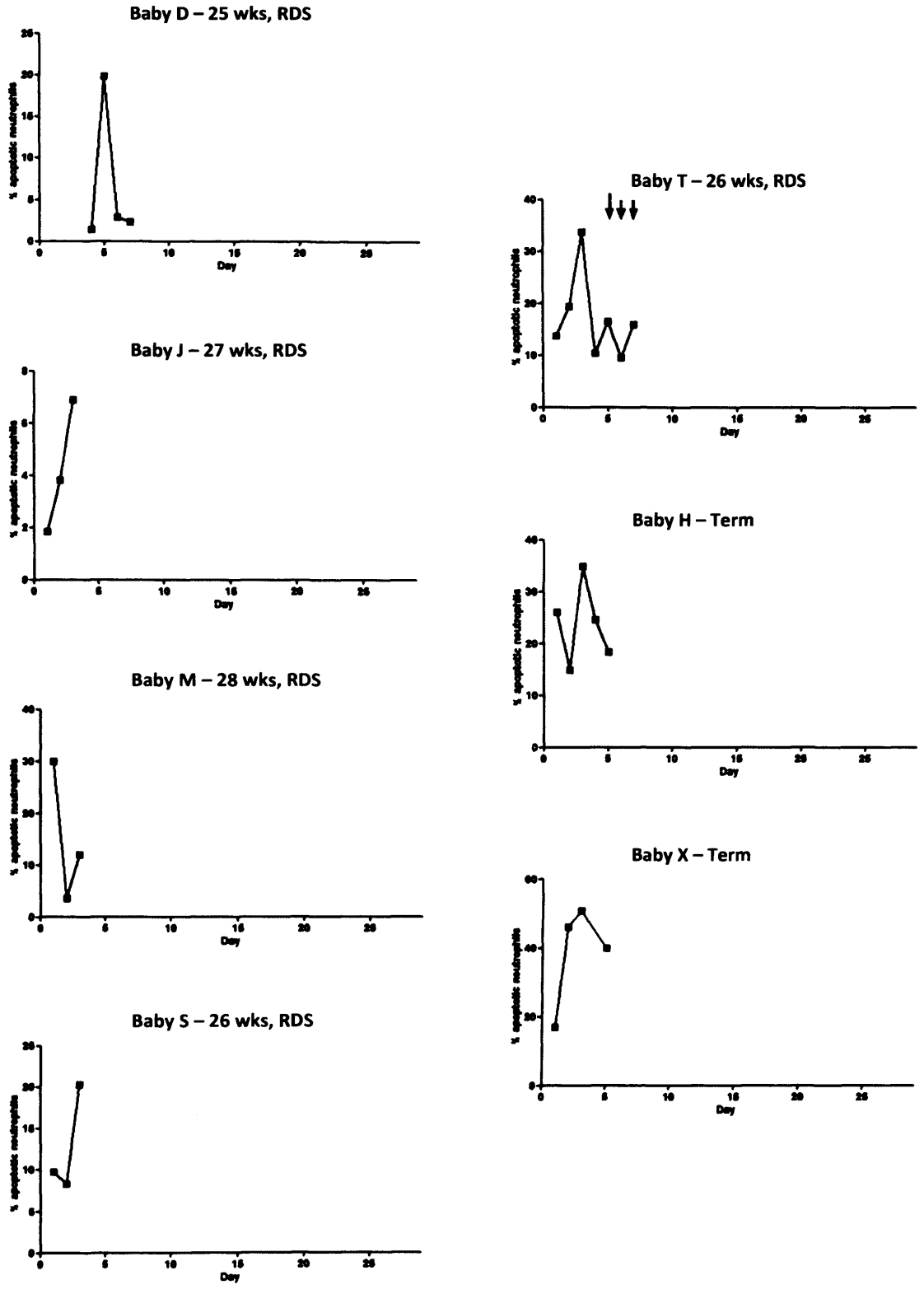


Figure 4.26 Graphs showing percentage of apoptotic neutrophils (black line) over time for individual infants in relation to the presence of 16S rRNA in BAL fluid (black arrows).

There were also no significant differences in apoptotic (p=0.2483), necrotic (p=0.3912) or viable (p=0.6198) neutrophil percentages between babies with or without *Ureaplasma* present in their BAL samples (Figure 4.27).

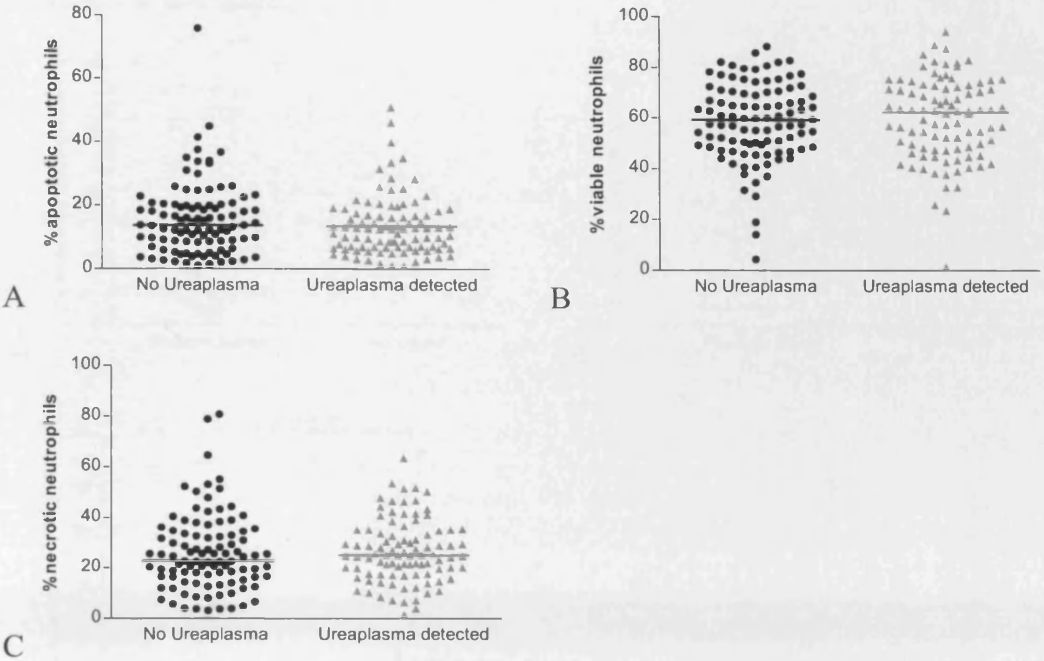


Figure 4.27 Scatterplots showing percentage of (A) apoptotic, (B) viable and (C) necrotic neutrophils in BAL samples of babies in whom Ureaplasma *was detected compared to those without.*

4.6 Neutrophil apoptosis in relation to mode of delivery

The process of labour has been demonstrated to prime/activate neutrophils and thus delay apoptosis (Molloy et al., 2004). In addition, it is possible that an undetected antenatal infection may precipitate the process of preterm labour, resulting in an inflammatory environment present in the lung at delivery and further delay to the apoptotic process. It could thus be expected that infants born following spontaneous preterm labour would have fewer apoptotic cells present initially than those born by elective Caesarean section. However, in our cohort there was no difference in the percentage of apoptotic cells between spontaneously and electively delivered preterm infants on day 1 (Mann-Whitney U-test, p=0.63) (Figure 4.28A) or on day 2 (p=0.74)

(Figure 4.28B). However, on day 3 the percentage of the neutrophil population that was apoptotic was significantly higher in the preterm labour group (p=0.019; Caesarean mean 2.18%, median 2.84%; labour mean 12.56%, median 7.37%) (Figure 4.28C) which is contrary to what might be expected. It is possible that the priming effect of labour on neutrophils is no longer active by the third day of life or another confounding factor, including small sample size, is producing this effect on neutrophil apoptosis at this stage.

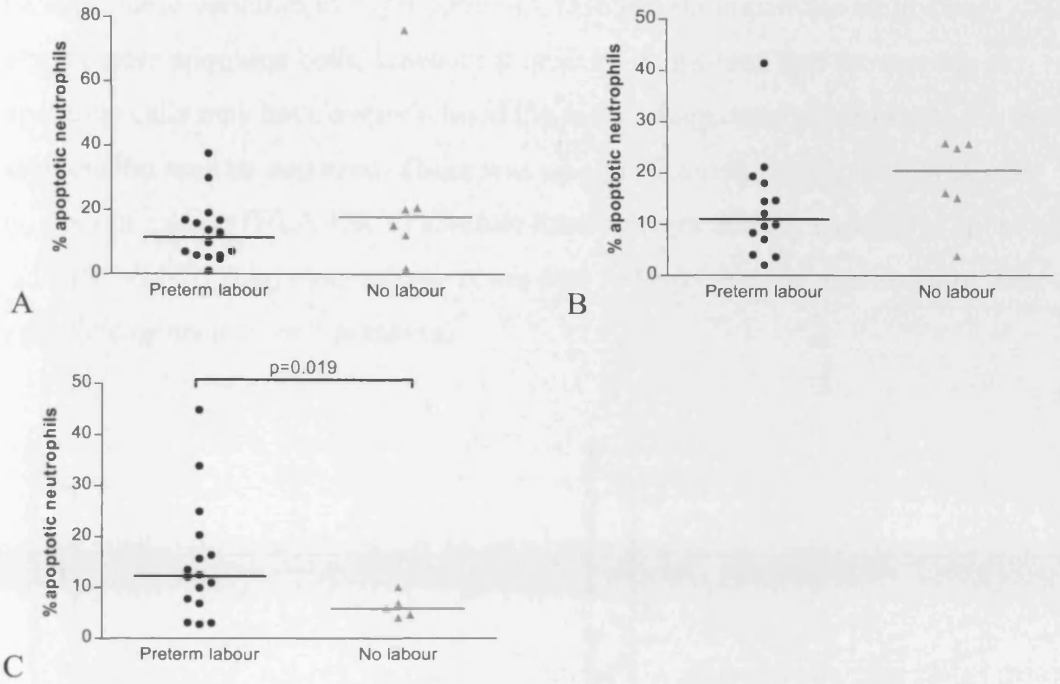


Figure 4.28 Scatterplots showing the percentage of apoptotic cells present in BAL samples of preterm infants (A) day 1, (B) day 2 and (C) day 3 of life, according to whether the baby was delivered by elective Caesarean section or following spontaneous onset of preterm labour.

4.7 Relationship of apoptotic neutrophils to the macrophage population

I have already discussed the positive correlations between the total neutrophil count and the number of apoptotic, necrotic and viable neutrophils. The neutrophil population is dependent on the presence of macrophages in the lung for neutrophil recruitment and also for removal of both apoptotic and necrotic neutrophils. The number and type of macrophages present in the lung may have an influence on either number or proportion of apoptotic or necrotic cells present. I would expect higher

numbers of macrophages, particularly mature macrophages, to reduce the number of apoptotic or necrotic cells present in the BAL samples due to phagocytosis of these cells.

When all babies are compared, there is a positive correlation between CD36+ macrophages and numbers of apoptotic (R^2=0.330, p<0.0001), necrotic (R^2=0.632, p<0.0001) and viable neutrophils. Intuitively there should be an inverse relationship between these variables as expression of CD36 should impart the ability to phagocytose apoptotic cells, however it must be considered that the number of apoptotic cells may have overwhelmed the macrophage response and thus the true relationship may be obscured. There was no significant relationship between the number of mature (HLA-DR +) alveolar macrophages and the number of apoptotic cells (R^2=0.13). Total macrophage count also did not correlate significantly with apoptotic or necrotic cell numbers.

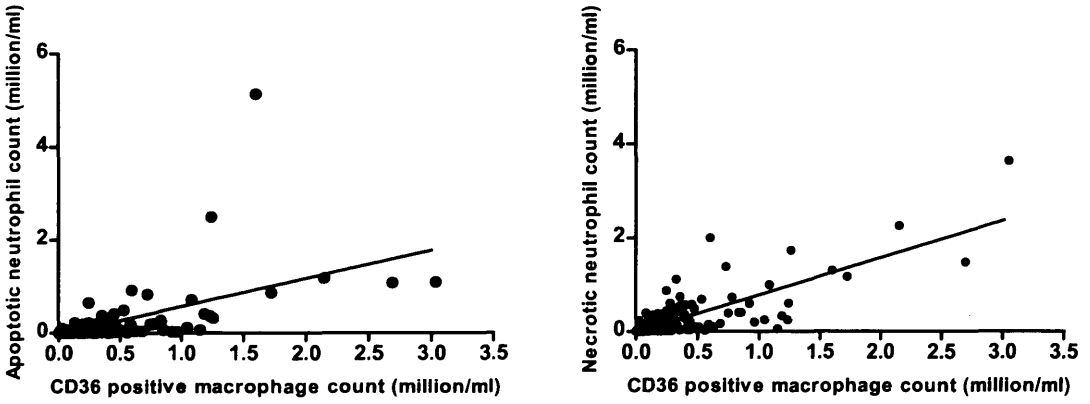
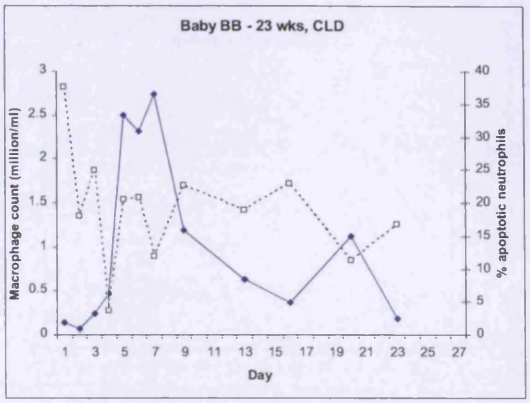


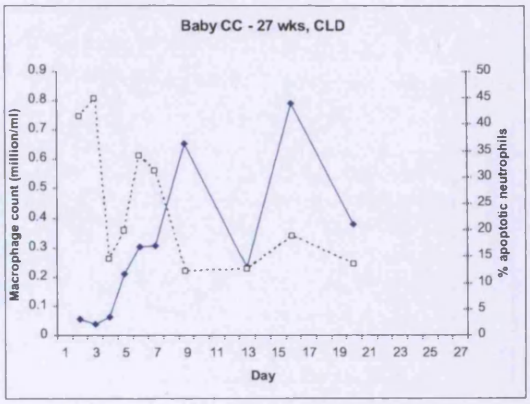
Figure 4.29 Scatterplots showing relationship between CD36 + macrophages and the number of (A) apoptotic and (B) necrotic neutrophils.

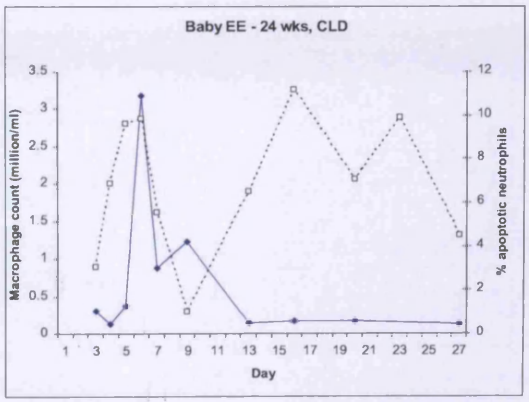
Although the relationship between macrophages and apoptotic cells has proved difficult to explore, one of the advantages of our study is the ability to look longitudinally at individual infants over their entire ventilatory course and observe any possible relationships between different cell types.

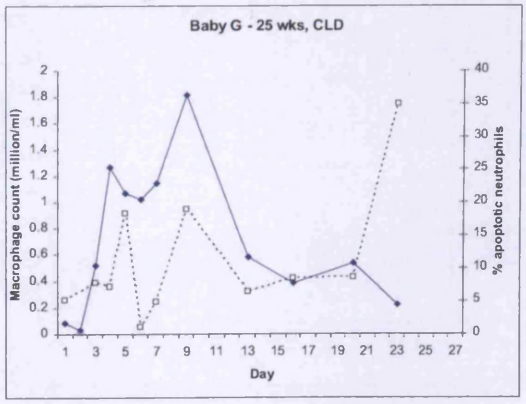
When individual baby data are reviewed, there is again no clear relationship between the macrophage count and the percentage of the neutrophil population that is apoptotic (Figure 4.30), however a much clearer relationship exists between the

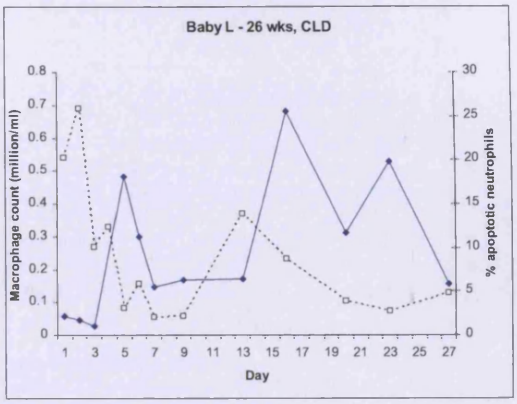
macrophage count and the number of apoptotic cells present, with the two absolute cell counts appearing to rise and fall almost simultaneously in many cases (Figure 4.31).

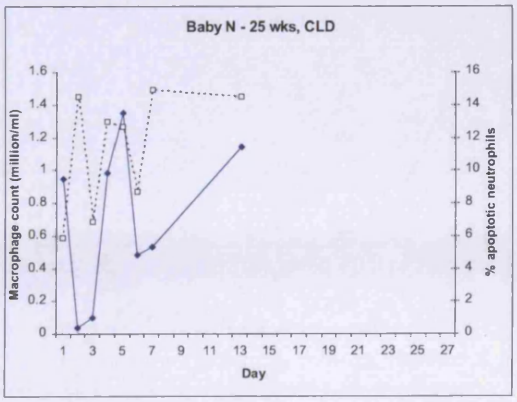

Baby BB - 23 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

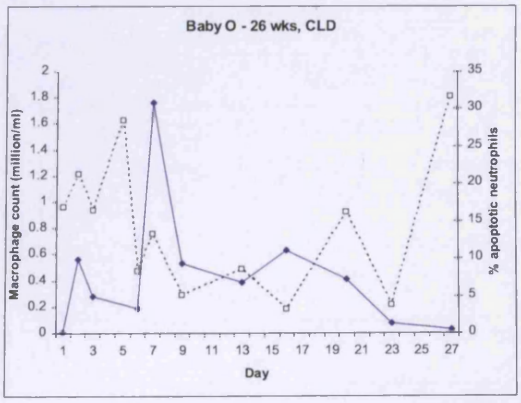

Baby CC - 27 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

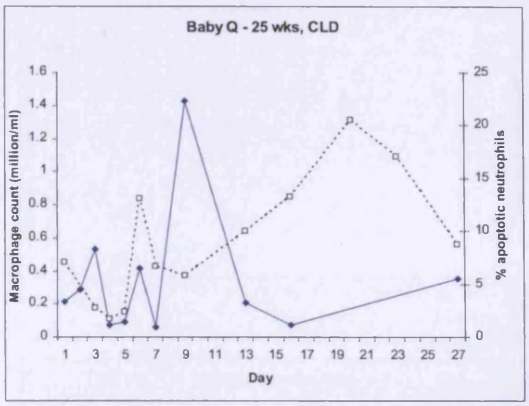

Baby EE - 24 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

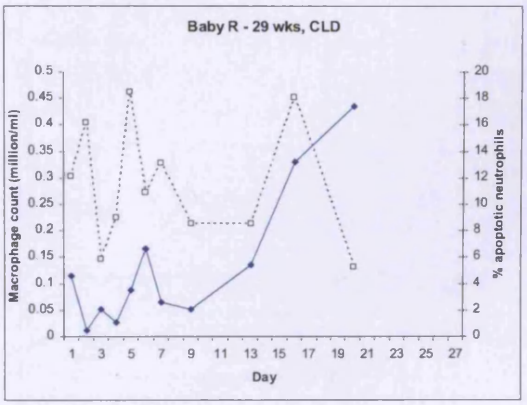

Baby G - 25 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

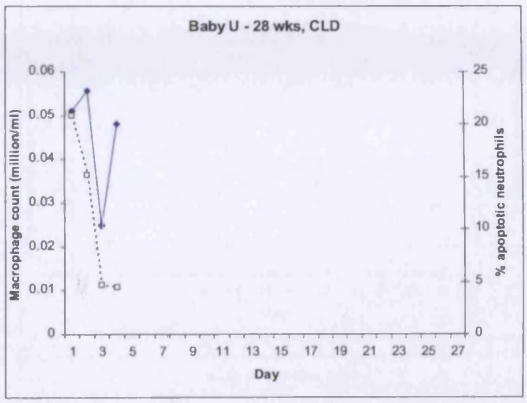

Baby L - 26 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

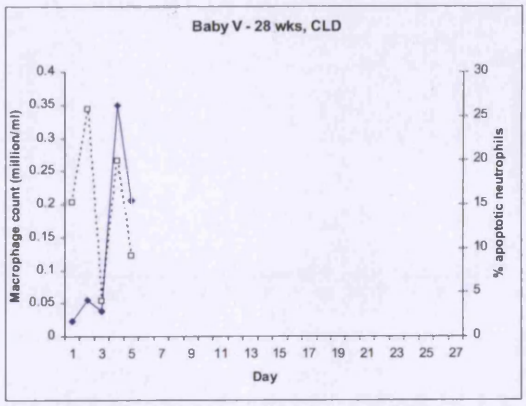

Baby N - 25 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

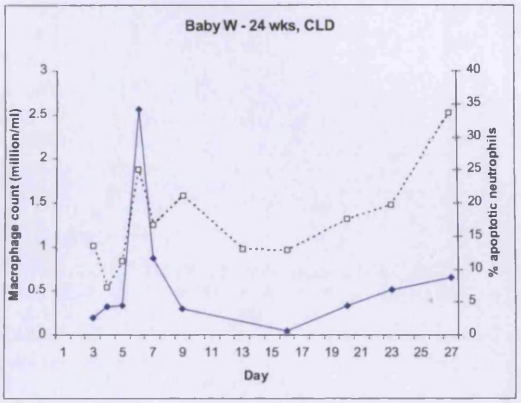

Baby O - 26 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

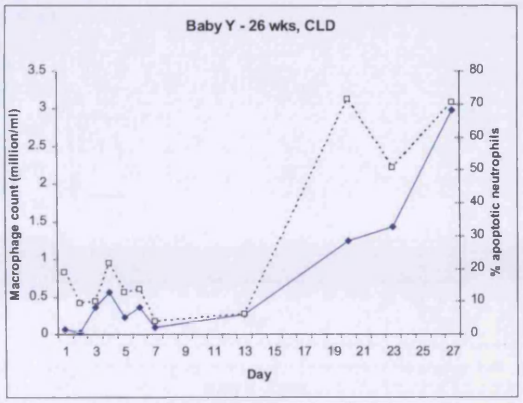

Baby Q - 25 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

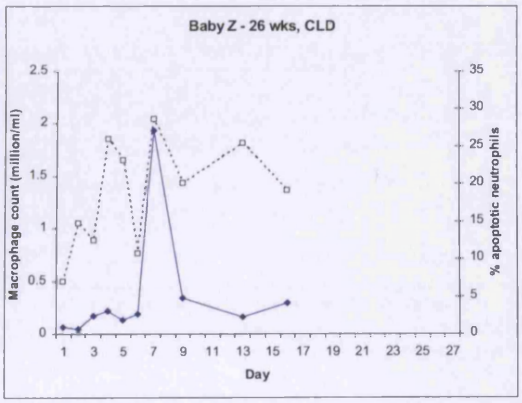

Baby R - 29 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

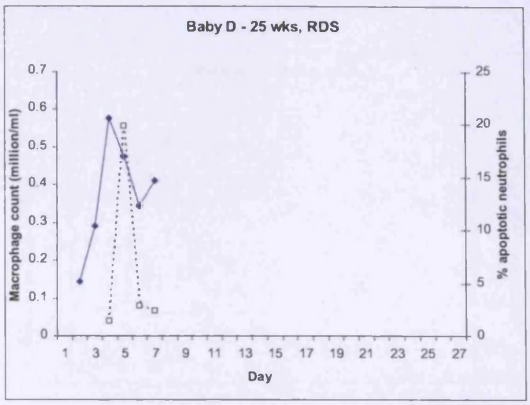

Baby U - 28 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

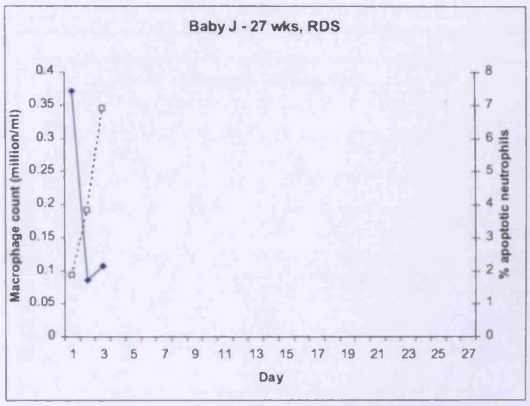

Baby V - 28 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

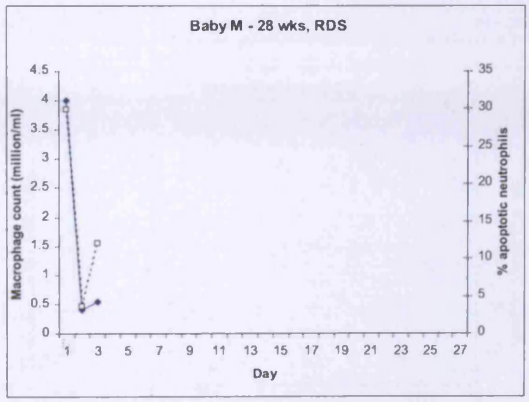

Baby W - 24 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

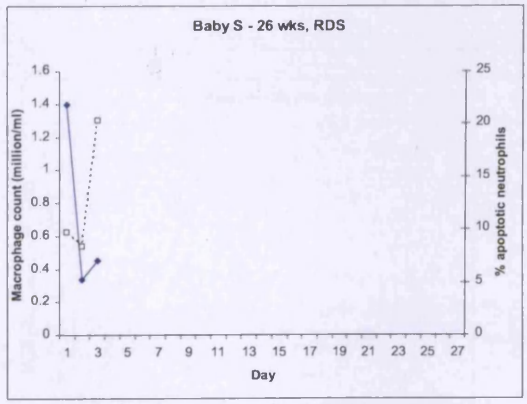

Baby Y - 26 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

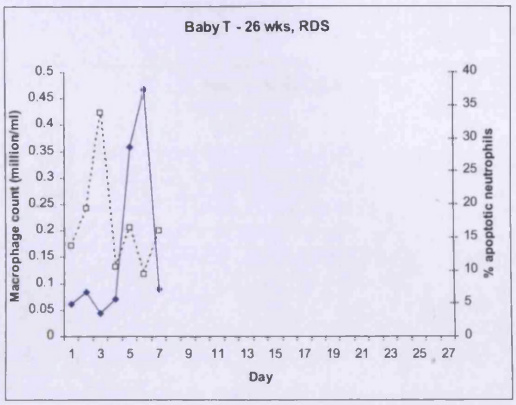

Baby Z - 26 wks, CLD
Macrophage count (million/ml)
% apoptotic neutrophils
Day

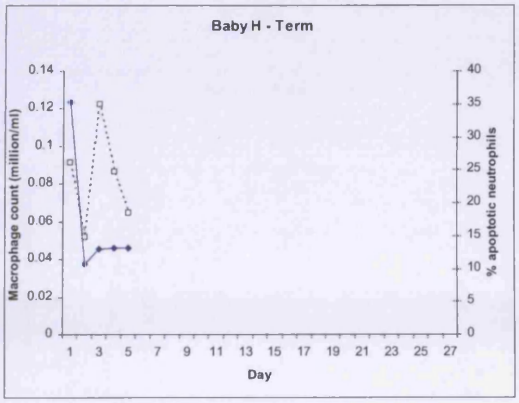












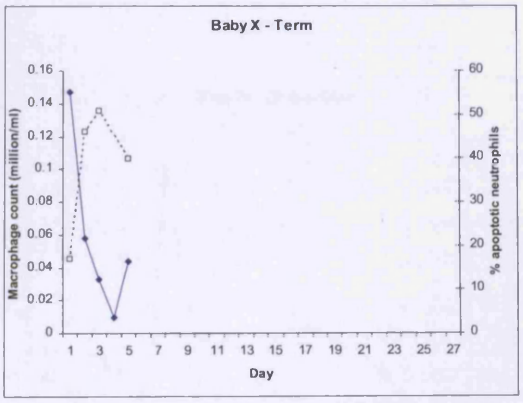
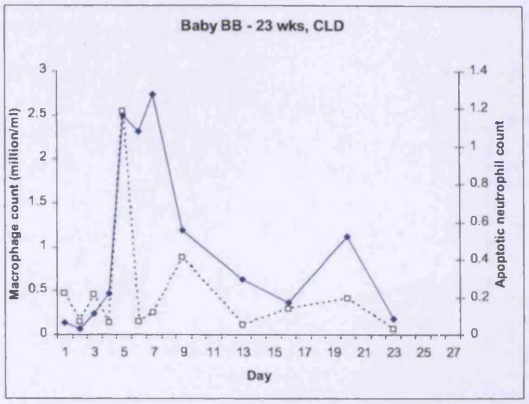
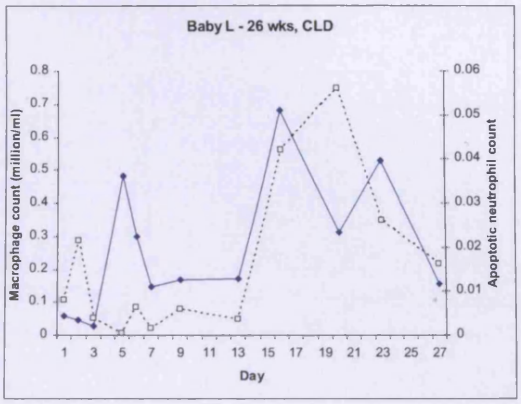
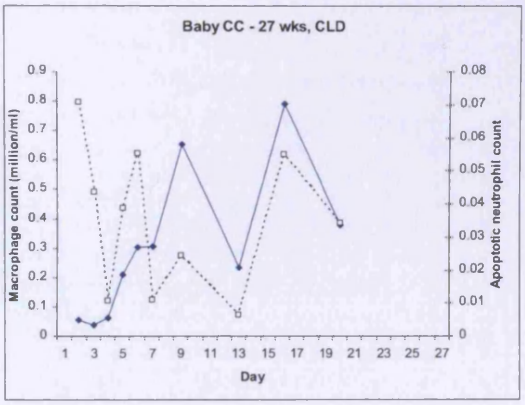
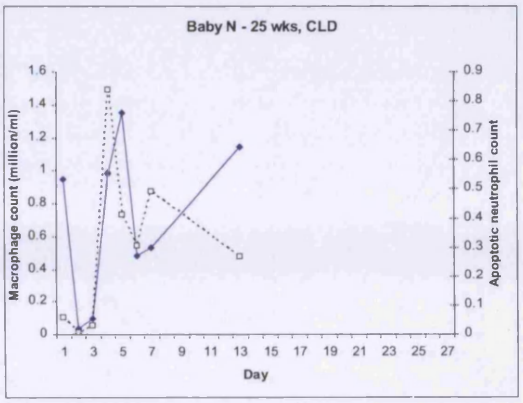


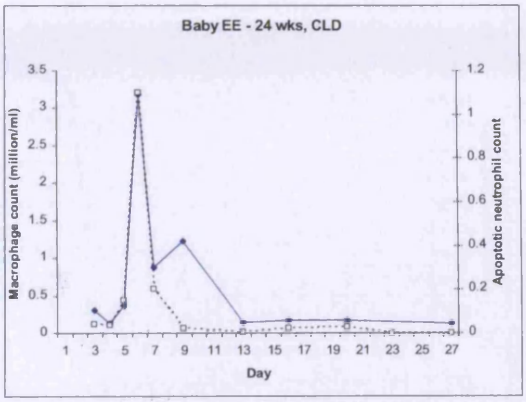
Figure 4.30 Longitudinal data for babies with 3 or more BAL samples showing the macrophage count (solid line) against the percentage of the neutrophil population which is apoptotic (dotted line).

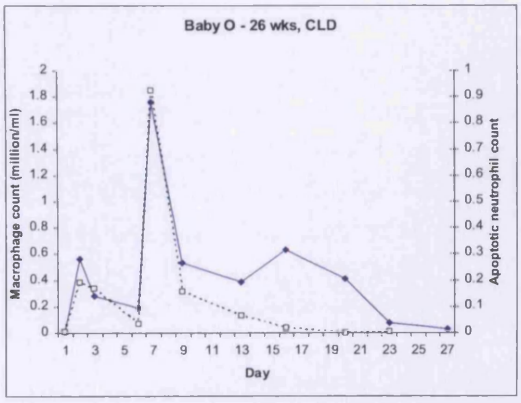

Baby BB - 23 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day

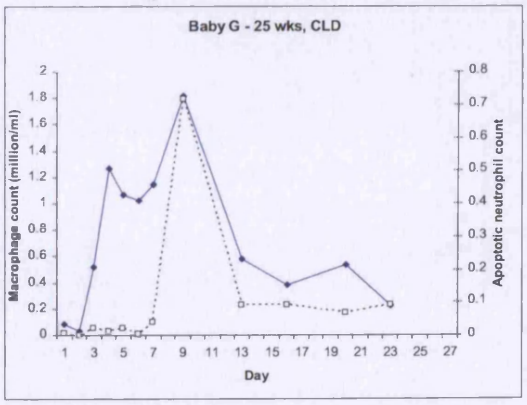

Baby L - 26 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day

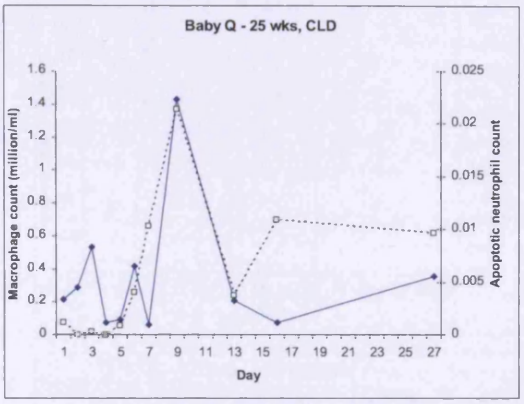

Baby CC - 27 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day

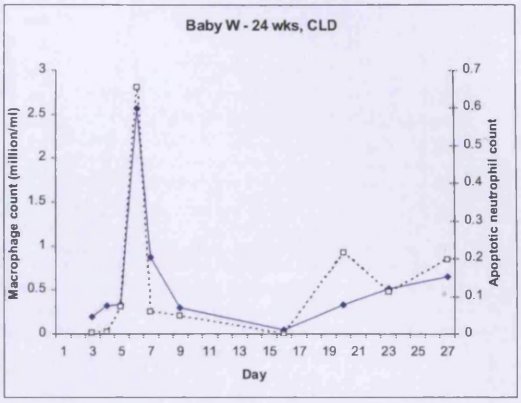

Baby N - 25 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day

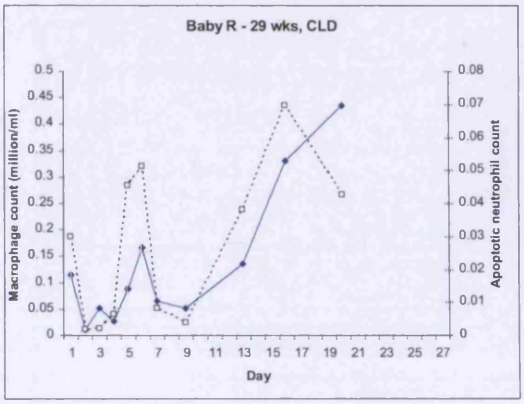

Baby EE - 24 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day

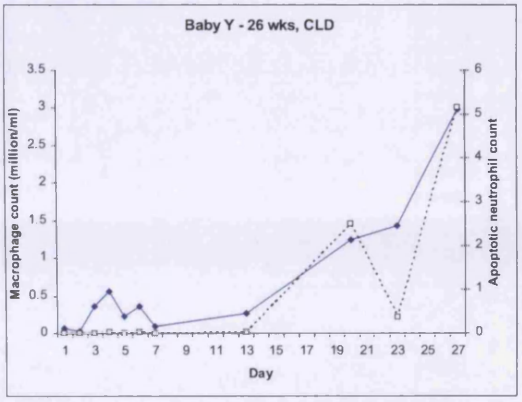

Baby O - 26 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day

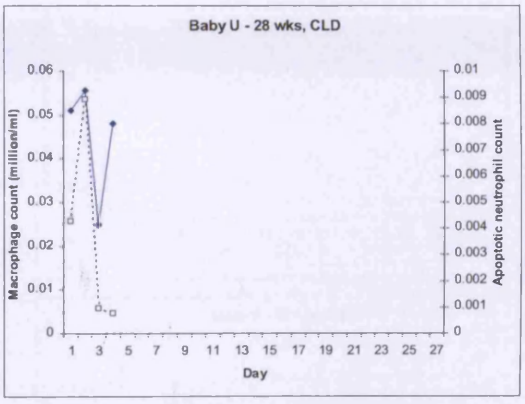

Baby G - 25 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day

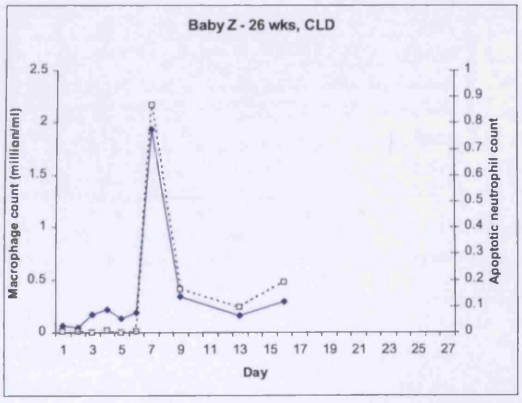

Baby Q - 25 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day

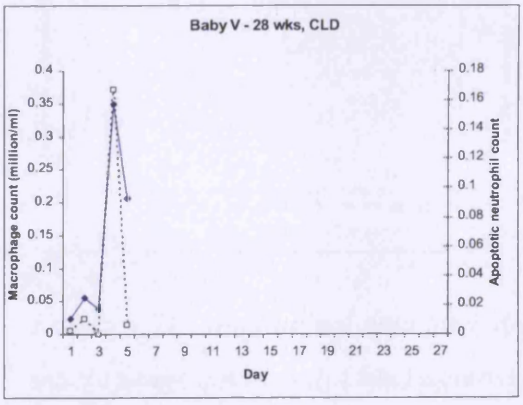

Baby W - 24 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day


Baby R - 29 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day


Baby Y - 26 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day


Baby U - 28 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day


Baby Z - 26 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day


Baby V - 28 wks, CLD
Macrophage count (million/ml)
Apoptotic neutrophil count
Day

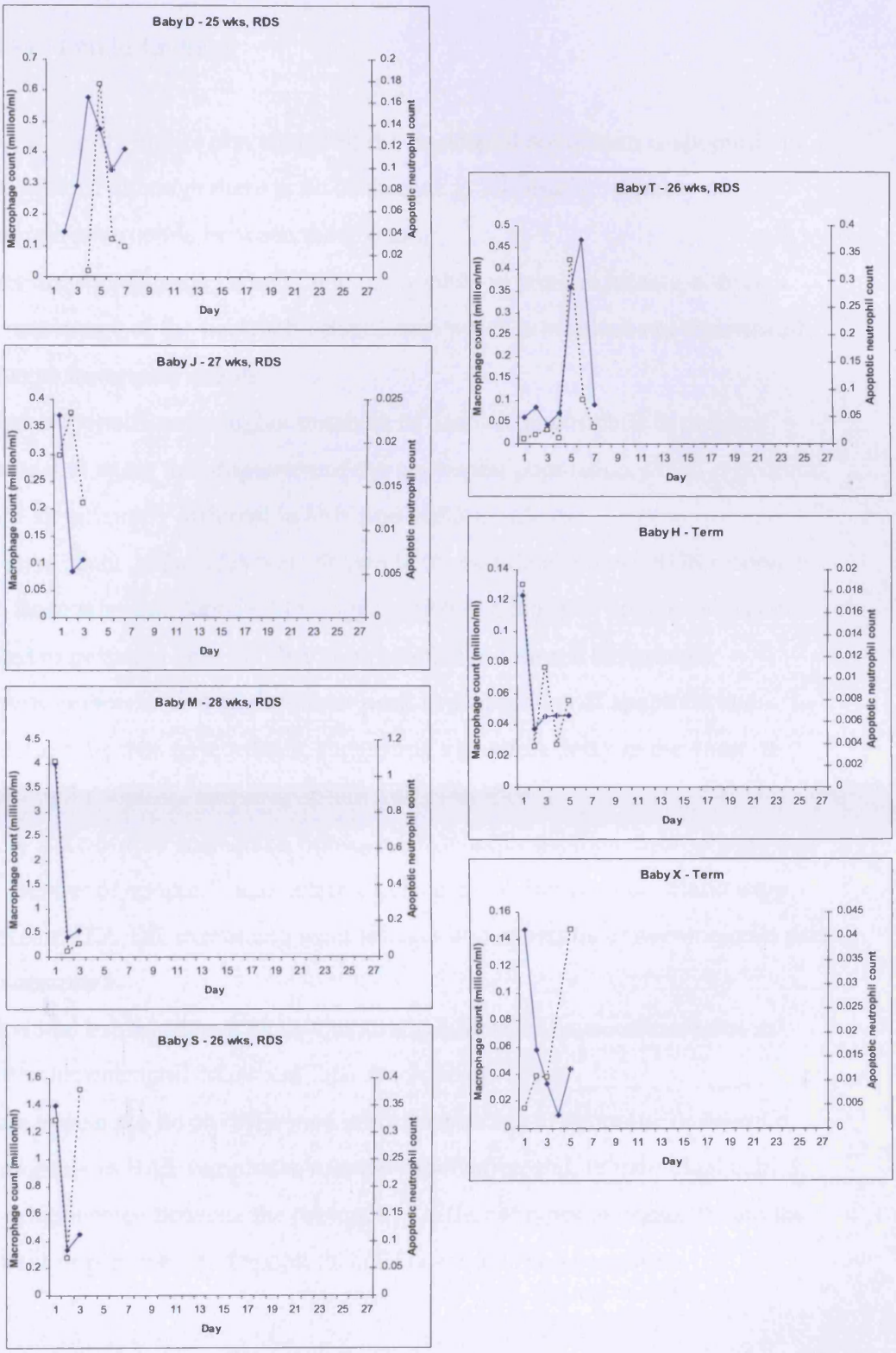


Figure 4.31 Longitudinal data for babies with 3 or more BAL samples showing the macrophage count (solid line) against the total number of apoptotic neutrophils present (dotted line).

4.8 Summary of main findings

1. A significantly higher percentage of the neutrophil population is apoptotic in term infants, although there is no difference in the absolute number of apoptotic neutrophils between the groups.
2. There are significantly more viable neutrophils in preterm infants, although the percentage of the neutrophil population which is viable is not significantly different from term infants.
3. There are significantly higher numbers of necrotic neutrophils in preterm infants, but again the proportion of the neutrophil population which is necrotic is not significantly different in term and preterm infants.
4. No significant differences were shown between infants whose RDS resolved and those who developed CLD, suggesting that neutrophil apoptosis may be linked to gestation or to another common factor between the groups.
5. Preterm infants have a slightly later peak in proportions of apoptotic and necrotic cells than term infants, suggesting a possible delay in the onset of neutrophil apoptosis and/or apoptotic cell clearance.
6. There is a positive correlation between CD36 expression on macrophages and the number of apoptotic and necrotic cells present, but no such relationship between HLA-DR expressing macrophages and apoptotic or necrotic cells was demonstrated.
7. Individual babies show a close temporal relationship between increases in apoptotic neutrophil count and total macrophage count.
8. There appears to be no difference in the percentage of apoptotic or necrotic neutrophils in BAL samples in relation to infection and, in individual babies, the relationships between the presence of different types of organism and the number or percentage of apoptotic cells is variable and complex.

4.9 Discussion

4.9.1 Overview

Ongoing inflammation is thought to be important in the pathogenesis of CLD. In chapter 3 I described the composition of the cellular component of BAL samples and patterns of influx of cells into the airways. Once in the airways, cells must be removed again in order for inflammation to be resolved. The removal of neutrophils from the airways in the newborn by apoptosis and then phagocytosis by macrophages was first described in 1991 by Grigg *et al* (Grigg et al., 1991). They identified, by microscopy, intact, apoptotic neutrophils within macrophages in BAL fluid of 8 ventilated infants but noted that their findings did not correlate with clinical outcome.

Since then several investigators (Kotecha et al., 2003, Oei et al., 2003) have proposed that reduced neutrophil apoptosis and increased neutrophil survival may be the mechanism by which neutrophils persist in the lungs of preterm infants and promote the development of CLD. However, this has not been a completely consistent finding with Cheah *et al* (Cheah et al., 2005a, Cheah et al., 2005b) finding that high numbers of apoptotic cells did not preclude progression to CLD.

This study is the only one to my knowledge which has looked at apoptosis using flow cytometry in BAL samples from the lungs of newborn ventilated infants. The study also benefits from repeated sampling of the same infants over time so that the natural history of the infant's lung injury and/or its resolution can be observed as it progresses. Previous studies of apoptosis in BAL fluid neutrophils (Kotecha et al., 2003, Oei et al., 2003) have not performed daily samples in the first week of life. Even despite this study's frequent sampling, comparison between term and preterm infants is hampered by the very short duration of ventilation in preterm infants.

The use of flow cytometry for assessment of neutrophil apoptosis in neonatal BAL samples is just as labour intensive as the preparation of cells for analysis of other surface markers as discussed in chapter 3. Again it requires immediate processing of BAL samples before any deterioration in the sample can occur and as such is not practical for routine use in clinical samples.

4.9.2 Neutrophils – apoptotic, necrotic and viable

As shown in chapter 3, preterm infants have higher neutrophil counts than their term counterparts, so it is perhaps not surprising that the numbers of viable neutrophils in BAL samples are significantly higher in preterm babies. In terms of the percentage of the neutrophil population that are viable, there is no significant difference between term and preterm infants. These higher absolute numbers of viable neutrophils may be responsible for the generation of proteases, ROS and other mediators which mediate lung damage. Analysis of these mediators in BAL supernatants is discussed further in chapter 5.

A similar picture is seen for the presence of necrotic cells – with preterm and term infants having a similar percentage of their BAL neutrophil population undergoing necrosis, however the preterm group of babies have a higher overall number of neutrophils and therefore a significantly higher number of necrotic neutrophils present. This higher absolute load of necrotic neutrophils may be involved in mediating lung injury through release of proteases and other neutrophil granule contents into the tissues. In addition, phagocytosis of necrotic neutrophil fragments directs macrophages to release more pro-inflammatory mediators, thus exacerbating the inflammatory response within the lung (Savill et al., 2002).

With the above in mind, one would expect that preterm infants would have a similar percentage of apoptotic cells to their term counterparts and higher absolute numbers of apoptotic cells in BAL samples due to higher cell counts. This is not the case. The absolute numbers of apoptotic neutrophils in preterm BAL samples are not significantly different to those in term babies, however term babies have a significantly higher percentage of their BAL neutrophils undergoing apoptosis than in preterm babies. This might imply that preterm neutrophils are in some way resistant to death by apoptosis, perhaps remaining viable for longer or preferentially undergoing death by necrosis instead. I will explore this hypothesis further in cord blood cells in chapter 6.

4.9.3 Relationship to macrophages

Following apoptosis, effete neutrophils must be taken up by macrophages in order to be cleared from the site of inflammation, which in turn facilitates the production of anti-inflammatory cytokines by the macrophages and further downregulation of the inflammatory response (Haslett, 1999, Fadok et al., 1998).

There appears to be a temporal relationship in individual infants between the number of apoptotic neutrophils and the number of macrophages present in the BAL sample. I have noted previously (in chapter 3) that neutrophil counts and macrophage counts tend to rise and fall in close relationship to each other and also (in this chapter) that there is a linear relationship between total neutrophil count and the number of apoptotic neutrophils which may partly explain this relationship.

The phagocytosis of apoptotic neutrophils may be dependent on the expression of various cell surface markers on the macrophage which allow the cell to recognise an apoptotic neutrophil. These surface markers include CD36 but there are a host of other molecules which play a role in this complex recognition process (Vandivier et al., 2006). Failing to demonstrate a clear relationship between macrophages with different surface markers implies that the process of recognition and phagocytosis of apoptotic cells is probably just more complex than cells expressing CD36 alone. The inflammatory response, particularly in preterm infants, may simply overwhelm the macrophage phagocytosis system's ability to deal effectively with apoptotic neutrophils, allowing more to become necrotic, resulting in further neutrophil and monocyte recruitment and obscuring (for the purposes of our study) the neutrophil/macrophage interactions that are taking place.

4.9.4 Relationship to infection

Current understanding of the role of inflammatory mediators in the process of apoptosis would suggest that the presence of infection would increase the longevity of neutrophils, reducing the percentage of the population that is apoptotic. Contrary to this, it is also known that certain organisms (e.g *P. aeruginosa*) are able to induce

apoptosis in host neutrophils as an "immune evasion" technique (Usher et al., 2002) and others eg *S. aureus* can induce rapid necrosis in host immune cells for similar reasons.

The majority of 16S rRNA genes detected in BAL samples were shown on sequencing to be from *S. epidermidis*, an organism that frequently colonises indwelling plastic medical devices such as endotracheal tubes and intravenous lines and cannulae. As previously discussed, the presence of *S. epidermidis* in BAL samples cannot simply be interpreted as infection in the lungs of the baby concerned as it may be a contaminant from the endotracheal tube, unavoidably picked up during BAL sampling. The role of *S. epidermidis* in neutrophil apoptosis is not well described, but it is known that *S. epidermidis* is unable to induce neutrophil apoptosis in the way that *S. aureus* does (Nilsdotter-Augustinsson et al., 2004). Additionally, more recently it was demonstrated that apoptotic neutrophils containing *S. epidermidis* produced a pro-inflammatory response by way of TNFα and IL-6 by the macrophage when ingested, rather than the expected anti-inflammatory one (Wilsson et al., 2008).

The presence of *Ureaplasma spp.* in a sub-group of the babies may also further complicate the interpretation of the relationship between apoptosis in BAL neutrophils and infection, as *Ureaplasma* has been shown to induce apoptosis in lung macrophages (Li et al., 2002) but its role in neutrophil apoptosis remains unclear. Five infants in our cohort were positive for 16S rRNA and had *Ureaplasma* detected in BAL samples, which is likely to further complicate interpretation of the FACS data on BAL cells.

The role of infection in neutrophil apoptosis in the lung is clearly far more complex than a simple "infection delays apoptosis" conclusion and merits further investigation in an *in vitro* setting where some of the many interacting factors in the process can be controlled or eliminated for study purposes. Some aspects of this process have been studied in cord blood neutrophils in chapter 6.

4.9.5 Conclusion

In this chapter I have described the BAL neutrophil population in terms of cell viability, apoptosis or necrosis and showed that preterm infants appear to have a high load of necrotic cells and a relatively low proportion of apoptotic neutrophils in BAL samples. Both of these factors can be understood to affect the development of CLD in preterm infants, although no difference between RDS and CLD groups was found, perhaps due to the relatively small number of infants studied and the low number of samples available from babies with resolving RDS as they were generally extubated very rapidly.

The apparent delay or reduction in neutrophil apoptosis in the preterm group of infants will be studied further in an *in vitro* setting in chapter 6 and possible mechanisms for this will be explored.

The relationship between macrophages and neutrophil apoptosis involves numerous factors, only a few of which have been studied here. This relationship may require further studies using different macrophage markers and possibly different methodology to more clearly elucidate the interactions that must be occurring.

The role of infection in neutrophil apoptosis is complex and no single effect of the presence of either 16S rRNA microbial genes or *Ureaplasma spp.* could be identified. The type of organism present may significantly alter the response of neutrophils entering apoptosis and the subsequent cytokine production by the macrophage after phagocytosis.

Chapter 5

BAL fluid supernatants

Chapter 5
BAL fluid supernatants

5.1 Introduction

In the preceding two chapters, the focus was on the cellular component of the BAL samples, however it is vital that the cells are not studied in isolation as the environment in which the cell or cells are found may not only reflect the production or release of various chemical mediators by the cell but may also have a profound effect on the behaviour of the cells. There is a complex and finely balanced interaction between various pro- and anti-inflammatory proteins, particularly chemokines, cytokines and proteases which influences the recovery from or progression of the inflammatory process in the lung.

Inflammatory mediators, such as cytokines, at sites of inflammation are critical to the development and functioning of both the innate and adaptive immune response. These mediators are most frequently secreted by immune cells that have encountered a pathogen, thus activating and recruiting further immune cells to increase the immune response to the pathogen. Cytokines may have different pro- or anti-inflammatory effects on different cell types in different situations

I sought to measure various inflammatory mediators within BAL supernatants and then attempted to relate these measurements to clinical outcomes of the infants, hypothesising that those infants with higher levels of pro-inflammatory mediators within the BAL fluid might be more likely to progress to CLD than those with lower levels. I also sought to relate levels of inflammatory mediators to the presence or absence of microbial genes in the BAL fluid. In light of a previously published finding (Kotecha et al., 2003) that BAL fluid may be anti-apoptotic in infants who develop CLD, I investigated the relative ability of the BAL supernatants to promote or inhibit neutrophil apoptosis.

5.2 Cytokine measurements

A cytometric bead array (CBA) was used to determine the amounts of 12 potentially relevant inflammatory mediators in BAL samples (as described in 2.1.7.3 above). CBA is a costly assay and it was not possible to analyse fluid from every one of the 207 BAL samples I had collected. From previous work (Kotecha et al., 1995, Kotecha et al., 1998, Kotecha et al., 1996a, Kotecha et al., 1996b) it was suggested that days 1, 2, 4 and 7 were likely to yield useful data for comparison with published data and between infants in this study cohort. These samples were analysed as well as samples from later time points in those babies who were ventilated for longer periods.

It is apparent that measurement of cytokines in BALsupernatants is difficult, complex and potentially inaccurate due to the mucousy nature of BAL supernatant even after centrifugation, the minute amount of BAL fluid returned from tiny preterm infants and the dilutional effect of the saline used for lavage. Attempts have been made in the past to correct cytokine concentrations for the dilutional effect of saline used for the lavage. The ERS guidelines (Haslam and Baughman, 1999, de Blic et al., 2000) suggest that no correction be made and this is the approach that has been used in the analysis and interpretation of the results of the CBA assay. Virtually all nucleated cells, including endothelial and epithelial cells are potent producers of cytokines such as IL-1, IL-6, and TNF-α and thus the amount of cytokine in a BAL sample cannot consistently be directly related to a variable such as cell count, making any form of "correction" of cytokine values of little value.

5.2.1 Interleukin-1 beta (IL-1β)

Levels of IL-1β rose steadily over the whole ventilated period in infants who developed CLD and by the end of the first week of life (day 7) were statistically higher than levels in the same babies on day 1 (Mann-Whitney U-test, $p=0.019$) (Figure 5.1). The small number of samples at later time points prevented similar analysis for the whole period.

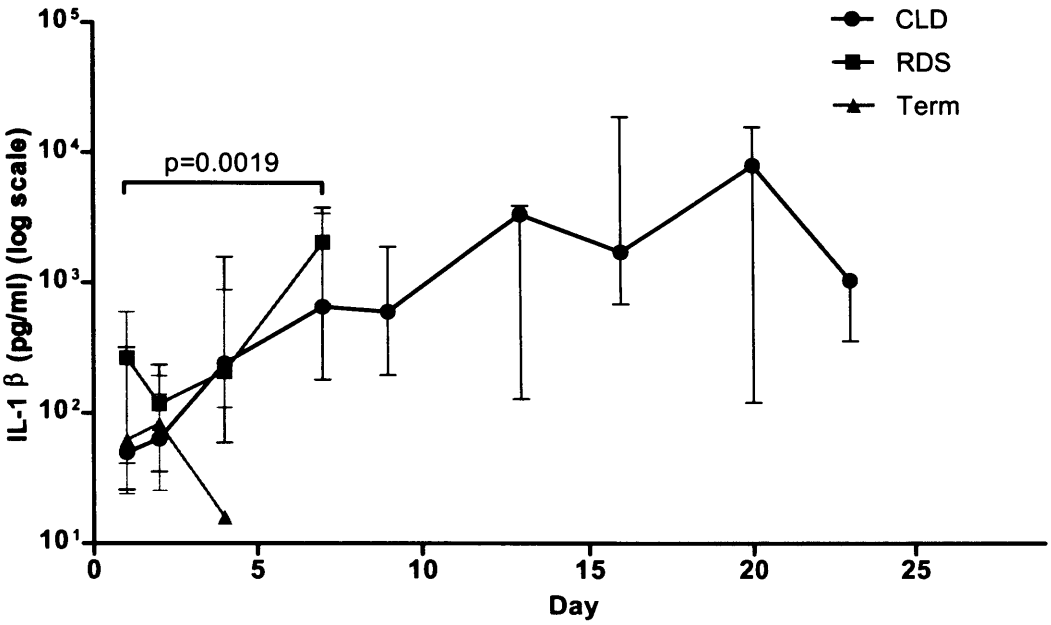


Figure 5.1 Graph of median IL-1β concentration against time in term, RDS and CLD infants. Error bars represent interquartile ranges. p=0.019 for day 1 vs day 7 values for infants developing CLD.

Increased concentrations of IL-1β in TAF have been associated with the development of CLD, possibly through the inhibition of neutrophil apoptosis (Kotecha et al., 1996b, Cayabyab et al., 2003). There appeared to be a close parallel between levels of IL-1β and percentage apoptotic neutrophils in BAL samples, with peaks occurring almost simultaneously in many patients, rather than a definite suppression of neutrophil apoptosis being seen when IL-1β levels were high (Figure 5.2).

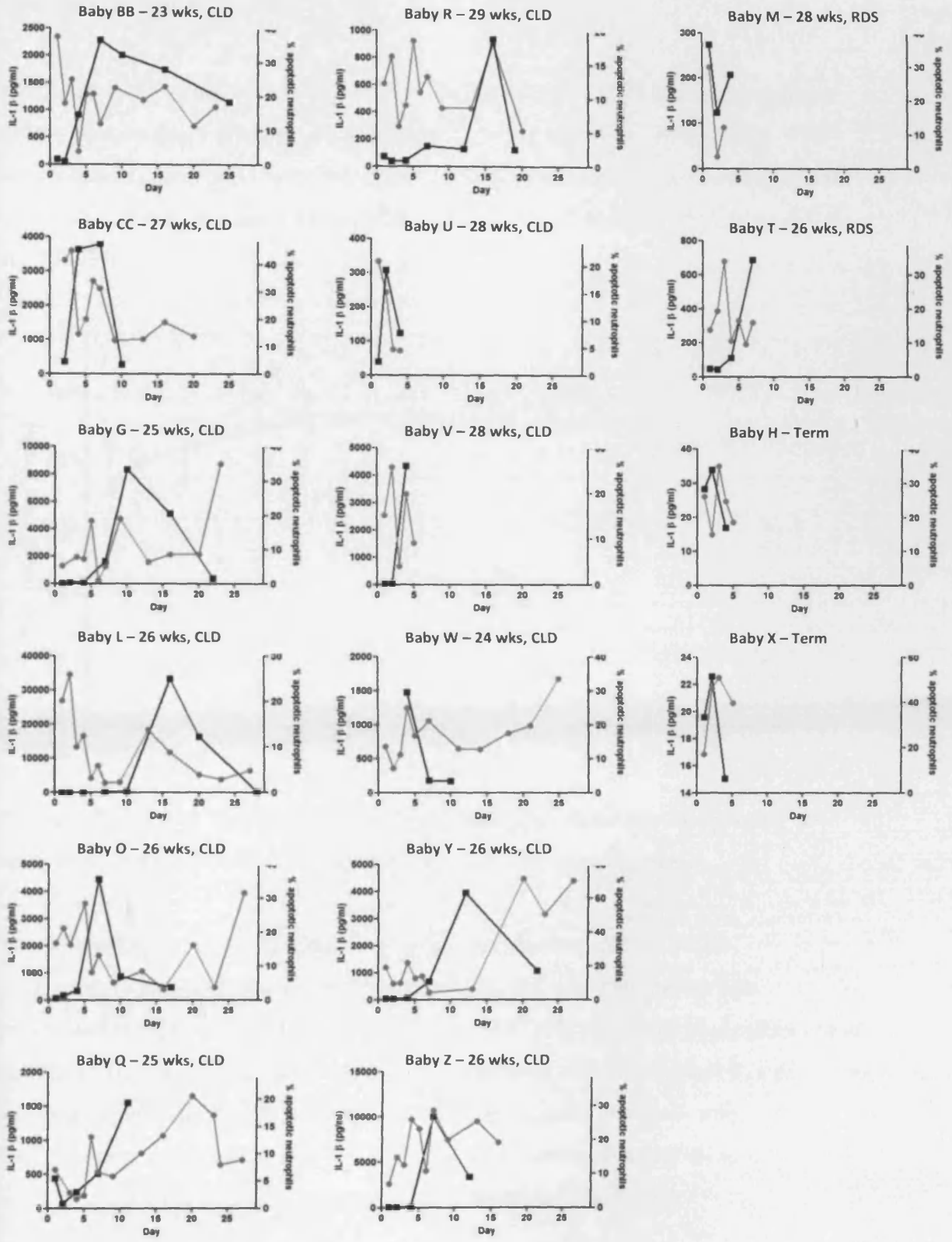


Figure 5.2 Graphs showing relationship between IL-1β (black line) and percentage of the neutrophil population which was apoptotic (grey line) for all babies with 3 or more results for each variable.

5.2.2 Interleukin-8 (IL-8)

There appear to be higher levels of IL-8 in both RDS and CLD infants compared to term babies on day 4 (Figure 5.3), although this is not statistically significant. IL-8 concentrations have previously been shown to be significantly higher in infants who develop CLD than in infants whose RDS resolves (Kotecha et al., 1995, Groneck et al., 1994).

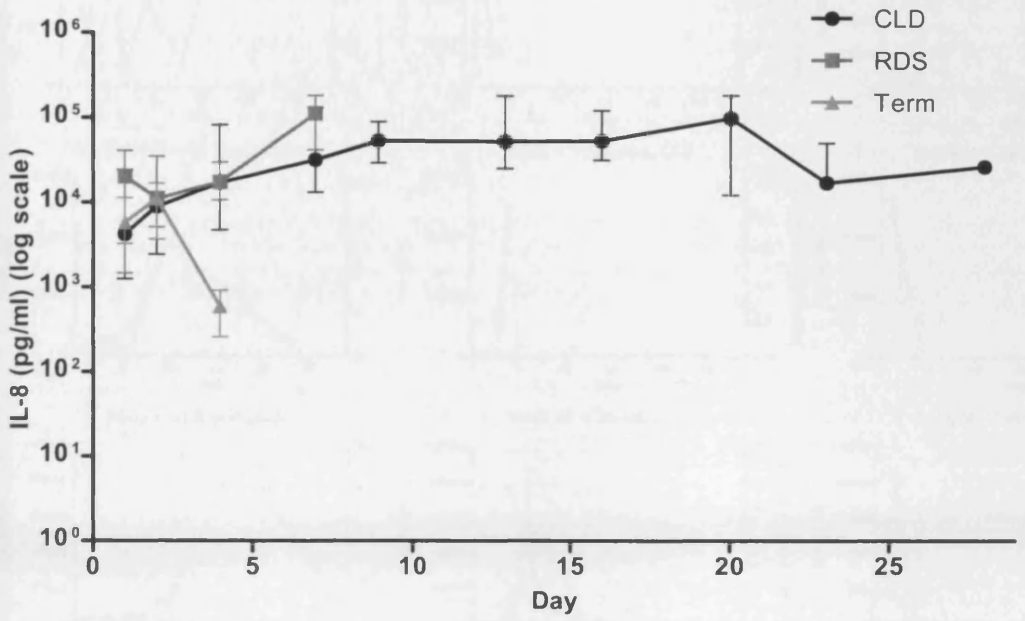


Figure 5.3 Graph of median IL-8 concentration (pg/ml, log scale) against time in term, RDS and CLD infants. Error bars represent interquartile ranges.

In published work, IL-8 was significantly elevated on the first day of life (immediately preceding the marked peak in neutrophil count) in babies who progressed to CLD in one study (Munshi et al., 1997) but in another study (Kotecha et al., 1995), IL-8 was elevated around day 10 coinciding with the neutrophil peak. From our data, in the majority of babies IL-8 and the neutrophil count track one another, peaking either in the same sample or the neutrophil count peaks shortly after the IL-8 peak, thus agreeing with previously published findings (Figure 5.4).

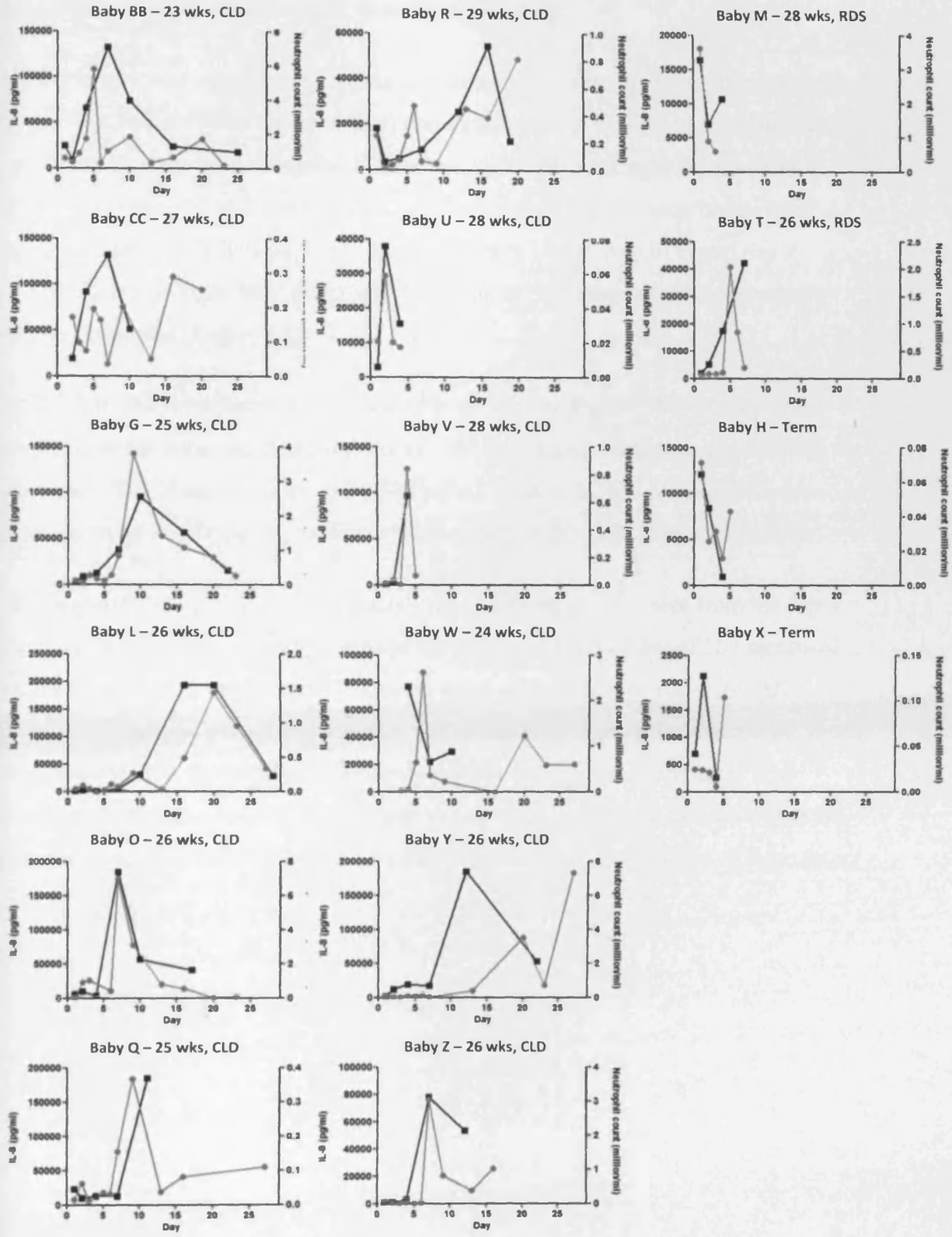


Figure 5.4 Graphs showing relationship between IL-8 (black line) and total neutrophil count (grey line) for all babies with 3 or more results for each variable.

5.2.3 Monocyte Chemoattractant Protein-1 (MCP-1)

MCP-1 levels were significantly higher in term babies compared to infants with RDS on day 1 (Kruskal-Wallis test, p=0.024) and in term babies compared to infants who developed CLD on day 2 (Kruskal-Wallis test, p=0.049). When the whole preterm group was compared with term infants, term infants had significantly higher MCP-1 levels than preterm babies on day 1 (Mann-Whitney U-test, p=0.011) and day 2 (p=0.018), but no significant difference was detected between any of the groups by the day 4 samples (Figure 5.5).

MCP-1 has also been found to be elevated in airway secretions where *Ureaplasma urealyticum* has been detected (Baier et al., 2001). No such relationship was found in our cohort (No *Ureaplasma* mean 20 140 pg/ml, median 31 880 pg/ml; *Ureaplasma* detected mean 35 870 pg/ml, median 35 110 pg/ml; p=0.53) (Figure 5.17 below).

Levels of MCP-1 measured, particularly in the CLD group of infants from the day 4 sample onwards, were very high (around 80 000 pg/ml) and 24 out of 103 measured values were on or exceeding the maximum range of the standard curve (60 000 pg/ml). For analysis purposes these data were included however, for this reason, mean or median MCP-1 levels may be falsely low in the CLD group as the assay was unable to measure accurately at such high values. Non-parametric statistical tests are rank tests and thus may still be a reasonably robust method for analysis of these data.

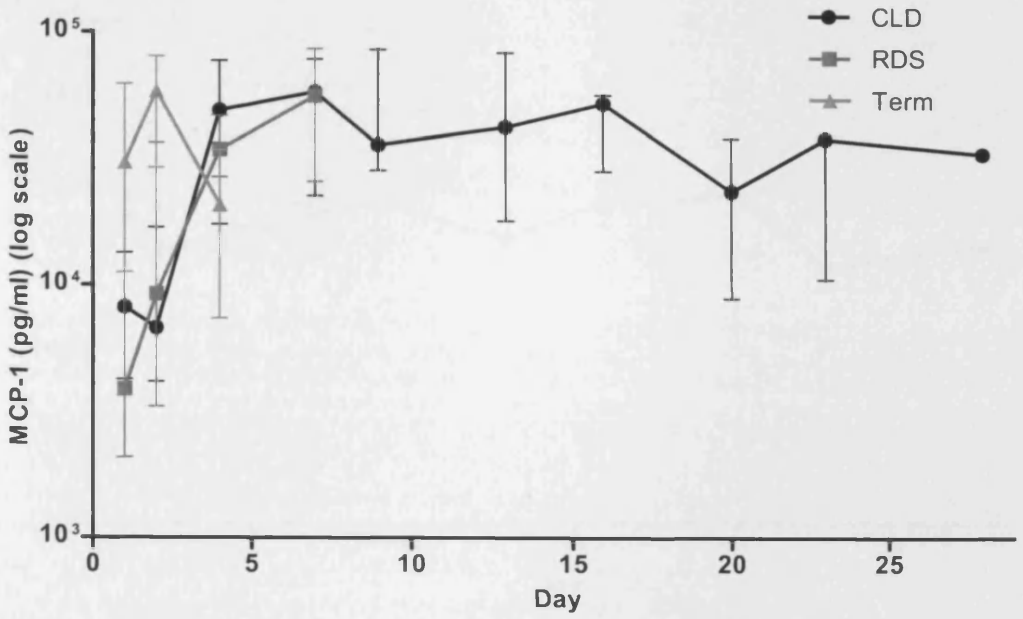


Figure 5.5 Graph of median MCP-1 concentration against time in term, RDS and CLD infants. Error bars represent interquartile ranges.

5.2.4 Interleukin-6 (IL-6)

IL-6 has previously been found to be dramatically elevated in preterm infants, particularly in infants developing CLD early in their ventilator course (Bagchi et al., 1994) and may be predictive for the development of CLD (Jonsson et al., 1997).

Levels of IL-6 measured in all 3 groups of infants in this study were very similar. Possible reasons for the difference between my results and previously published data may be due to methodological differences (neither previous study used CBA methodology on BAL samples) or because both previous studies were performed early on in the "surfactant era" and even the most recent of these two papers, had only two thirds antenatal steroid coverage and used surfactant as "rescue" therapy only in infants requiring more than 60% inspired oxygen which implies a slightly different study population from the one in our study.

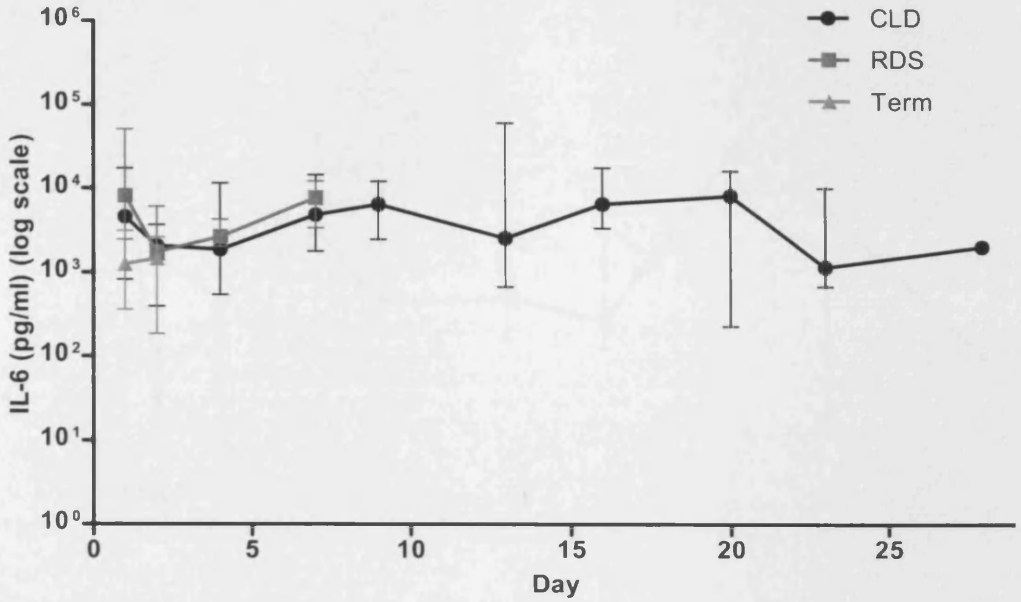


Figure 5.6 Graph of median IL-6 concentration against time in term, RDS and CLD infants. Error bars represent interquartile ranges.

5.2.5 Interleukin-10 (IL-10)

There was a large number of samples (44/108) with undetectable IL-10. There were only 4 samples with detectable IL-10 from a total of 3 babies in the term group so statistical comparison with the term group was not possible, however it appears that levels of IL-10 were markedly lower in term babies than in either of the preterm groups.

In contrast to this observation, IL-10 has been reported to rise rapidly over the first five days of life (Beresford and Shaw, 2002) but to be significantly lower or even undetectable in preterm infants (Jones et al., 1996, Whicher and Evans, 1990) and in infants developing CLD (Beresford and Shaw, 2002, McColm et al., 2000). Only 40.7% of our samples yielded results for IL-10 and the sensitivity of the assay was around 300 pg/ml. Lower IL-10 levels in term infants (as seen in median values shown in Figure 5.7) are extrapolated and should be interpreted with caution.

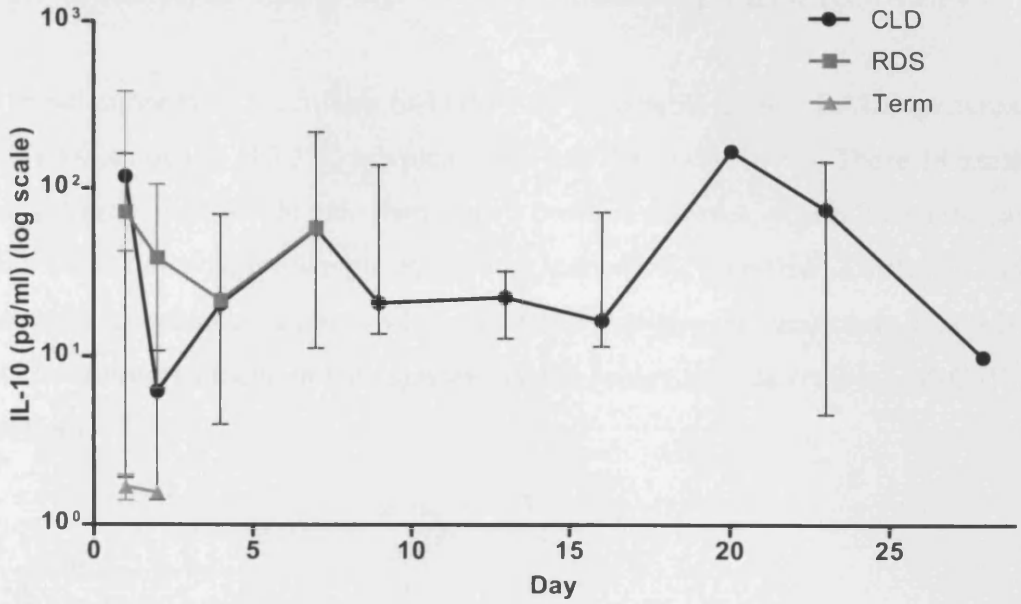


Figure 5.7 Graph of median IL-10 concentration against time in term, RDS and CLD infants. Error bars represent interquartile ranges.

5.2.6 Granulocyte - Colony Stimulating Factor (G-CSF)

There were no significant differences in G-CSF among the groups. Further discussed in 5.2.7, below.

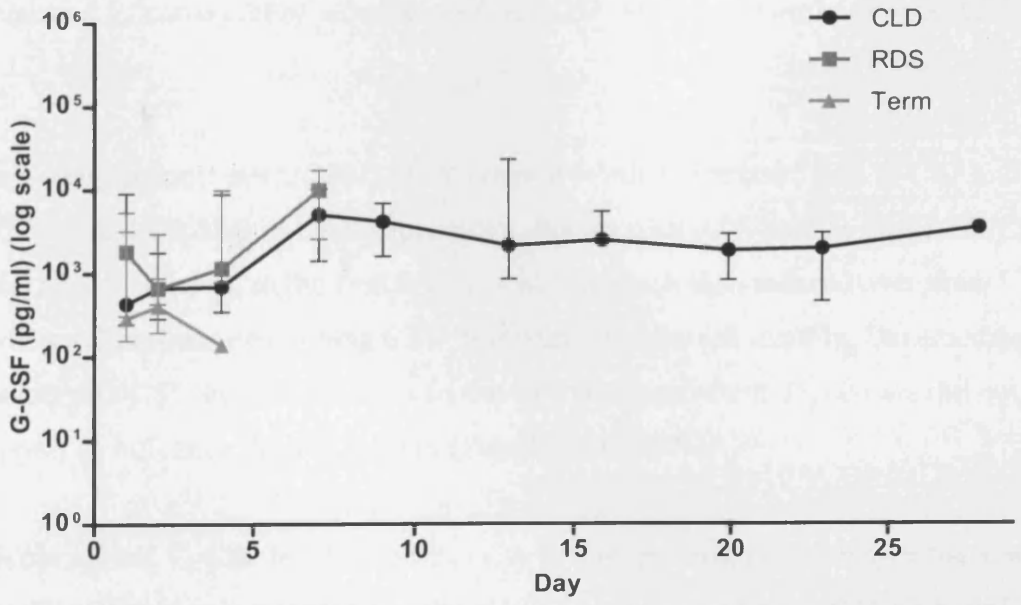


Figure 5.8 Graph of median G-CSF concentration against time in term, RDS and CLD infants. Error bars represent interquartile ranges.

5.2.7 Granulocyte Macrophage – Colony Stimulating Factor (GM-CSF)

A small minority of the infants had GM-CSF detectable in their BAL supernatant. Only 14 out of 104 (13.5%) samples tested had detectable levels. These 14 samples came from 8 individuals (one baby with 3 positive samples, 4 with 2 positive samples and 3 with one positive sample each). Four samples (2 each from 2 different babies) had only just detectable levels which fell just below the standard curve for GM-CSF but have been included in the analysis. All the babies with detectable GM-CSF were preterm.

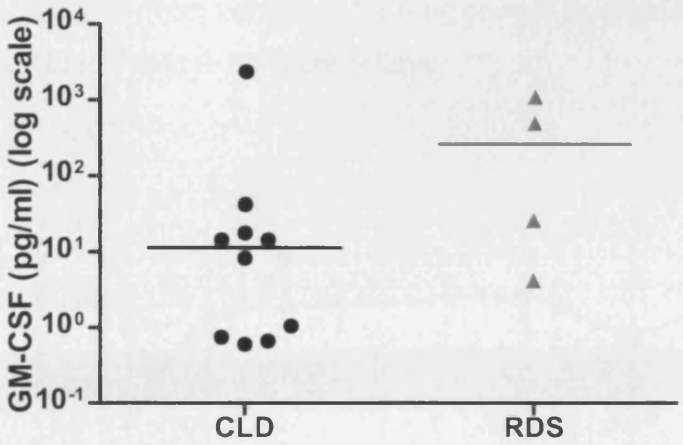


Figure 5.9 Scatterplot of all measured GM-CSF concentrations in term, RDS and CLD infants.

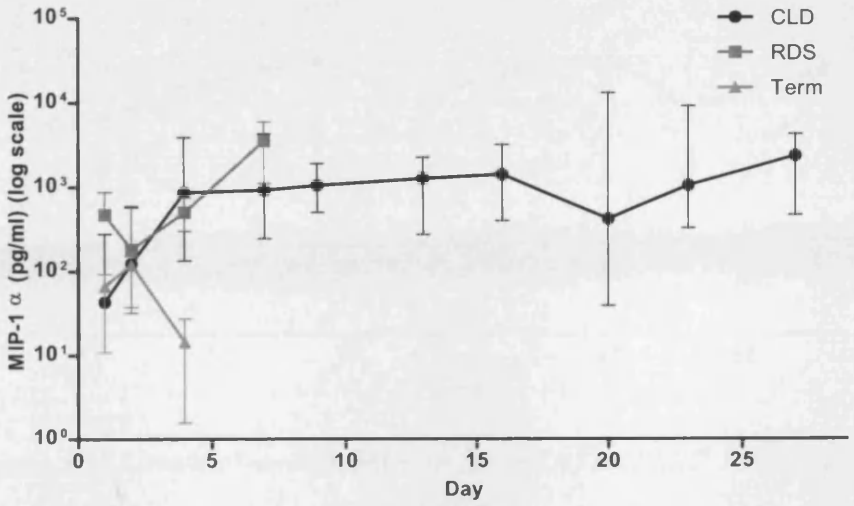
In a study (Papoff et al., 2001) of 18 preterm ventilated infants, both G-CSF and GM-CSF were detectable in BAL supernatant. Babies with RDS had the highest G-CSF and GM-CSF levels in the first few days of life which then reduced over time, whereas in infants developing CLD, these levels increased steadily. Dexamethasone reduced G-CSF and GM-CSF levels but LPS increased them. Hyperoxia did not appear to influence these cytokines (Papoff et al., 2001).

In our cohort, G-CSF levels appear to rise in both preterm groups before those with RDS are extubated and those developing CLD appear to plateau. GM-CSF was detected in too few infants for a trend to be observed.

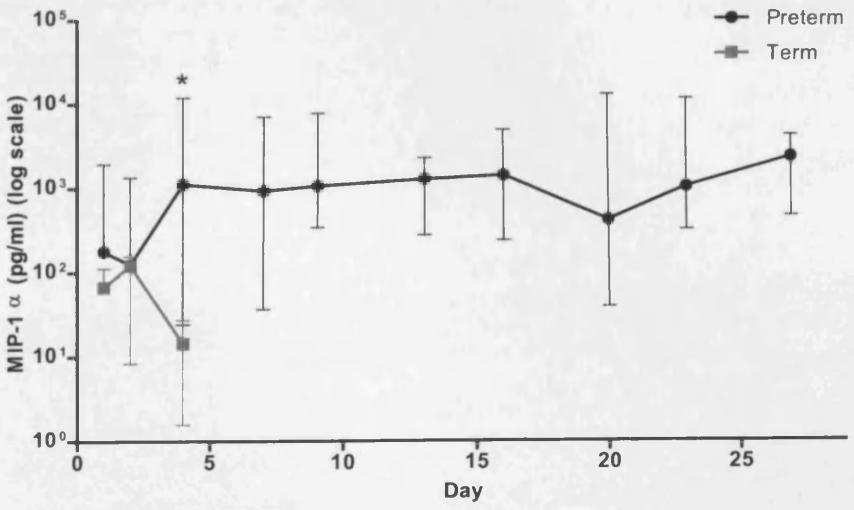
5.2.8 Macrophage inflammatory proteins (MIP-1α and MIP-1β)

Both these cytokines are present at significantly higher levels in preterm infants than term babies on the fourth day of life (Mann-Whitney U-test, MIP-1α p=0.041; MIP-1β p=0.028) (Figure 5.10 B and D). Levels of both cytokines rise steadily in infants developing CLD, throughout their ventilated period.

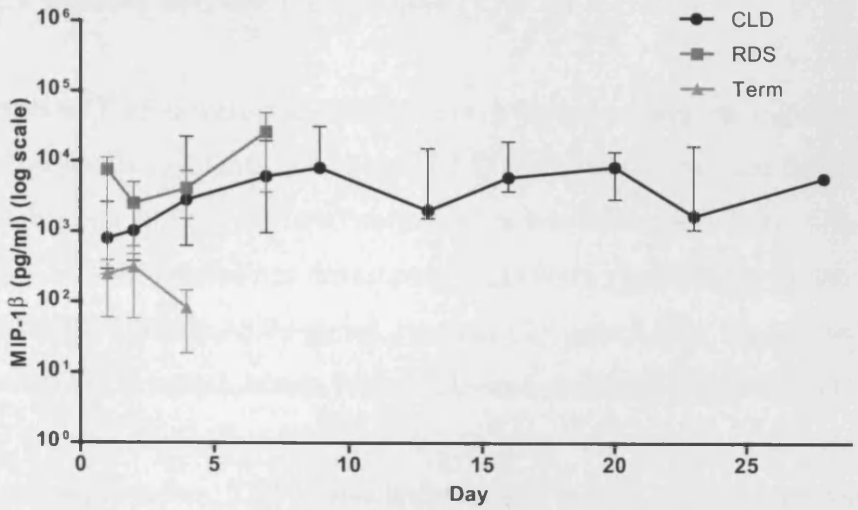
MIP-1α and MIP-1β were expected to be elevated in preterm infants, particularly those developing CLD (Baier et al., 2004) and those exposed to hyperoxic conditions (D'Angio et al., 1998). Our data confirm statistically significantly higher levels of both these cytokines on day 4 of life but unfortunately there were no term or RDS infants who were ventilated for long enough to enable comparisons to be made later in the clinical course for these infants.



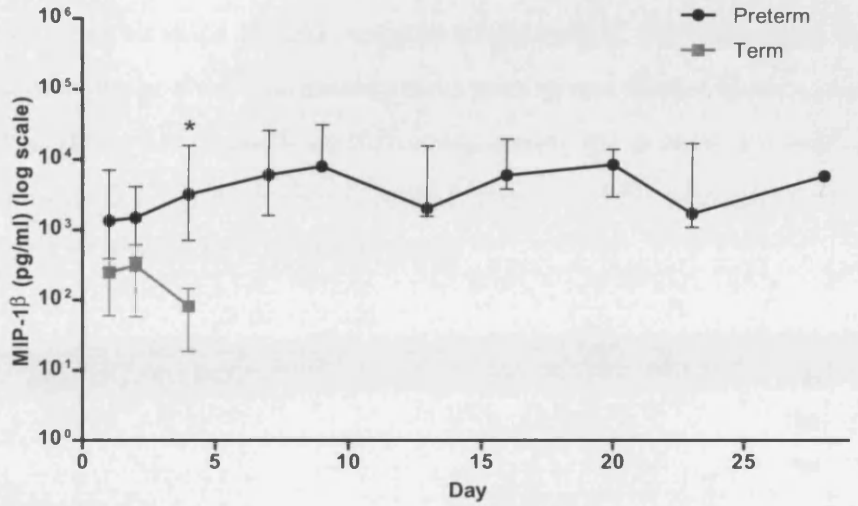
A



B



C



D

*Figure 5.10 Graph of median MIP-1α (A and B) and MIP-1β (C and D) concentrations according to (A and C) diagnosis and (B and D) gestation against time. Error bars represent interquartile ranges. In (B) *p=0.041 and in (D) *p=0.028.*

5.2.9 Tumour necrosis factor alpha (TNF-α)

Levels of TNF-α were not significantly different among the 3 groups of infants but TNF-α levels in infants developing CLD rose sharply over the first few days of life and throughout the ventilated period, such that by the end of the first week of life, levels of TNF-α in babies developing CLD were significantly higher than on day 1 (CLD Day 1, mean 45.99 pg/ml, median 7.29 pg/ml; Day 7 mean 361.70 pg/ml, median 81.32 pg/ml; Mann-Whitney U-test, p=0.016) (Figure 5.11).

In previous studies, TNF-α was undetectable in term controls and low in infants with RDS but peaked at day 14 in babies who progressed to CLD (Bagchi et al., 1994). TNF-α was elevated in BAL samples taken early in the ventilatory course of preterm infants with the worst pulmonary outcomes in one study (Mahieu et al., 2005). Our cohort showed no significant difference among the groups, although peak TNF-α levels (see 5.2.12) were significantly higher in babies who developed CLD and peaked on around day 20 in this group.

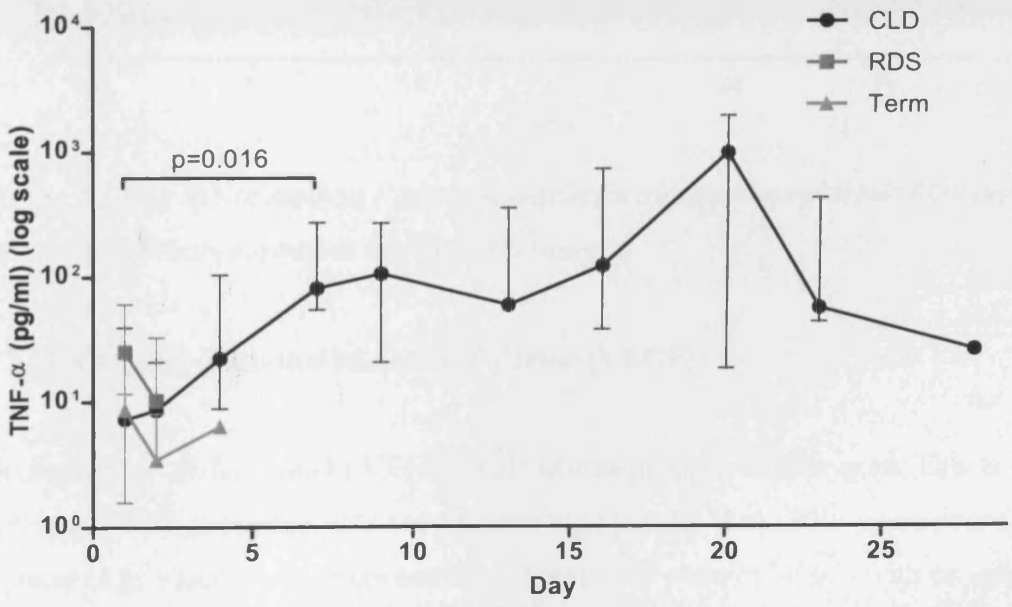


Figure 5.11 Graph of median TNF-α concentration (pg/ml, log scale) against time in term, RDS and CLD infants. Error bars represent interquartile ranges. p=0.016 for day 1 vs day 7 levels in infants developing CLD.

5.2.10 Fas ligand (FasL)

No significant differences could be observed between levels of FasL among the groups. FasL has been studied in neonatal and paediatric blood samples (Ennaciri et al., 2006, Hanna et al., 2005, Sarandakou et al., 2003), but not in lung lavage samples in this age group. No significant changes in FasL were noted and FasL remained relatively constant after the end of the first week of life in the CLD group.

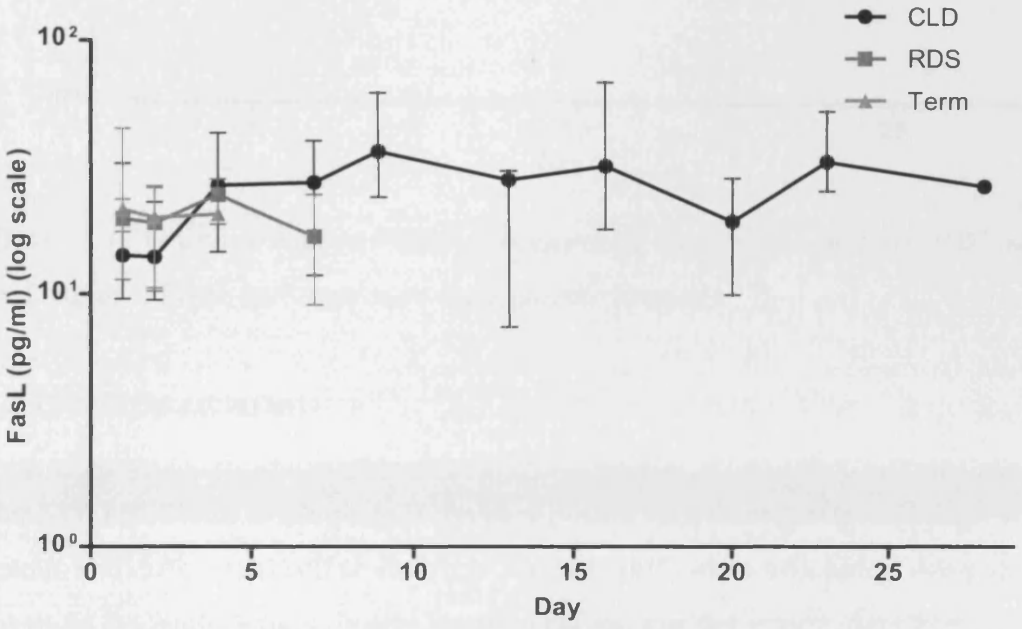


Figure 5.12 Graph of median FasL concentration against time in term, RDS and CLD infants. Error bars represent interquartile ranges.

5.2.11 Vascular Endothelial Growth Factor (VEGF)

No significant differences in VEGF levels among groups could be seen. This is in agreement with published data from Currie *et al* (Currie et al., 2001) who found no difference in VEGF levels between term infants and preterm infants with or without CLD, but in contrast to the reduced levels found in infants developing CLD by Lassus *et al.* (Lassus et al., 1999). It has been postulated that either increased or decreased VEGF levels can mediate lung injury (Voelkel et al., 2006).

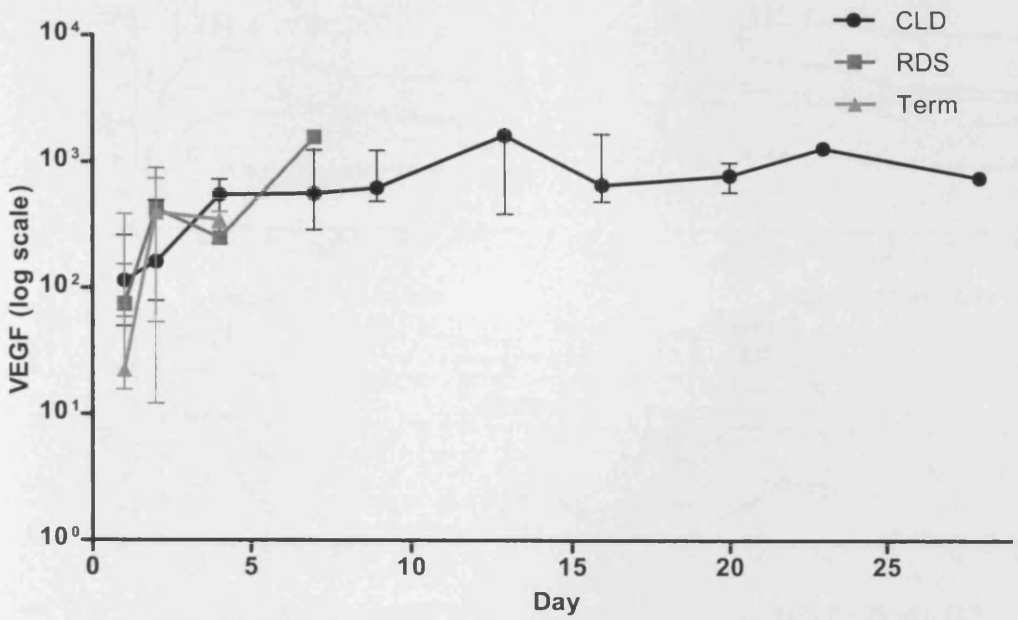
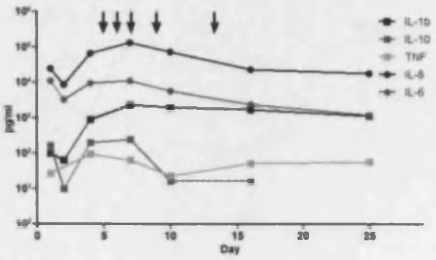


Figure 5.13 Graph of median VEGF concentration against time in term, RDS and CLD infants. Error bars represent interquartile ranges.

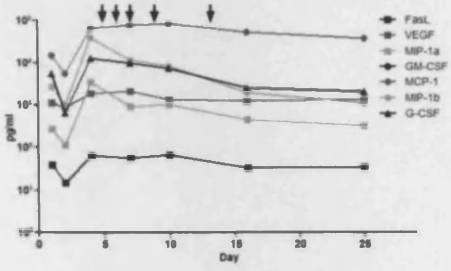
5.2.12 Individual infants

Just as it is difficult to adequately compare pooled data from groups of infants for cell counts and differential cell counts, it is similarly difficult to adequately compare levels of cytokines longitudinally between babies. For this reason, data from individual babies was reviewed to identify patterns or relationships between various cytokines and clinical diagnosis or infection (Figure 5.14).

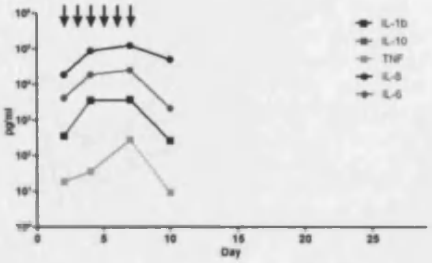
Baby BB – 23 wks, CLD



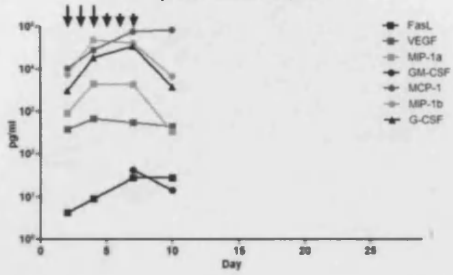
Baby BB – 23 wks, CLD



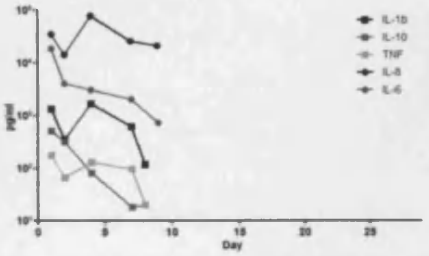
Baby CC – 27 wks, CLD



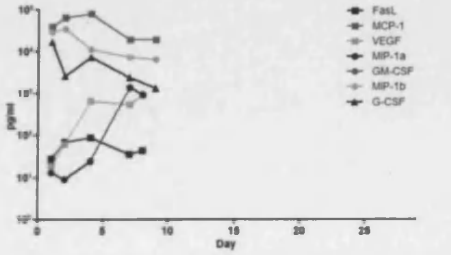
Baby CC – 27 wks, CLD



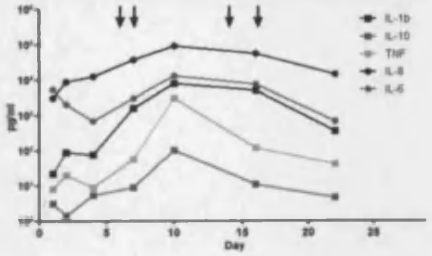
Baby B – 25 wks, CLD



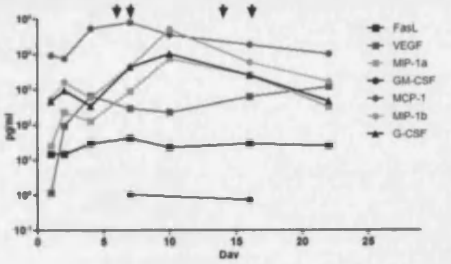
Baby B – 25 wks, CLD



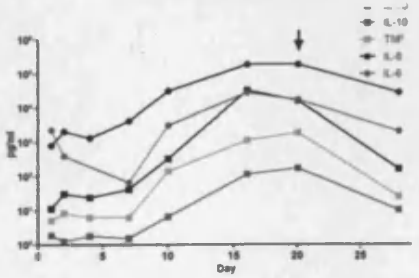
Baby G – 25 wks, CLD



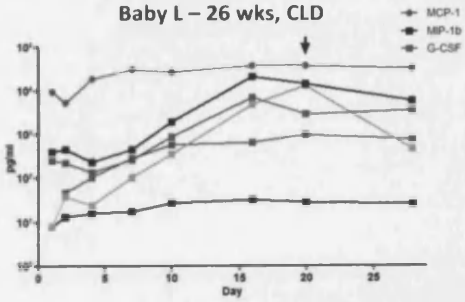
Baby G – 25 wks, CLD



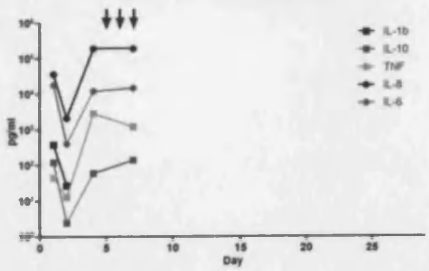
Baby L – 26 wks, CLD



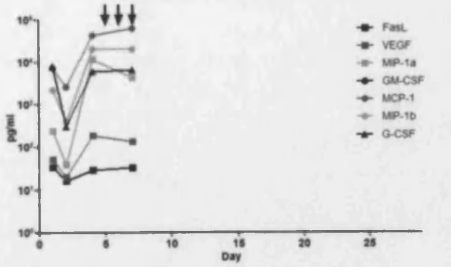
Baby L – 26 wks, CLD



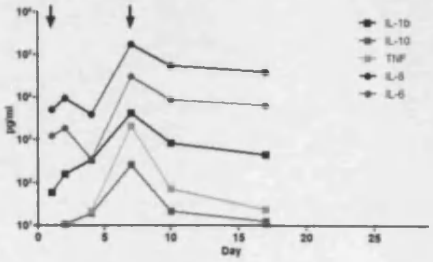
Baby N – 25 wks, CLD



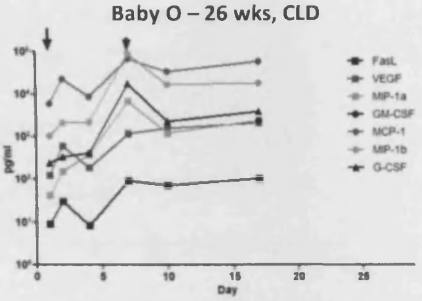
Baby N – 25 wks, CLD



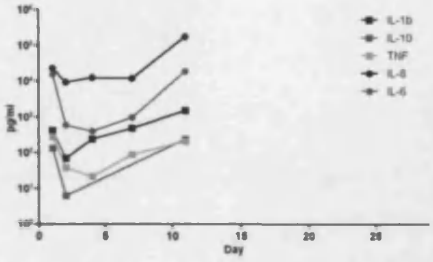
Baby O – 26 wks, CLD



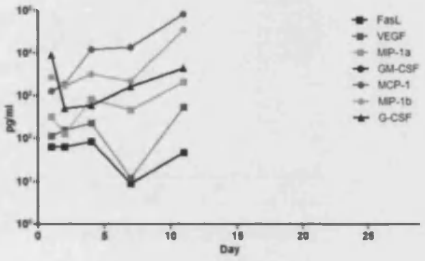
Baby O – 26 wks, CLD



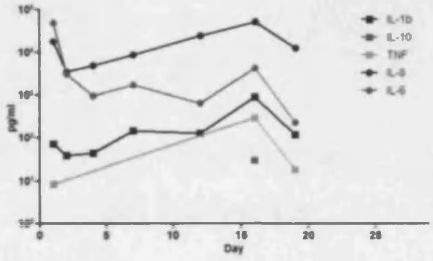
Baby Q – 25 wks, CLD



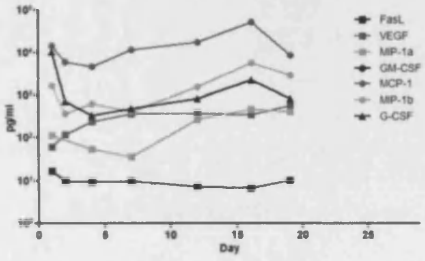
Baby Q – 25 wks, CLD



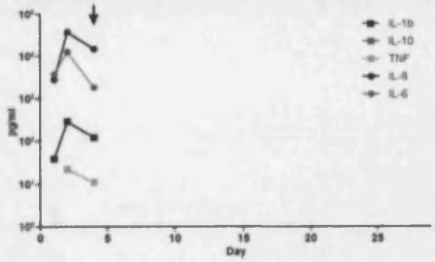
Baby R – 29 wks, CLD



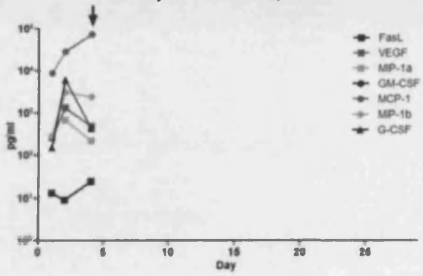
Baby R – 29 wks, CLD



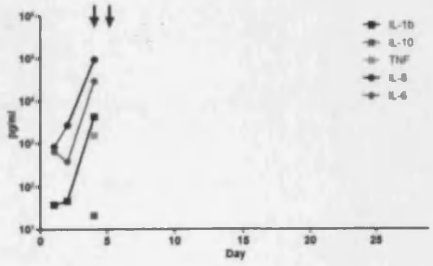
Baby U – 28 wks, CLD



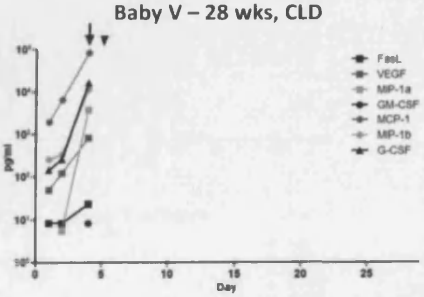
Baby U – 28 wks, CLD



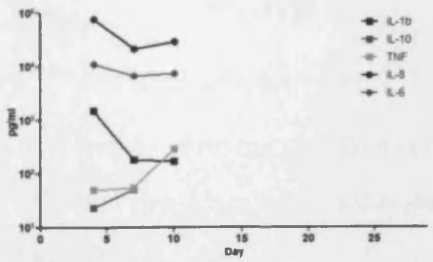
Baby V – 28 wks, CLD



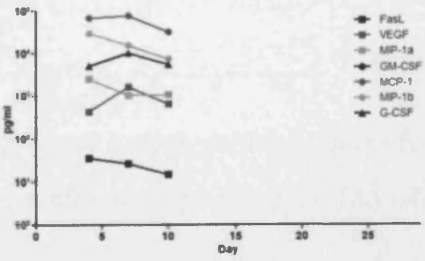
Baby V – 28 wks, CLD



Baby W – 24 wks, CLD



Baby W – 24 wks, CLD



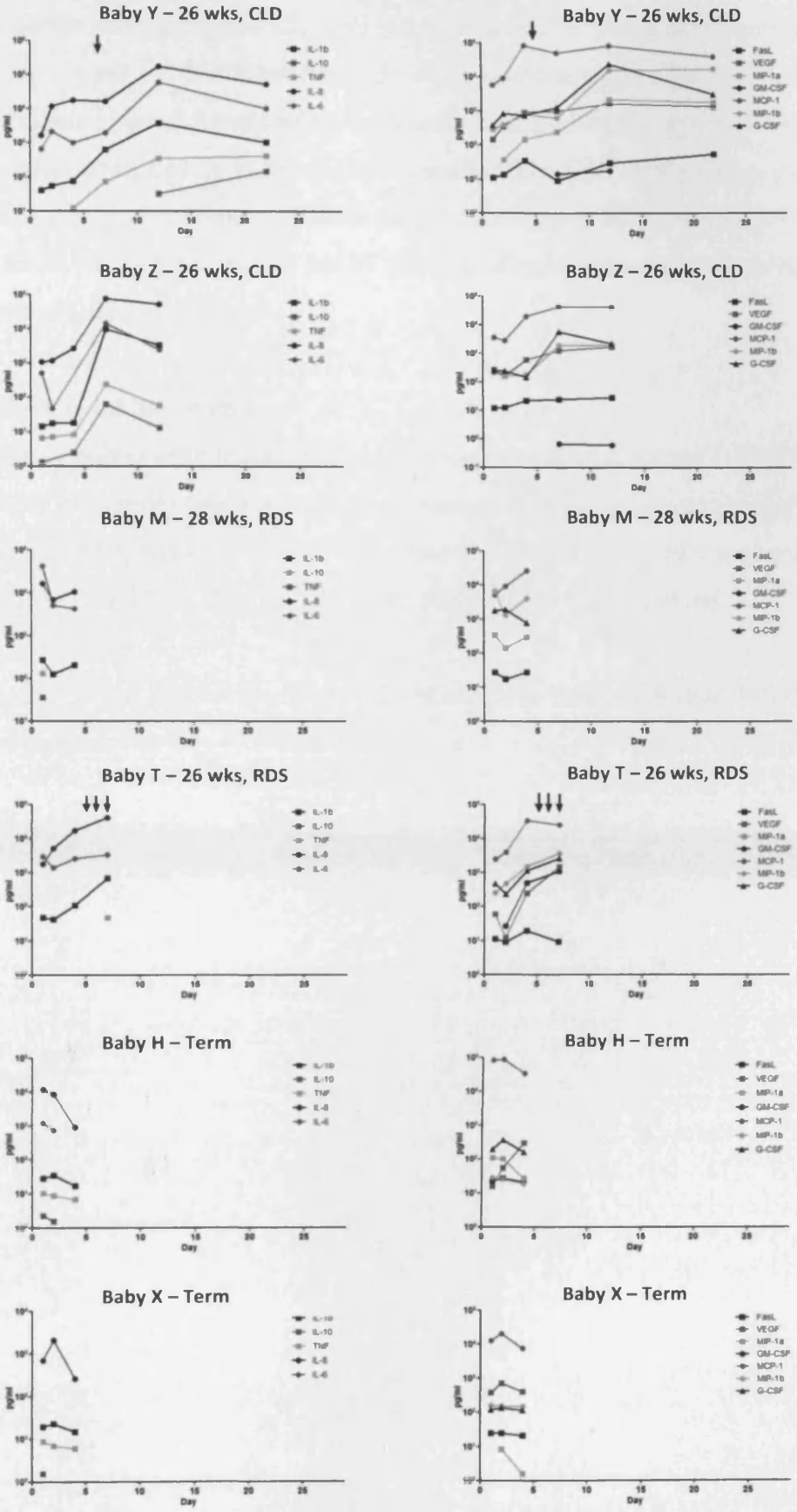


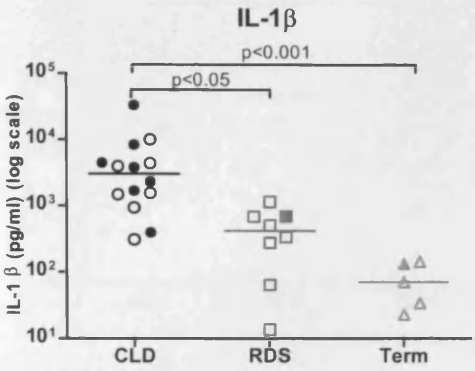
Figure 5.14 Graphs showing cytokine levels (pg/ml) for all babies with 3 or more BAL samples in which cytokines were measured. Samples which were positive for 16S rRNA are indicated by arrows.

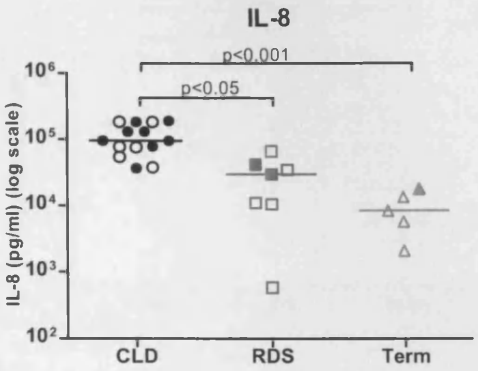
In most babies who developed CLD, an increase in all the cytokines was observed over the first week of life. Thereafter high levels were maintained for almost the entire ventilated period. Levels of all the interleukins studied, along with TNF, had begun to decline by day 28 or extubation in most infants (left hand panel in Figure 5.14), whereas levels of other cytokines remained elevated. In the two term infants (H and X) all levels are beginning to fall by the time of extubation and well within the first week of life.

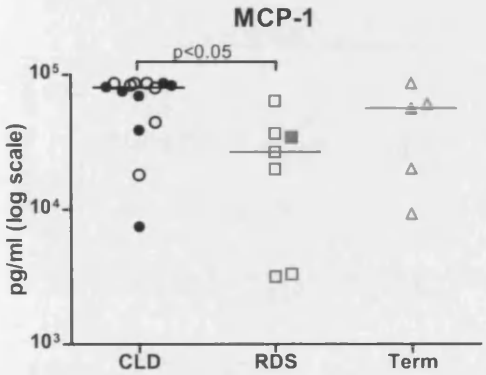
5.2.13 Peak cytokine levels

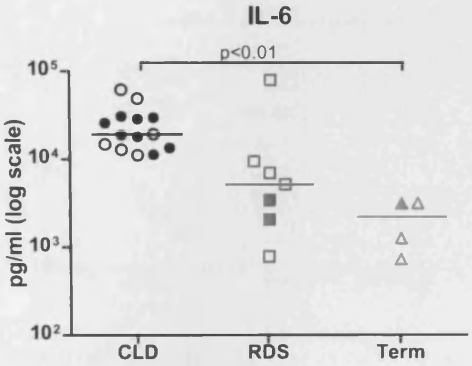
Significantly higher peak levels of all the cytokines measured, except GM-CSF where the number of samples was too small, were present in preterm infants compared to their term counterparts. Infants who developed CLD had significantly higher levels of IL-1β, MIP-1α and TNF (Figure 5.15) than those whose RDS resolved.

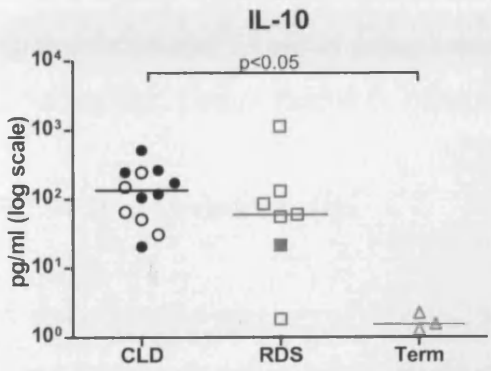
The majority of the highest cytokine peaks occurred in infants who had 16S rRNA detected (see 5.2.14).

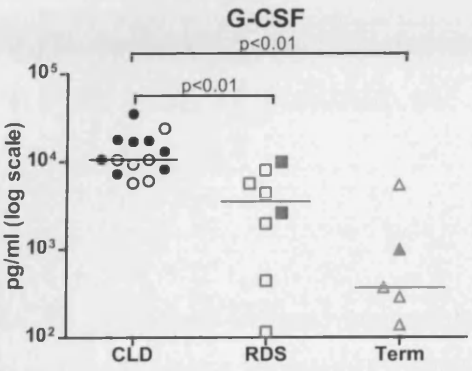

IL-1β
p<0.001
p<0.05
IL-1 β (pg/ml) (log scale)
CLD
RDS
Term

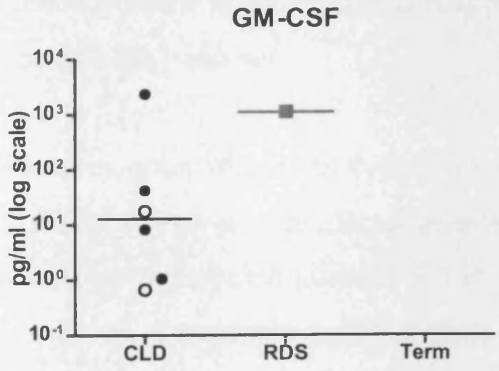

IL-8
p<0.001
p<0.05
IL-8 (pg/ml) (log scale)
CLD
RDS
Term

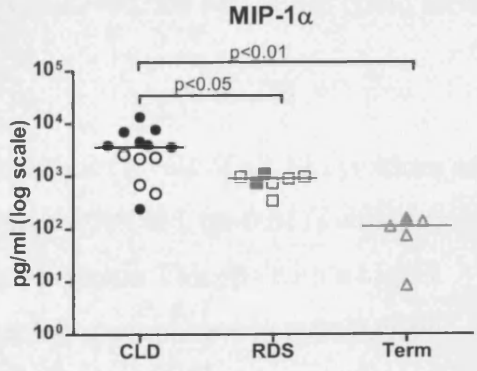

MCP-1
p<0.05
pg/ml (log scale)
CLD
RDS
Term


IL-6
p<0.01
pg/ml (log scale)
CLD
RDS
Term


IL-10
p<0.05
pg/ml (log scale)
CLD
RDS
Term


G-CSF
p<0.01
p<0.01
pg/ml (log scale)
CLD
RDS
Term


GM-CSF
pg/ml (log scale)
CLD
RDS
Term


MIP-1α
p<0.01
p<0.05
pg/ml (log scale)
CLD
RDS
Term

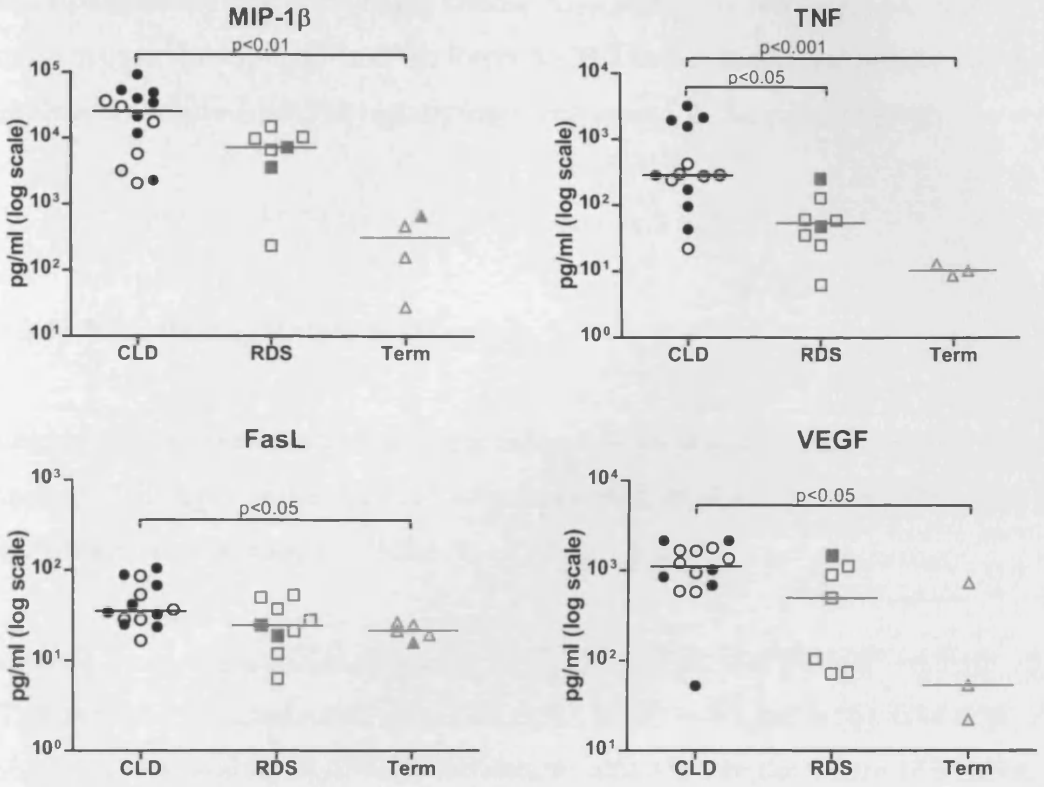


Figure 5.15 Scatterplots of peak cytokine concentrations (pg/ml, log scale) in term, RDS and CLD infants. Horizontal lines represent medians. Filled markers indicate infants in whom 16S rRNA genes were detected in that sample or an immediately adjacent one. Empty markers reflect babies in whom infection was not detected.

5.2.14 Day 1 cytokine data

A more detailed analysis of cytokine levels on the first day of life may be useful as these levels may reflect antenatal conditions, such as infection in utero. It could be clinically useful if cytokine values in BAL fluid on the first day of life could serve as a prognostic indicator.

However, comparing term against preterm infants for levels of all 12 cytokines on day 1 of life only gives a significant difference only for MCP-1 (p=0.011) with term infants having much higher MCP-1 levels than preterm. This fits with a higher proportion of macrophages being mature alveolar macrophages in term babies (although absolute numbers of macrophages and mature macrophages are similar) (section 3.3.4.4). More MCP-1 in the term infants on day 1 could allow resolution of

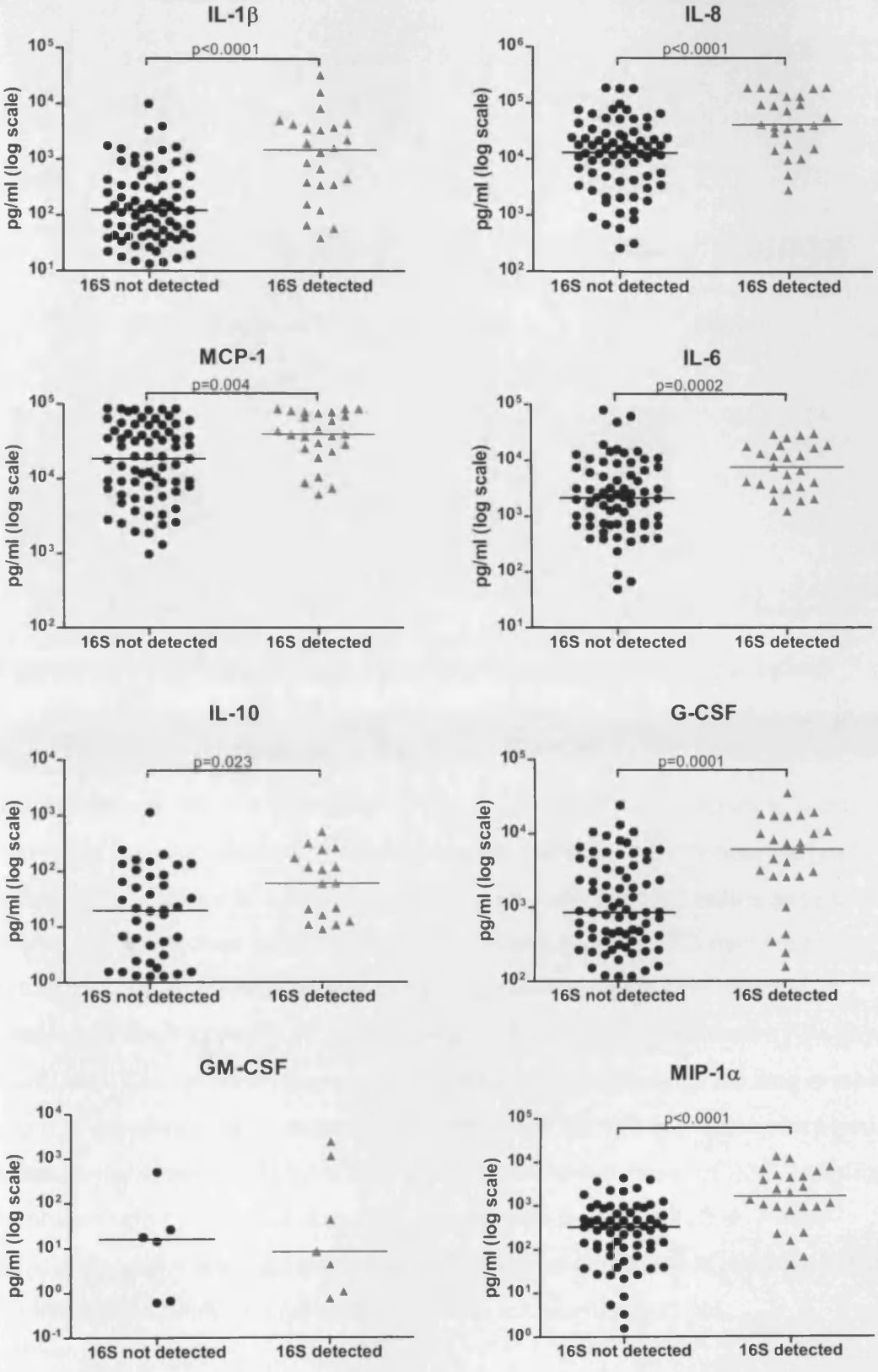
the inflammatory process to begin almost immediately by promoting recruitment of more mature macrophages and the lower MCP-1 levels in preterm infants may help to explain the relative delay in macrophage recruitment in the preterm group.

No child had detectable levels of GM-CSF on day 1 of life.

5.2.15 BAL fluid cytokines in infection

Once again the role of infection in the inflammatory process in the neonatal lung was studied in the light of the cytokine values obtained. PCR for the 16S rRNA gene was undertaken as described in 2.1.5.2.

Overall, there was significantly more IL-1β, IL-8, IL-6, G-CSF, MIP-1α, MIP-1β and TNF detected in samples that were 16S rRNA positive (Figure 5.16). GM-CSF, FasL and VEGF showed no significant differences between samples where 16S rRNA genes were detected and samples in which they were not found (Figure 5.16).


IL-1β
p<0.0001
pg/ml (log scale)
16S not detected
16S detected
IL-8
p<0.0001
pg/ml (log scale)
16S not detected
16S detected
MCP-1
p=0.004
pg/ml (log scale)
16S not detected
16S detected
IL-6
p=0.0002
pg/ml (log scale)
16S not detected
16S detected
IL-10
p=0.023
pg/ml (log scale)
16S not detected
16S detected
G-CSF
p=0.0001
pg/ml (log scale)
16S not detected
16S detected
GM-CSF
pg/ml (log scale)
16S not detected
16S detected
MIP-1α
p<0.0001
pg/ml (log scale)
16S not detected
16S detected

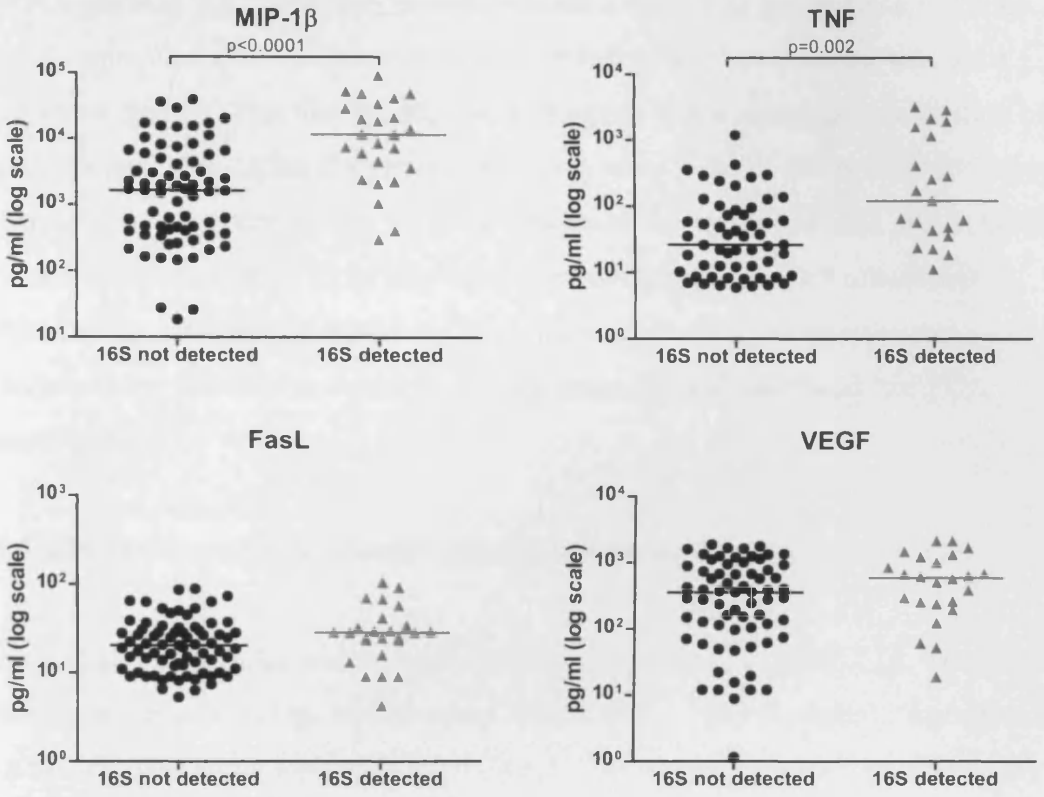


Figure *5.16 Scatterplots showing levels of inflammatory mediators in infants according to whether 16S rRNA genes were or were not detected in BAL samples.*

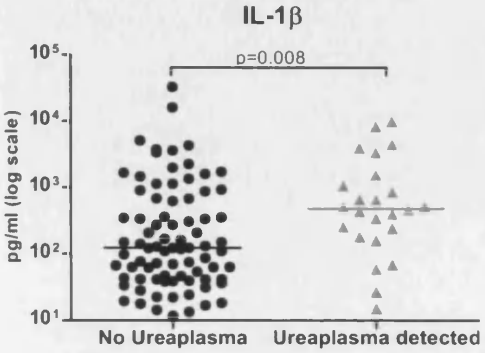
This mixture of pro- and anti-inflammatory cytokines in most samples may reflect that while there are attempts at resolution of the inflammatory process, the pro-inflammatory actions of microbes in the lung are superimposed on this picture. The timing of the detection of 16S rRNA genes in BAL samples PCR may lag behind the actual appearance of the organism in the lung (BAL samples were obtained at intervals of approximately 24 hours during the first week and intervals of 2-4 days thereafter). The cytokine response to the presence of a microbe in the lung is rapid and it is impossible for us to determine exactly how far into the course of a possible infection our observations have been made. The snapshot nature of BAL sampling, combined with the fact that only BAL supernatants from days 1, 2, 4, 7 were subjected to CBA analysis, may mean that important alterations in cytokine balance were missed at times or days when there were no samples analysed.

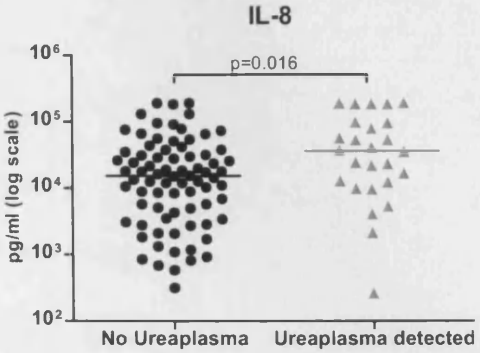
When data from individual babies were reviewed (Figure 5.14) there did not appear to be a consistent relationship between the level of cytokines and the detection of 16S

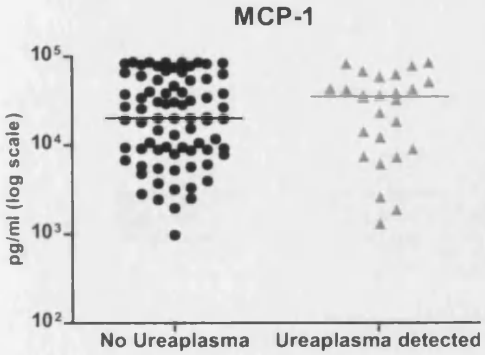
rRNA genes in BAL samples. In some infants, a sharp rise in almost all cytokine levels coincided with the detection of 16S rRNA genes, however this was not a universal finding. This may be because 16S rRNA genes represent colonisation of the endotracheal tube, rather than true infection in some infants, or that 16S rRNA genes were detected in some infants before an infection became established and a cytokine response was mounted. There may have been other factors which altered this relationship, such as administration of antibiotics, some of which have anti-inflammatory effects e.g. erythromycin, or drugs like indomethacin (for PDA treatment).

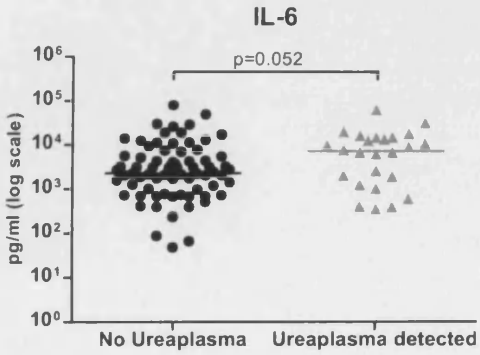
5.2.16 Cytokines in *Ureaplasma* colonisation or infection

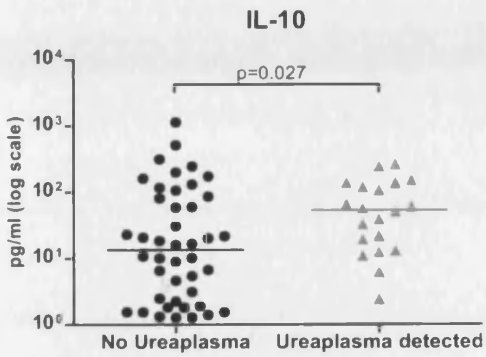
Almost all of the inflammatory mediators tested, apart from MCP-1 (in which the result may be affected by around 1 in 4 values being above or close to the upper limit of the standard curve for the assay), GM-CSF (in which there were too few results to make adequate comparisons), MIP-1α and VEGF, show significantly higher levels of the inflammatory mediators in samples in which *Ureaplasma* was detectable in the BAL fluid. IL-6 does not quite reach significance.

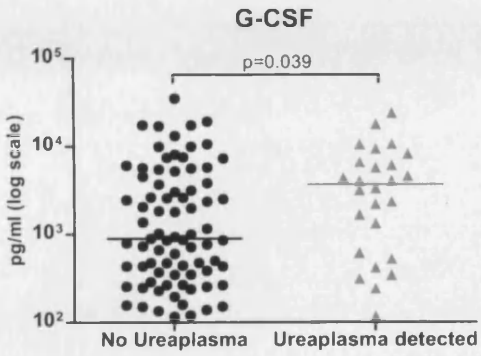

IL-1β
p=0.008
pg/ml (log scale)
No Ureaplasma
Ureaplasma detected

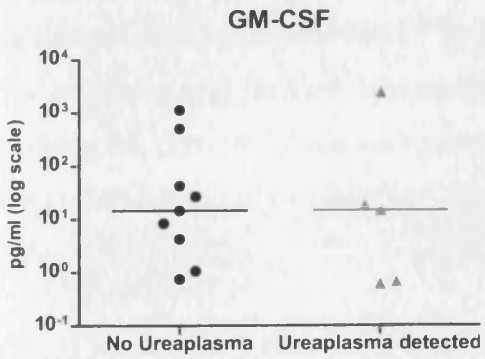

IL-8
p=0.016
pg/ml (log scale)
No Ureaplasma
Ureaplasma detected

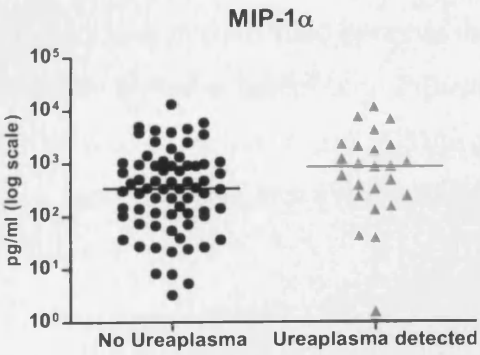

MCP-1
pg/ml (log scale)
No Ureaplasma
Ureaplasma detected


IL-6
p=0.052
pg/ml (log scale)
No Ureaplasma
Ureaplasma detected


IL-10
p=0.027
pg/ml (log scale)
No Ureaplasma
Ureaplasma detected


G-CSF
p=0.039
pg/ml (log scale)
No Ureaplasma
Ureaplasma detected


GM-CSF
pg/ml (log scale)
No Ureaplasma
Ureaplasma detected


MIP-1α
pg/ml (log scale)
No Ureaplasma
Ureaplasma detected

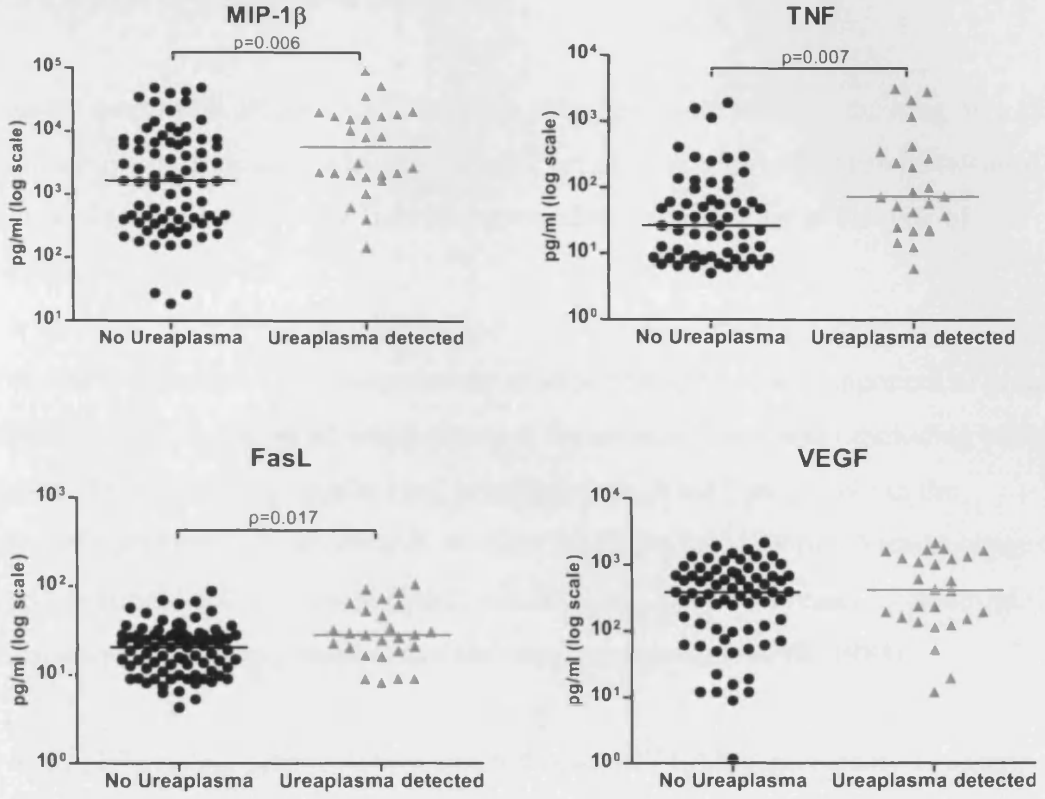


Figure 5.17 Scatterplots showing levels of measured inflammatory mediators in neonatal BAL samples according to whether Ureaplasma was detected in the sample. Horizontal lines represent medians. Significant p values shown.

5.2.17 Cytokines and mode of delivery

Comparing babies born by elective Caesarean section where the mother was not in labour with babies born as a result of spontaneous preterm labour, in whom antenatal infection could not be ruled out as a cause for preterm labour, there were no significant differences in levels of the selected cytokines in BAL fluid between the two groups, except for FasL which was significantly higher in babies born following spontaneous preterm labour compared to those delivered electively (p=0.028). Again, this is difficult to fully explain and there may be more than one factor responsible for this.

5.3 Elastase in BAL supernatants.

Human neutrophil elastase is the most abundant protease found in the lung. It is stored in intra-cytoplasmic azurophilic neutrophil granules from where it can be released into a phagocytic vacuole or into the surrounding environment at the time of neutrophil activation.

Elastase is capable of digesting elastin, an important structural component of lung tissue, as well as almost all components of the extracellular matrix including collagen types I-IV, fibronectin, laminin and proteoglycans. It may play a role in the physiological turnover of connective tissue when produced by tissue macrophages and certain stromal cells, however hydrolysis of matrix macromolecules by neutrophil elastase most probably represents a pathological process (Janoff, 1983).

Neutrophil elastase activity was measured in all 207 BAL supernatants as described in section 2.1.7.1.

Elastase activity was detected in 50/207 (24.2%) BAL samples and in a total of 19 of the 32 infants studied. In 13/15 (86.7%) infants who developed CLD there was elastase detected in at least one lavage sample, which was significantly more than 4/11 (36.4%) in preterm infants whose RDS resolved (Chi square test, p=0.0077). When all preterm infants are compared to term infants, where only 1/5 had free elastase activity in their BAL fluid, this was not significantly different (p=0.0596), probably on account of the small numbers of infants in the term control group.

	Elastase detected (%)	No elastase detected (%)	TOTALS
CLD	13 (86.7)	2 (13.3)	15
RDS	4 (36.4)	7 (63.6)	11
Term	1 (20)	4 (80)	5
Died	1 (100)	0 (0)	1
TOTALS	19 (59.4)	13 (40.6)	32

Table 5.1 Table showing numbers of infants with and without elastase activity detected in at least one BAL sample.

Peak elastase activity was also significantly greater in infants who went on to develop CLD compared to both infants with resolved RDS (Mann-Witney U-test, p=0.0131) and term controls (p=0.028), as demonstrated in figure 5.16. There was no significant difference in the peak elastase activity between preterm infants whose RDS resolved and term infants.

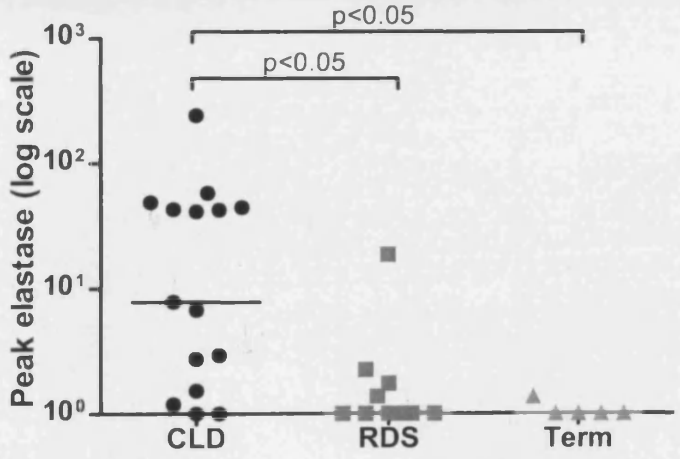
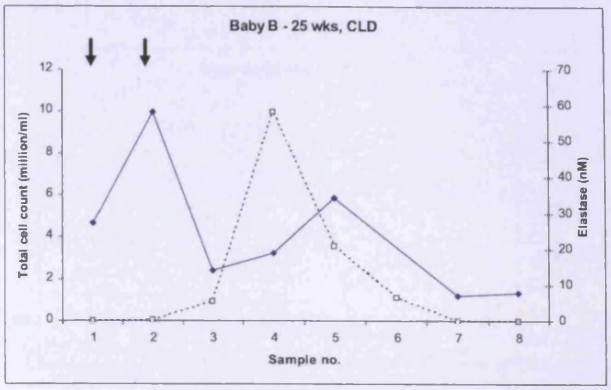


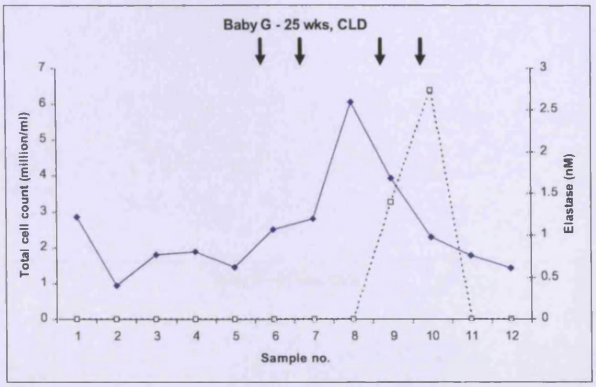
Figure 5.18 A scatterplot of the peak elastase concentrations for infants with CLD, resolved RDS and control infants, demonstrating significantly higher peaks in infants developing CLD.

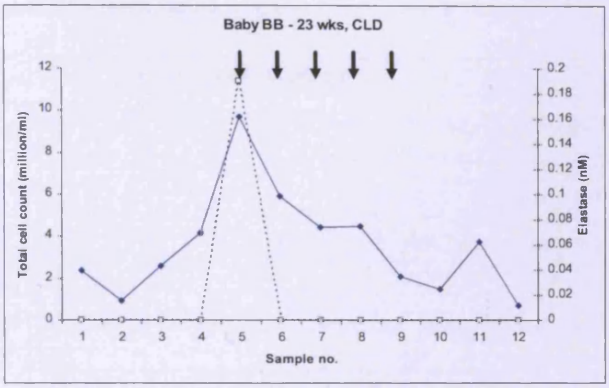
If the presence of elastase is considered in terms of the 50 samples in which elastase activity was detected out of the total of 207 samples collected, rather than individual patients, the following results are found: in those infants who developed CLD,

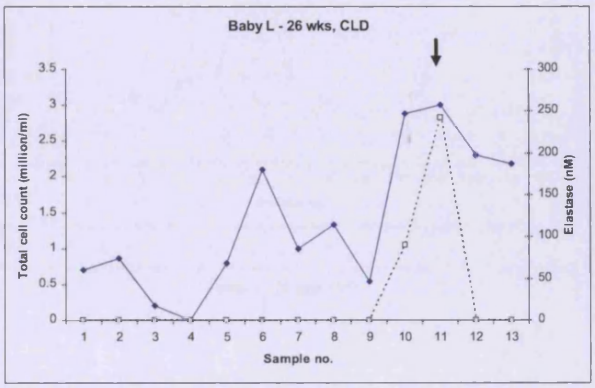
elastase activity was detected in 37/152 (24.3%) samples and 8/32 (25%) samples from infants developing RDS, compared to 1/19 (5.3%) of term control infants. All 4 samples from the infant who died had elastase present, albeit in very small amounts. When elastase activity was present, it was episodic in nature and was only present for a small number of samples for each infant. The timing of the activity spikes was also extremely variable, with the initial spike of elastase activity (when present) being a median of 7.5 days (range 3-27 days, IQR 5-19 days) after birth. No baby had elastase present on day 1.

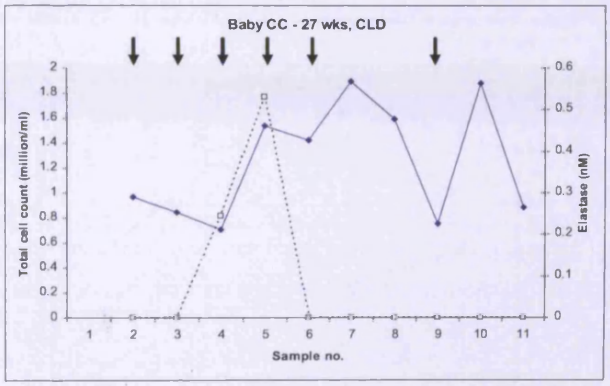
When longitudinal data for individual infants is analysed (figure 5.19) the episodic spiking nature of increases in elastase activity can be appreciated. It also appears that the timing of increases in elastase activity correlates well with the timing of increases in cell count and with the presence of 16S rRNA genes in the sample.

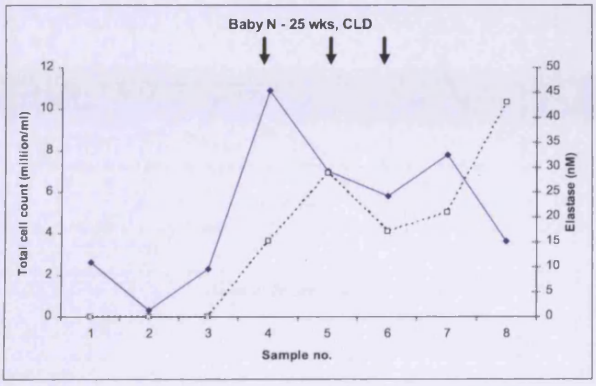

Baby B - 25 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.

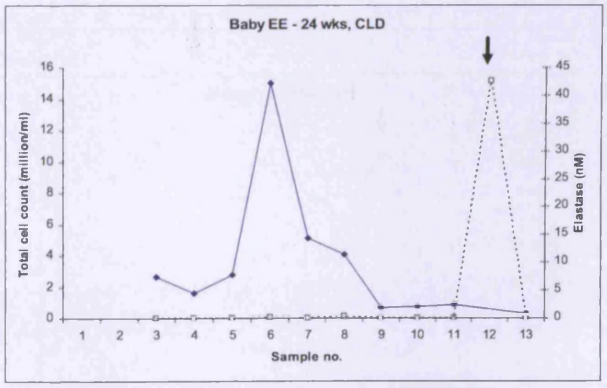

Baby G - 25 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.

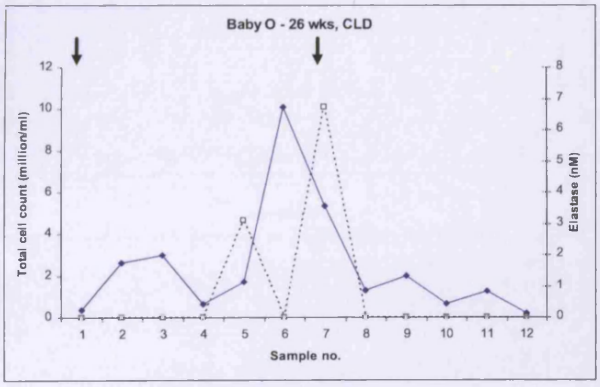

Baby BB - 23 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.

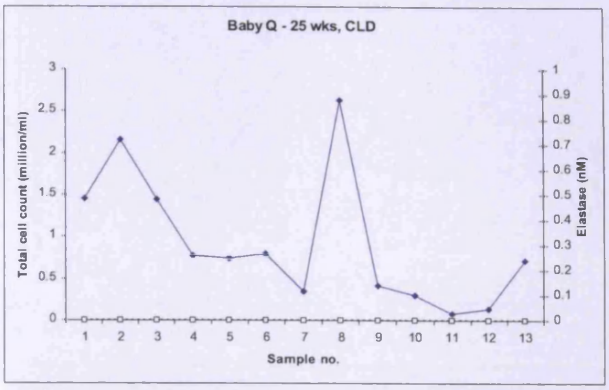

Baby L - 26 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.

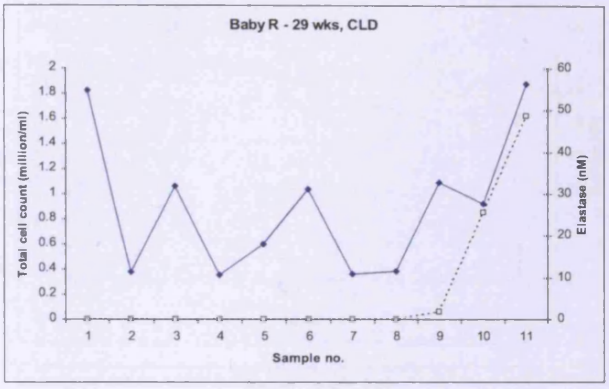

Baby CC - 27 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.

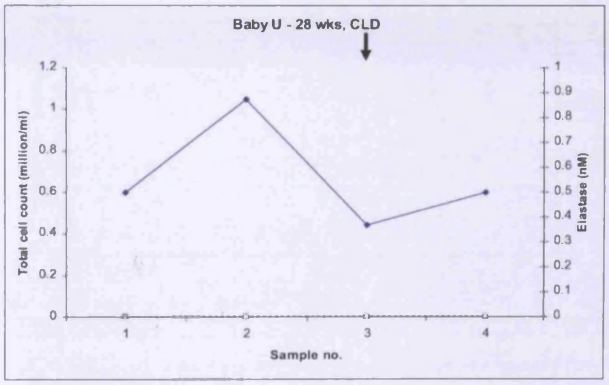

Baby N - 25 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.

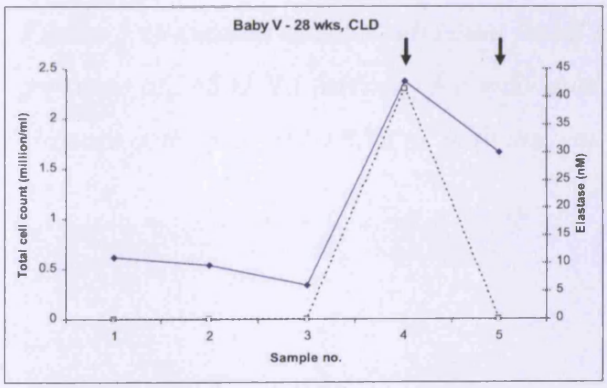

Baby EE - 24 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.

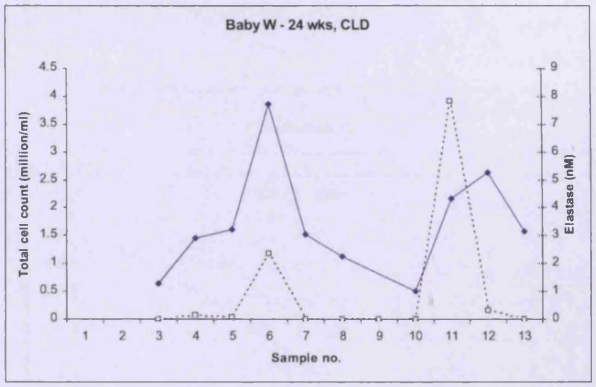

Baby O - 26 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.

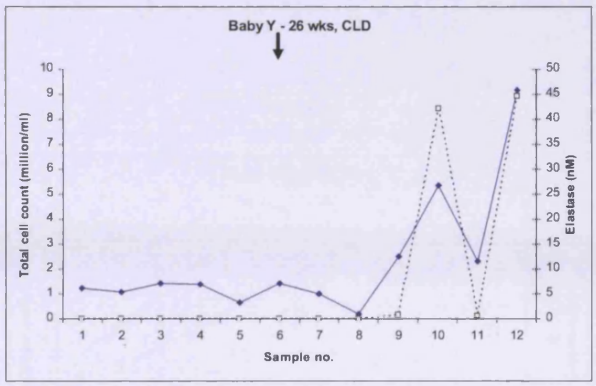

Baby Q - 25 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.

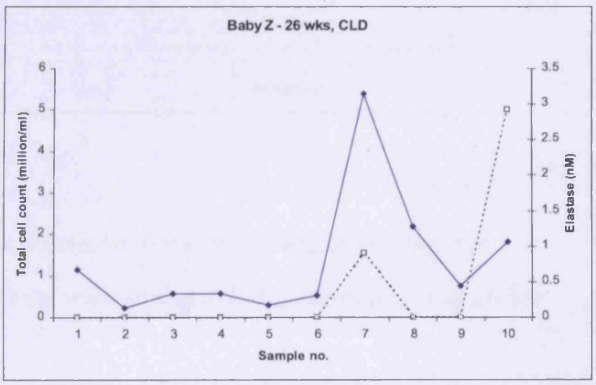

Baby R - 29 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.


Baby U - 28 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.


Baby V - 28 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.


Baby W - 24 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.


Baby Y - 26 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.


Baby Z - 26 wks, CLD
Total cell count (million/ml)
Elastase (nM)
Sample no.

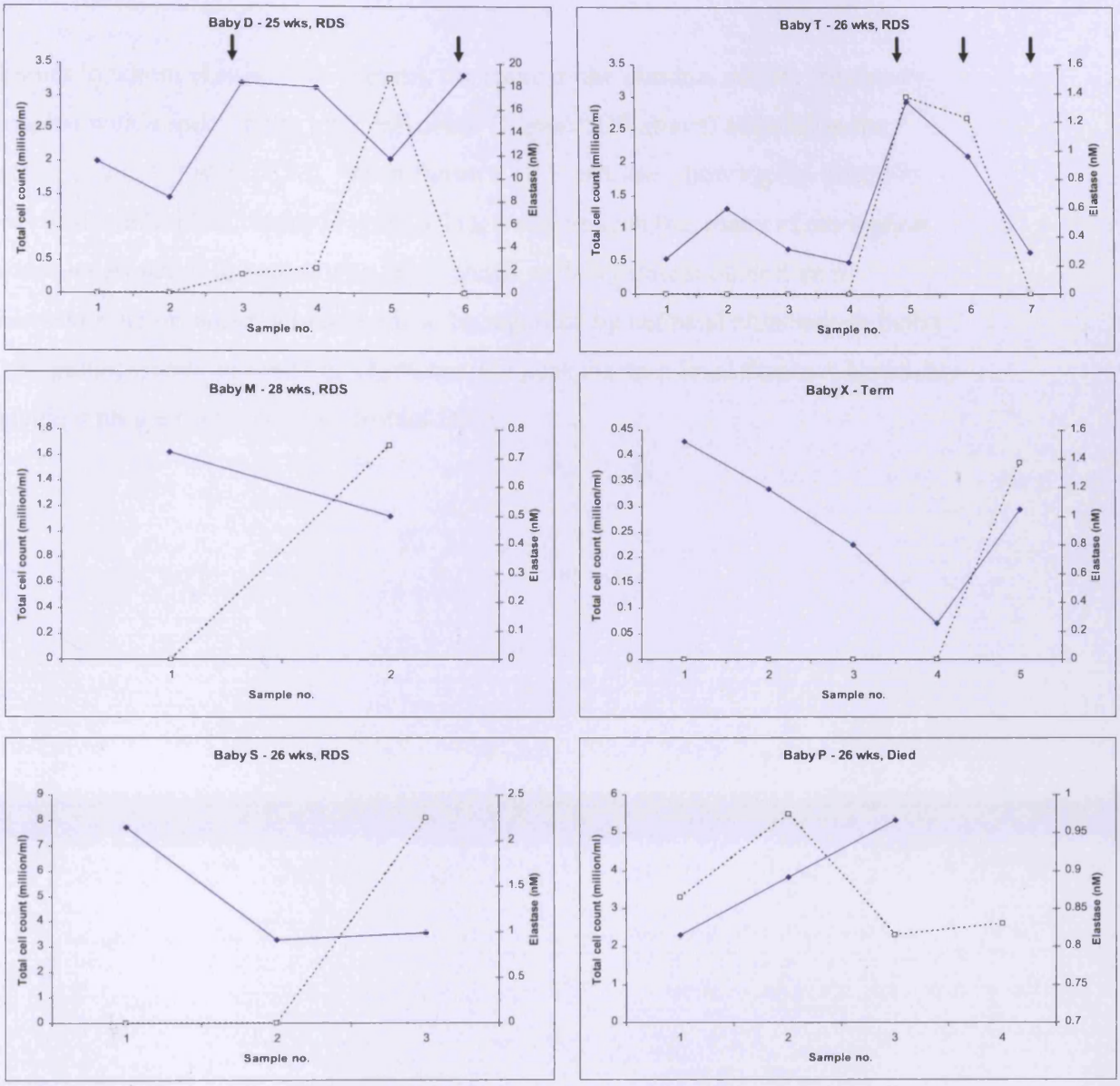
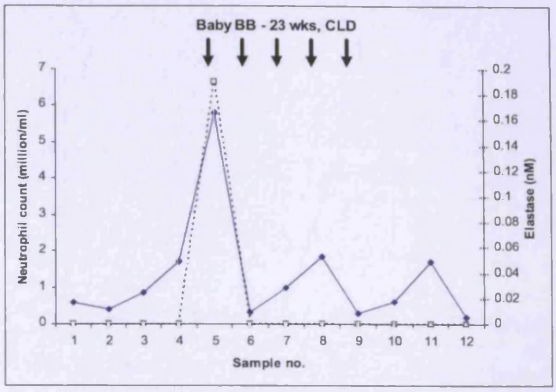
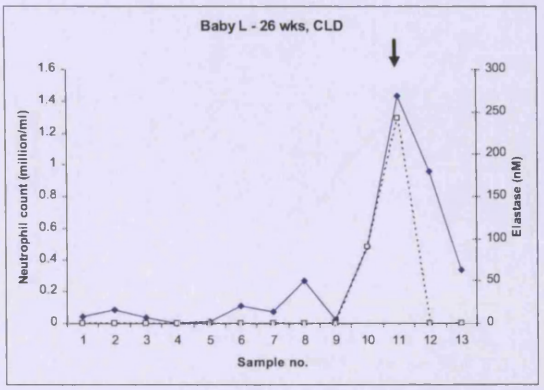


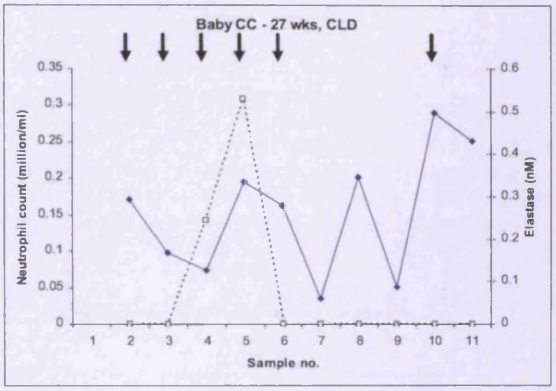
Figure 5.19 Graphs of total cell count (solid line), elastase activity (dotted line) and the presence of 16S rRNA (arrows) for individual infants with at least 3 BAL samples and either elastase activity or 16S rRNA or both present.

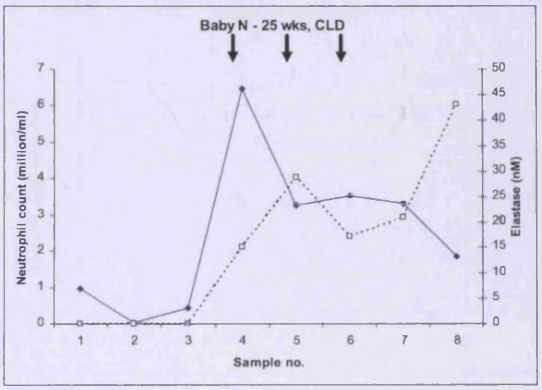
5.3.1 Elastase and infection

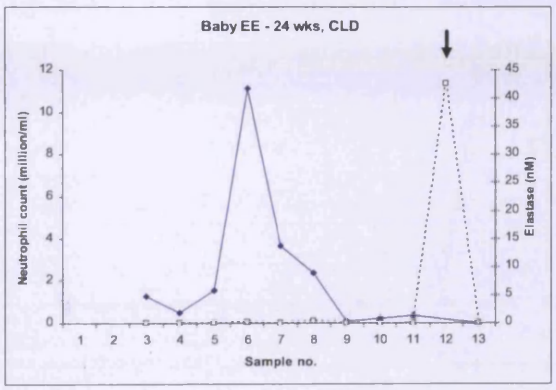
In babies in whom elastase was present, the spike in the elastase activity frequently coincided with a spike in the total cell count (Figure 5.19 above) and also in the neutrophil count (Figure 5.20). When figure 5.18 is redrawn showing the infection status of the individual babies (Figure 5.21), it can be seen that many of the highest elastase levels occur in babies who are infected with organisms other than *S. epidermidis,* an organism which tends to be regarded by neonatal clinicians as being of low pathogenicity in the lung. However, the peak elastase level does not invariably coincide with the presence of microbial DNA.

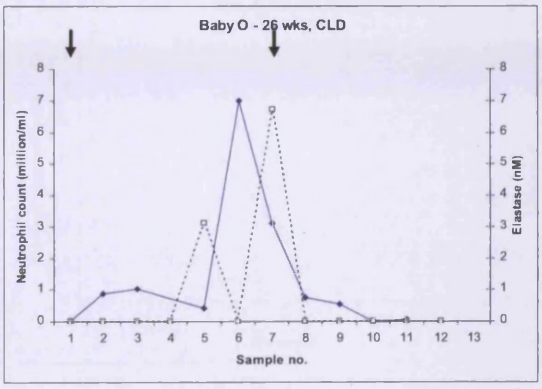

Baby BB - 23 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

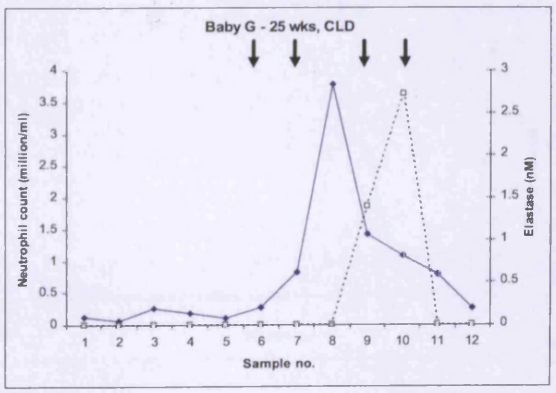

Baby L - 26 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

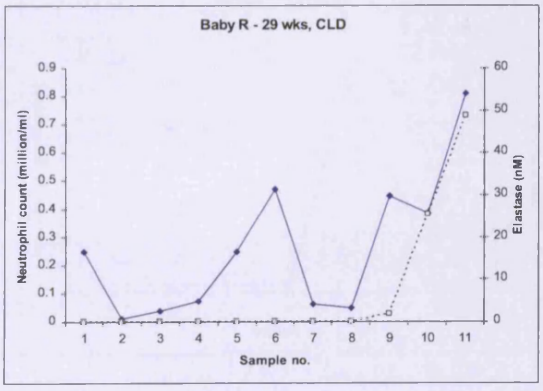

Baby CC - 27 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

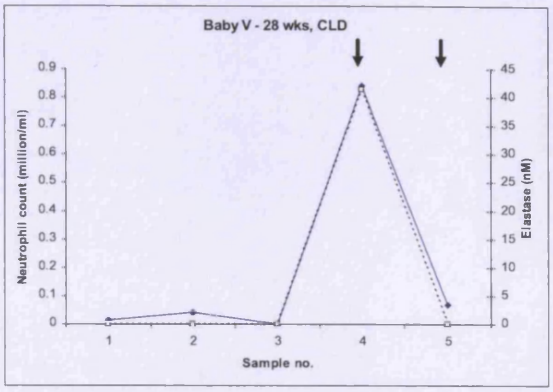

Baby N - 25 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

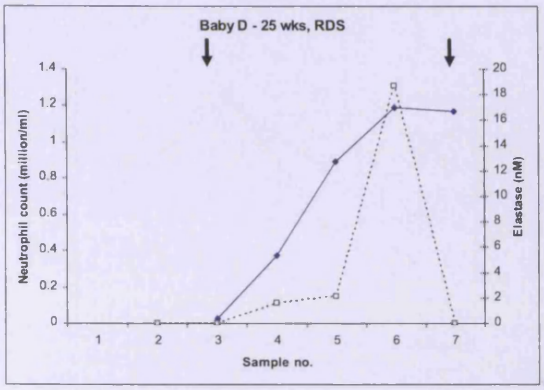

Baby EE - 24 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

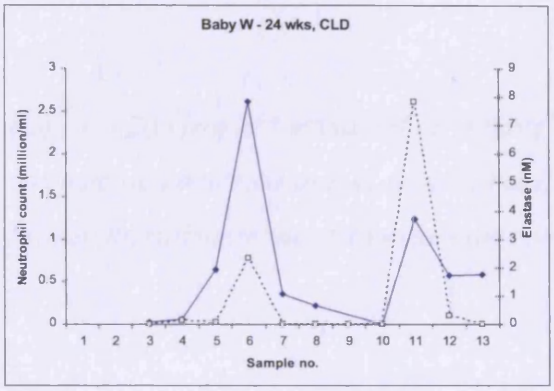

Baby O - 26 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

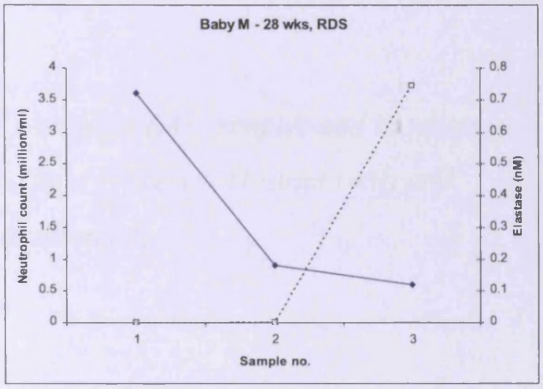

Baby G - 25 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

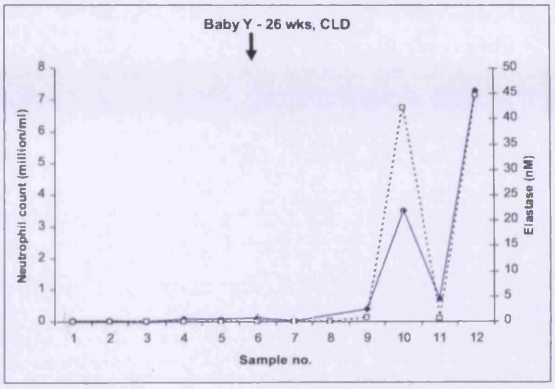

Baby R - 29 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

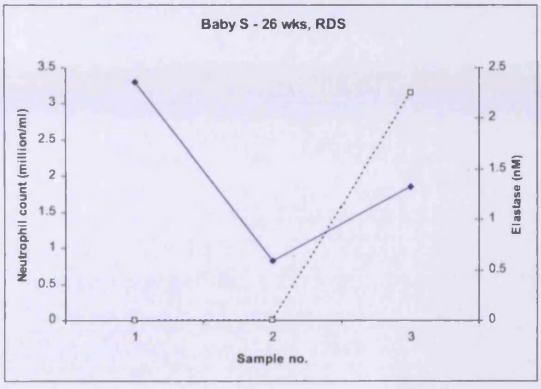

Baby V - 28 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

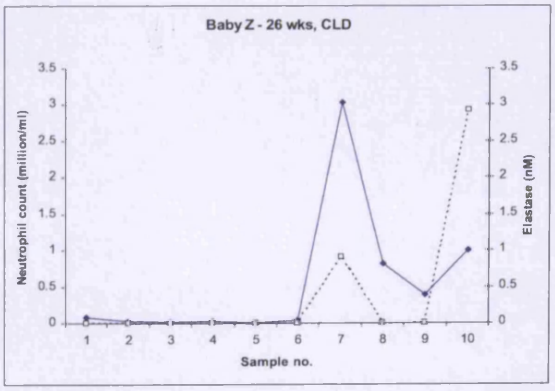

Baby D - 25 wks, RDS
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

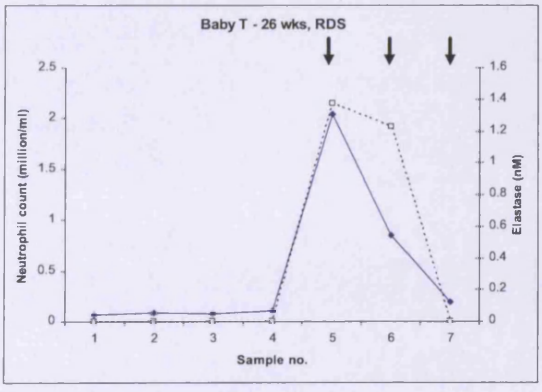

Baby W - 24 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.


Baby M - 28 wks, RDS
Neutrophil count (million/ml)
Elastase (nM)
Sample no.


Baby Y - 26 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.


Baby S - 26 wks, RDS
Neutrophil count (million/ml)
Elastase (nM)
Sample no.


Baby Z - 26 wks, CLD
Neutrophil count (million/ml)
Elastase (nM)
Sample no.


Baby T - 26 wks, RDS
Neutrophil count (million/ml)
Elastase (nM)
Sample no.

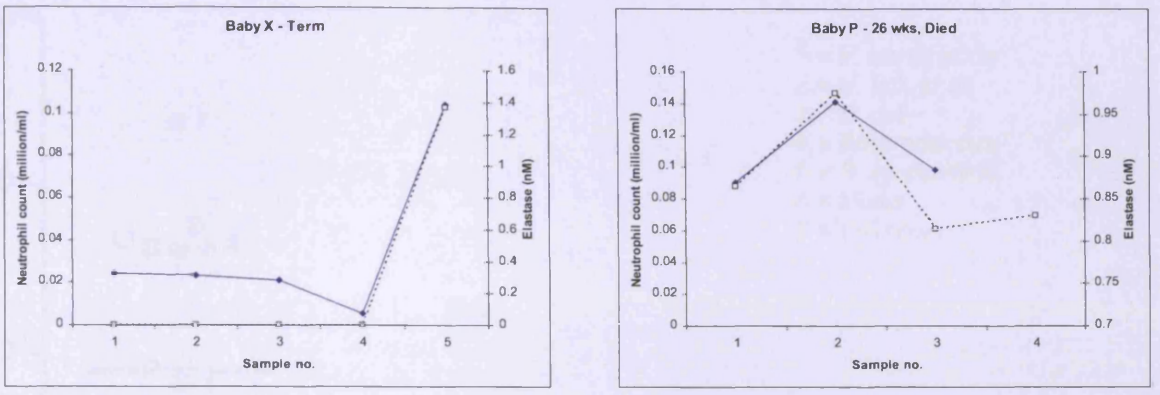


Figure 5.20 Graphs for individual infants with 3 or more BAL samples and in whom elastase was detected in BAL supernatant. Solid lines represent elastase (nM) and broken lines denote the total neutrophil count (million/ml).

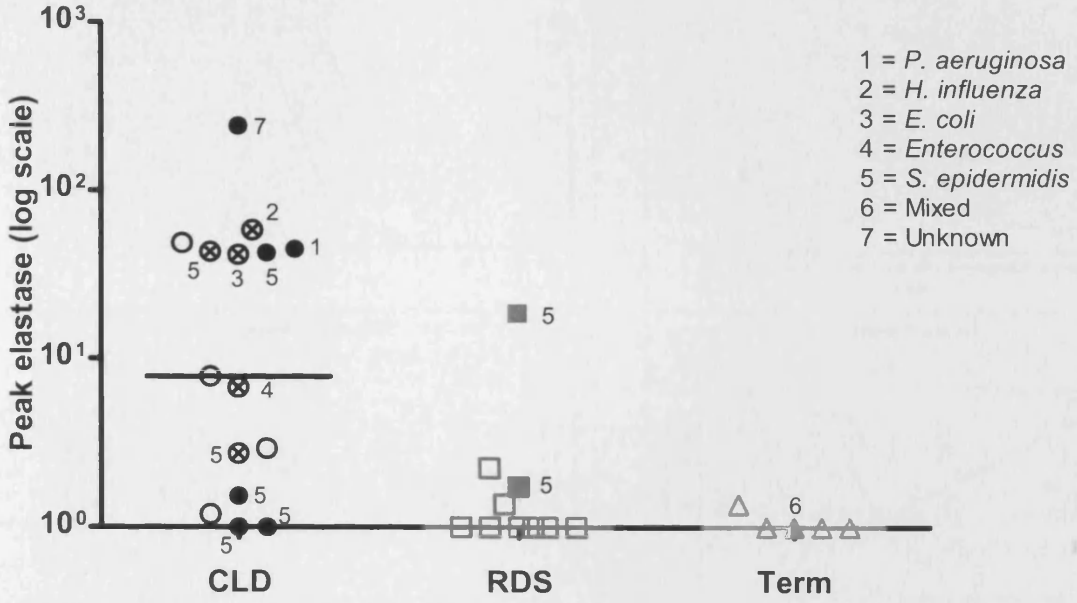
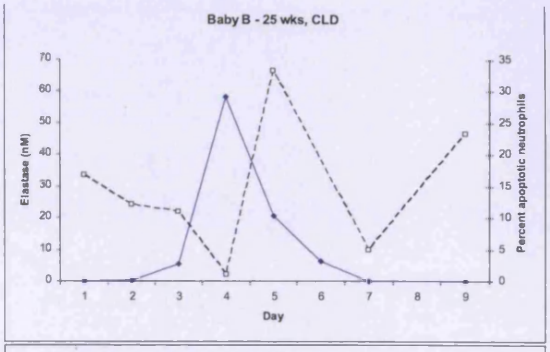
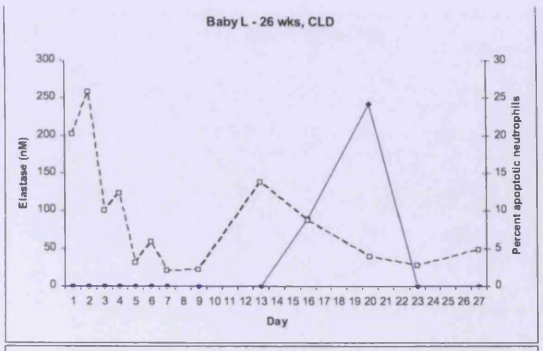


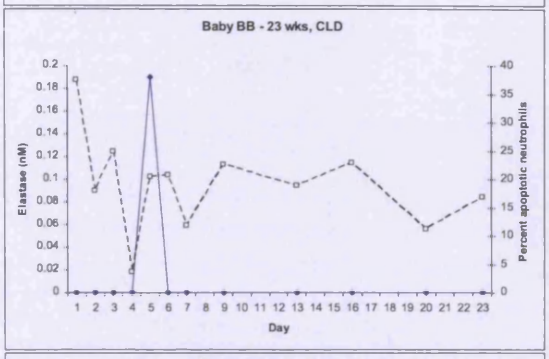
Figure 5.21 Scatterplot showing log of peak elastase activity level for each infant in whom elastase was detected. Solid markers indicate babies in whom peak elastase level co-incided with detection of 16S rRNA genes in the BAL sample. Markers with a cross in the centre indicate infants in whom 16S rRNA was detected but this was not coincident (or one day before or after) with the peak elastase level. Open markers indicate that 16S rRNA genes were not detected in that infant.

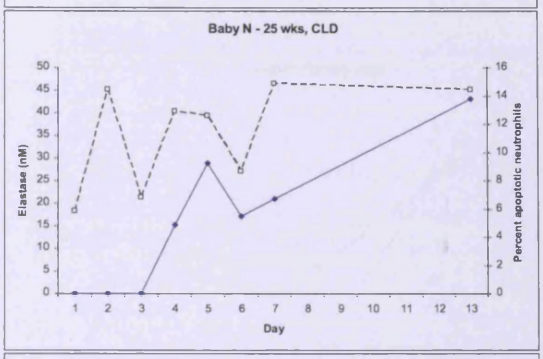
5.3.2 Elastase and apoptosis or necrosis

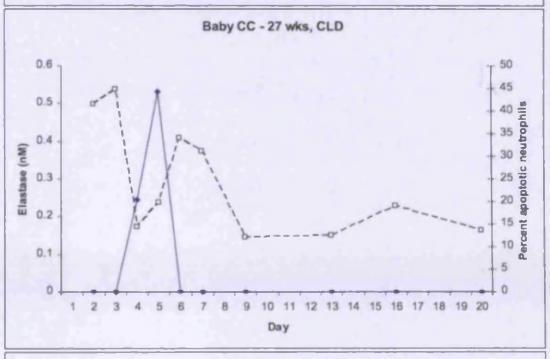
Elastase peaks are frequently temporally associated with peaks in the neutrophil count (Figure 5.20). Elastase has been implicated in the process of apoptosis, through its ability to stimulate or induce apoptosis (Trevani et al., 1996, Yang et al., 1996) and through cleavage of cell surface antigens eg CD16 (Middelhoven et al., 2001) and is released during cell death by necrosis, so elastase levels were plotted against both apoptotic and necrotic neutrophil percentages in order to assess a possible relationship between elastase and either apoptosis or necrosis of lung neutrophils (Figures 5.22 and 5.23). In some infants a temporal relationship between apoptosis or necrosis of neutrophils and the presence of elastase could be observed but this pattern was not as consistent as the relationship between elastase and the neutrophil count.

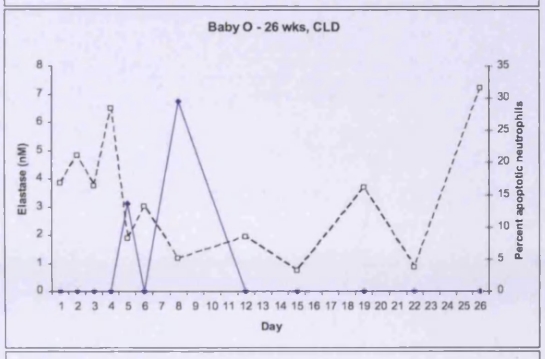

Baby B - 25 wks, CLD
Elastase (nM)
Percent apoptotic neutrophils
Day

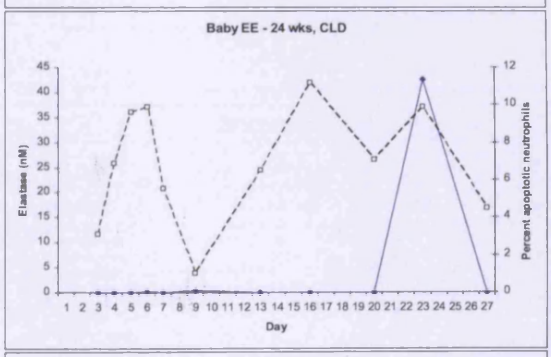

Baby L - 26 wks, CLD
Elastase (nM)
Percent apoptotic neutrophils
Day

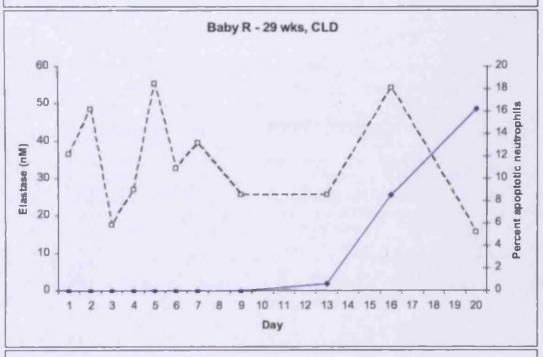

Baby BB - 23 wks, CLD
Elastase (nM)
Percent apoptotic neutrophils
Day

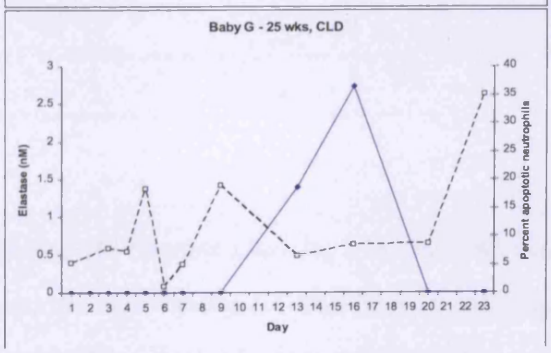

Baby N - 25 wks, CLD
Elastase (nM)
Percent apoptotic neutrophils
Day

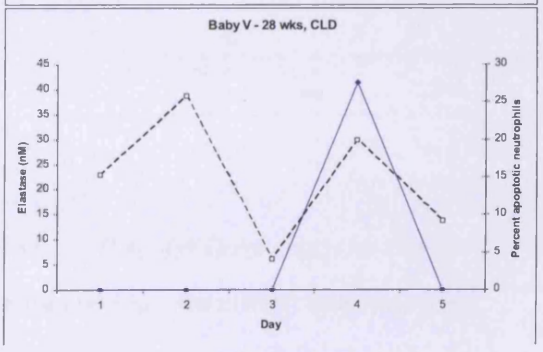

Baby CC - 27 wks, CLD
Elastase (nM)
Percent apoptotic neutrophils
Day


Baby O - 26 wks, CLD
Elastase (nM)
Percent apoptotic neutrophils
Day


Baby EE - 24 wks, CLD
Elastase (nM)
Percent apoptotic neutrophils
Day


Baby R - 29 wks, CLD
Elastase (nM)
Percent apoptotic neutrophils
Day


Baby G - 25 wks, CLD
Elastase (nM)
Percent apoptotic neutrophils
Day


Baby V - 28 wks, CLD
Elastase (nM)
Percent apoptotic neutrophils
Day

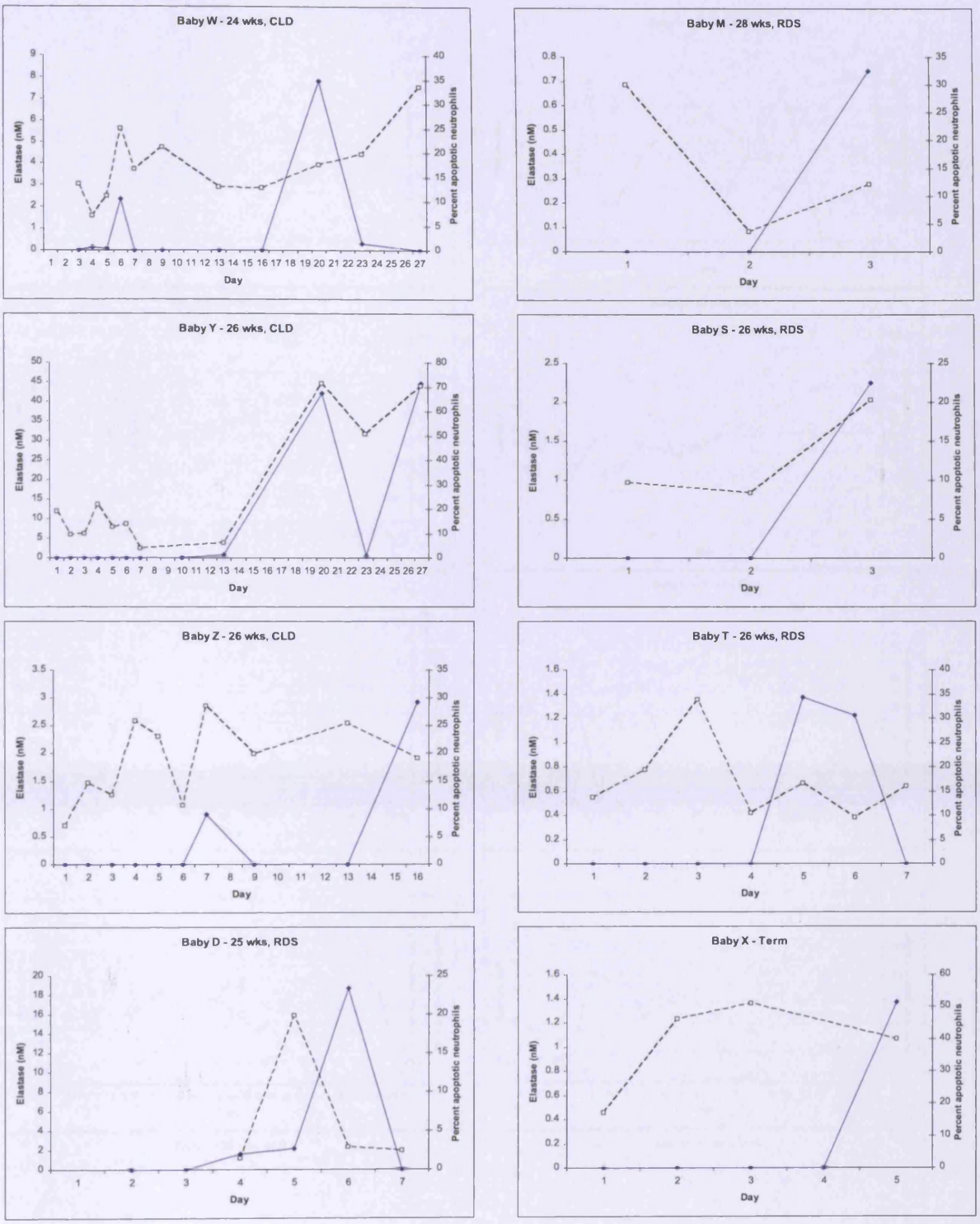
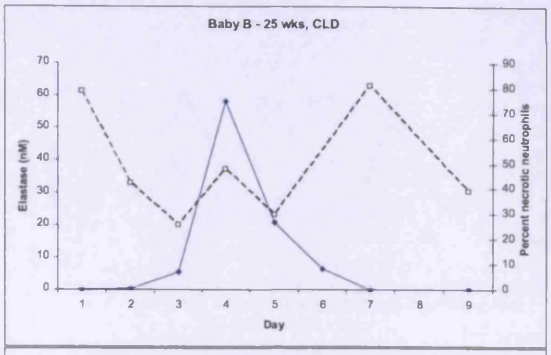
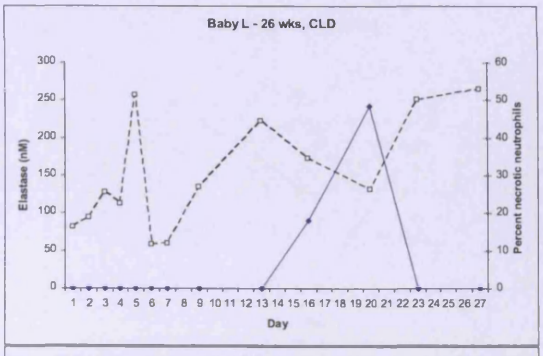
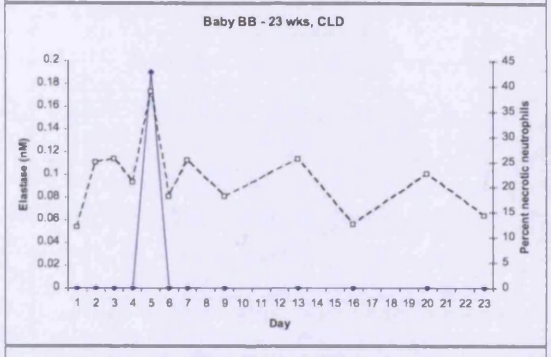
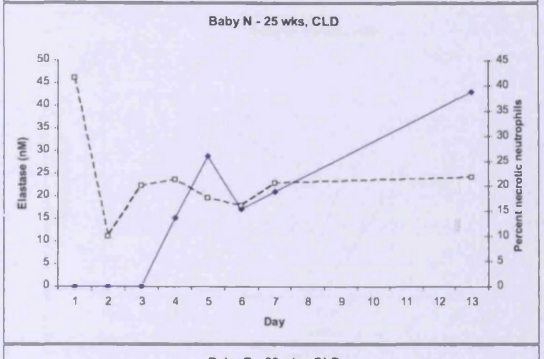


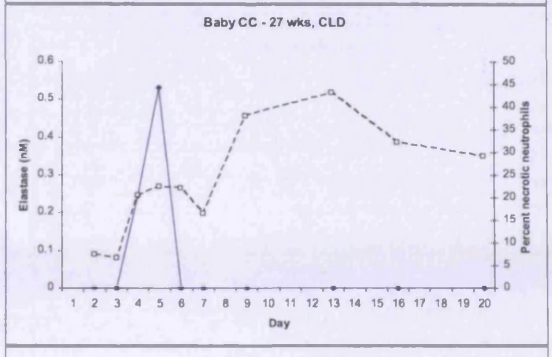
Figure 5.22 Graphs showing elastase concentration (nM) (solid line) and percentage apoptotic neutrophils (dotted line) in infants from whom 3 or more BAL samples were taken and in which elastase was detected.

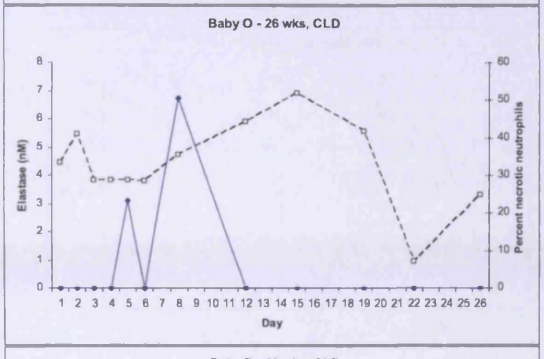

Baby B - 25 wks, CLD
Elastase (nM)
Percent necrotic neutrophils
Day

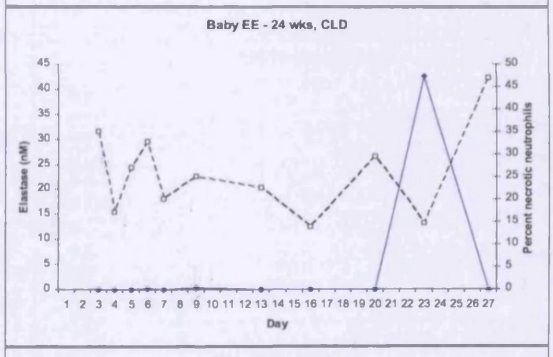

Baby L - 26 wks, CLD
Elastase (nM)
Percent necrotic neutrophils
Day

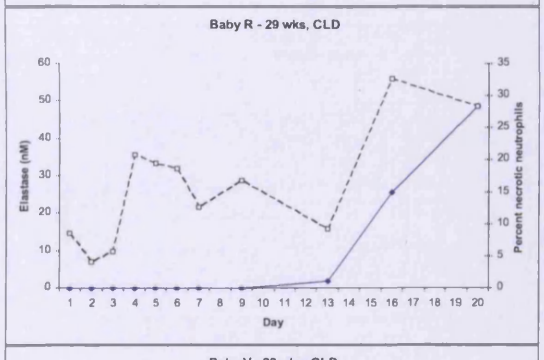

Baby BB - 23 wks, CLD
Elastase (nM)
Percent necrotic neutrophils
Day

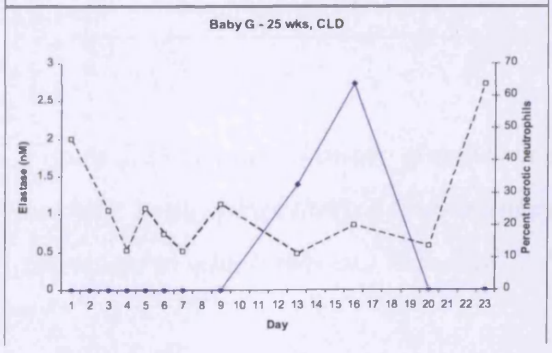

Baby N - 25 wks, CLD
Elastase (nM)
Percent necrotic neutrophils
Day

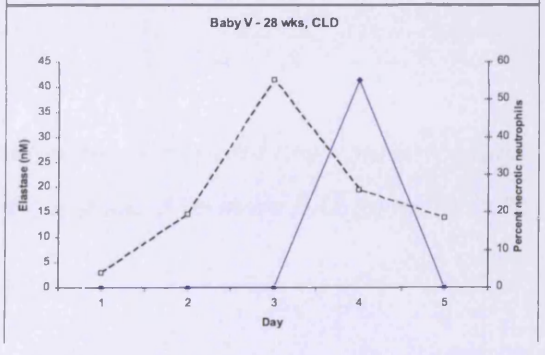

Baby CC - 27 wks, CLD
Elastase (nM)
Percent necrotic neutrophils
Day


Baby O - 26 wks, CLD
Elastase (nM)
Percent necrotic neutrophils
Day


Baby EE - 24 wks, CLD
Elastase (nM)
Percent necrotic neutrophils
Day


Baby R - 29 wks, CLD
Elastase (nM)
Percent necrotic neutrophils
Day


Baby G - 25 wks, CLD
Elastase (nM)
Percent necrotic neutrophils
Day


Baby V - 28 wks, CLD
Elastase (nM)
Percent necrotic neutrophils
Day

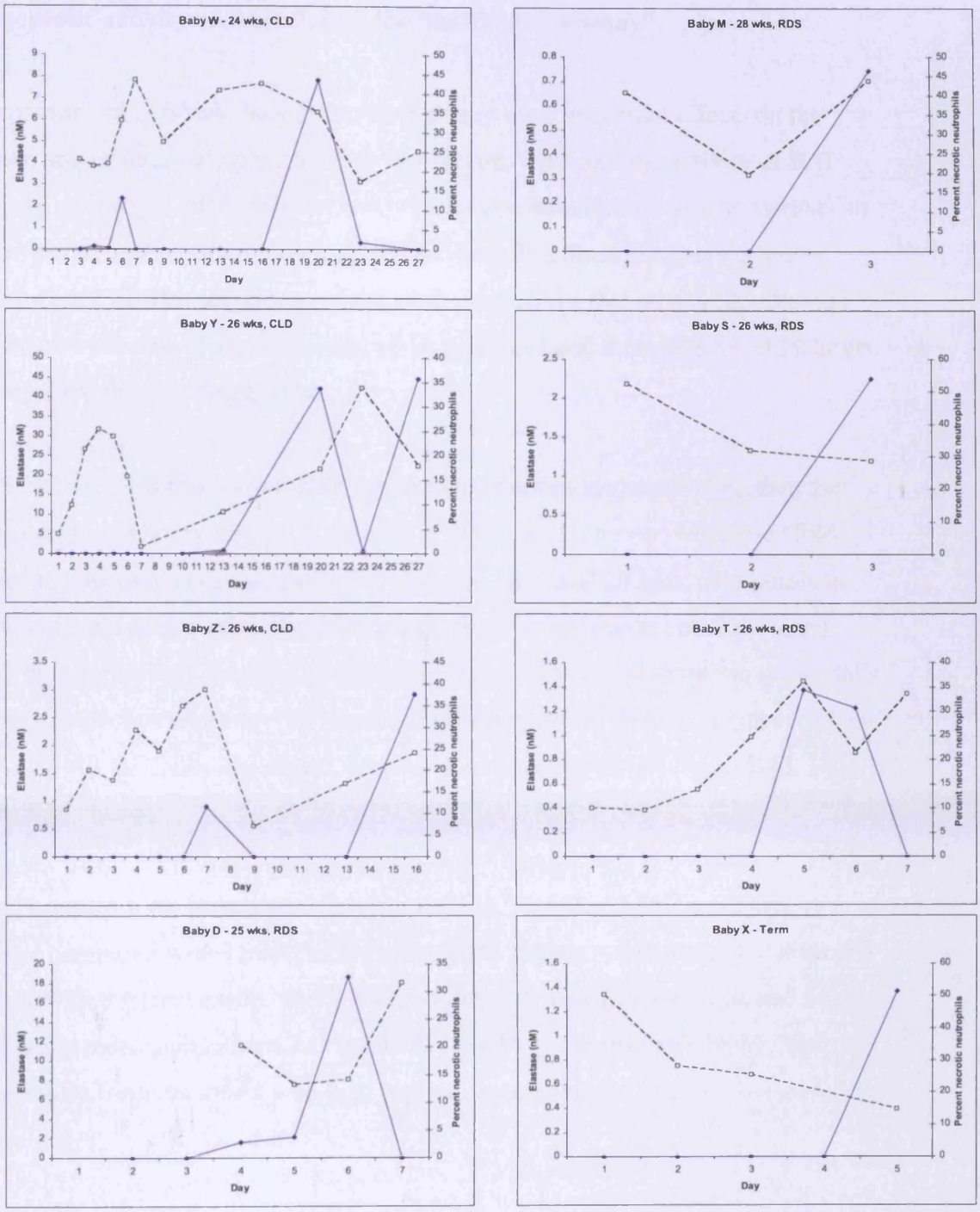


Figure 5.23 Graphs showing elastase concentration (nM) (solid line) and percentage necrotic neutrophils (dotted line) in infants from whom 3 or more BAL samples were taken and in which elastase was detected.

5.4 Apoptotic activity of BAL fluid – the Sheffield "bioassay"

The environment in which the cells are found may have important effects on the initiation and progress of apoptosis. For this reason, the apoptotic activity of BAL fluid (182 samples in total) against freshly isolated adult neutrophils was assessed in an assay performed by Sharon Gill, in Sheffield, as described in materials and methods (2.1.7.2). The percentage of the adult neutrophils that were apoptotic was assessed on cytospins by light microscopy at the outset and then after 5 and 20 hours of exposure to the BAL supernatant.

Only 9/182 samples showed a higher percentage of apoptotic neutrophils than the saline control at 5 hours and just 3 samples at 20 hours. However, when the "fold change" in apoptotic cells was measured between the 5 and 20 hour time points in each sample and compared to the fold change in the saline control over the same period, 96 samples had greater fold change in the percentage of apoptotic neutrophils than their respective controls – i.e relatively proapoptotic BAL fluid (Figure 5.24 and Table 5.2). All the following results for pro- or anti-apoptotic activity of BAL fluid refer to fold change.

Among samples from babies who developed CLD, 71/135 (52.6%) were pro-apoptotic compared with 11/28 (39.3%) from RDS infants – this was not statistically significant. In the term group, 14/15 (93.3%) samples were pro-apoptotic and this was significantly more than either CLD ($p=0.0025$) or RDS groups ($p=0.0006$). None of the 4 samples from the infant who died was pro-apoptotic.

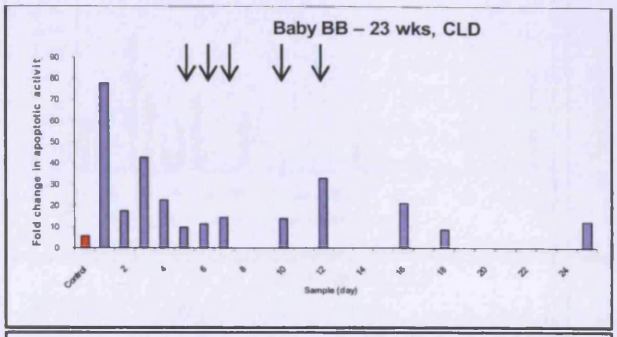
	Pro-apoptotic BAL (%)	Anti-apoptotic BAL (%)	TOTALS
CLD	71 (52.6)	64 (47.4)	135
RDS	11 (39.3)	17 (60.7)	28
Term	14 (93.3)	1 (6.7)	15
Died	0 (0)	4 (100)	4
TOTALS	96 (52.7)	86 (47.3)	182

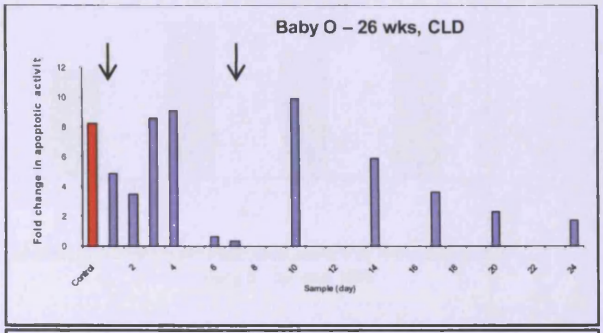
Table 5.2 Table showing the number of BAL supernatants in each diagnostic group which displayed either relative pro- or anti-apoptotic activity compared to saline controls.

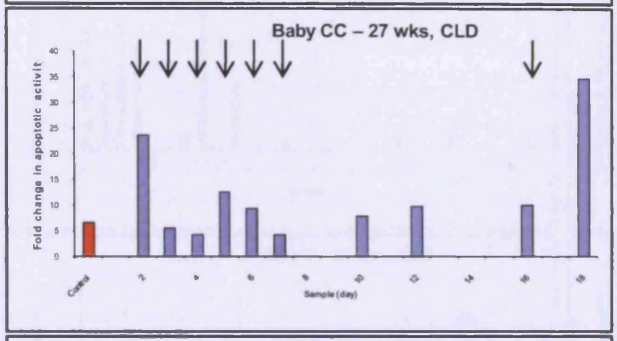
The pattern of pro- and anti-apoptotic samples in individual infants can be seen in the bar graphs in Figure 5.24. Term infants (H and X) appear to have BAL fluid that is consistently relatively pro-apoptotic when compared to their saline control. Preterm infants tend to have a more mixed picture with some samples relatively pro-apoptotic and others relatively anti-apoptotic. It is noticeable in Figure 5.24 that in many of the preterm infants, particularly babies BB, CC, L, N, O, W and Y, although early samples are strongly pro-apoptotic, later samples become more anti-apoptotic.

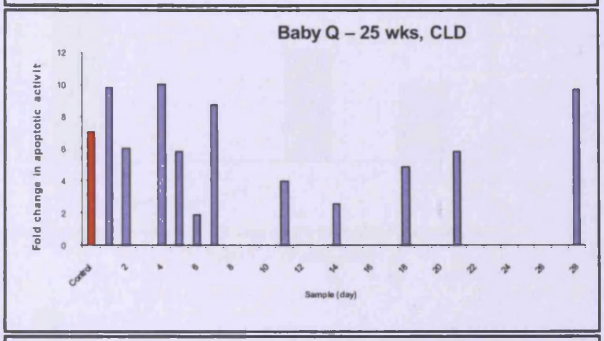
This experiment was conducted using adult neutrophils *in vitro*. It is known and will be shown again in chapter 6 that neutrophils from newborn infants have different apoptotic rates and responses to adult neutrophils, so data from this series of experiments must be interpreted in light of this.

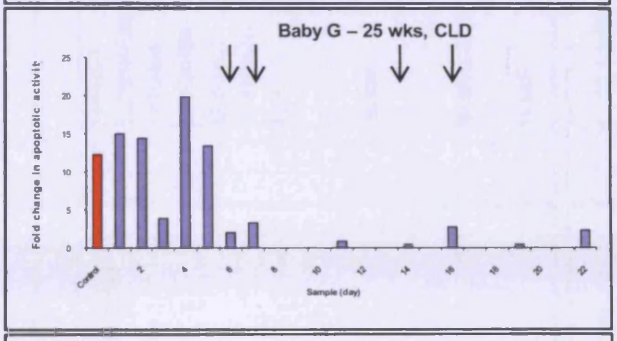
In Figure 5.24, it is also noticeable that in samples from which 16S rRNA microbial genes were isolated, the levels of apoptotic activity are among the most anti-apoptotic for that individual infant.

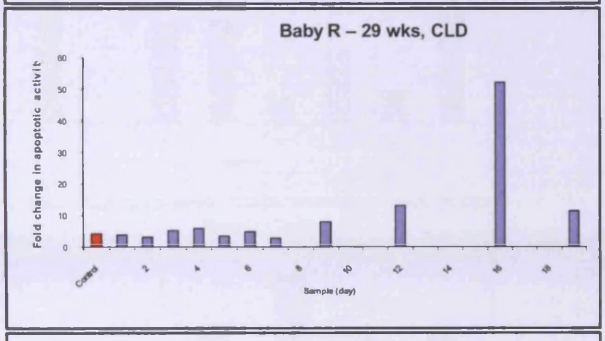

Baby BB – 23 wks, CLD
Fold change in apoptotic activit
Sample (day)

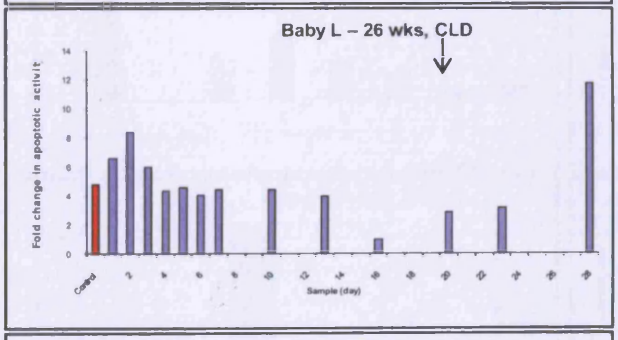

Baby O – 26 wks, CLD
Fold change in apoptotic activit
Sample (day)

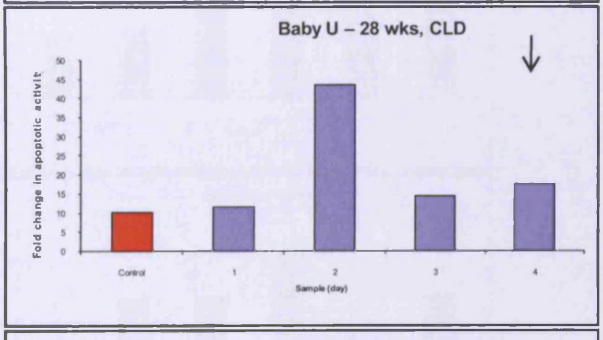

Baby CC – 27 wks, CLD
Fold change in apoptotic activit
Sample (day)

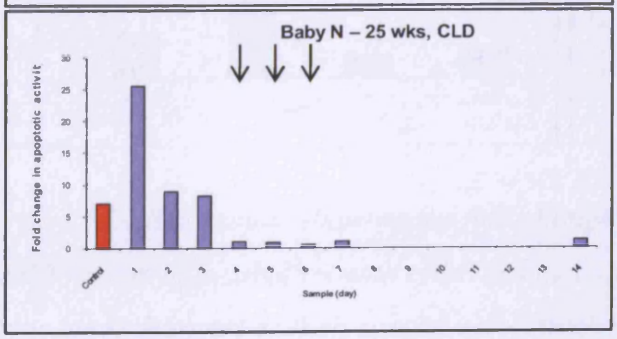

Baby Q – 25 wks, CLD
Fold change in apoptotic activit
Sample (day)

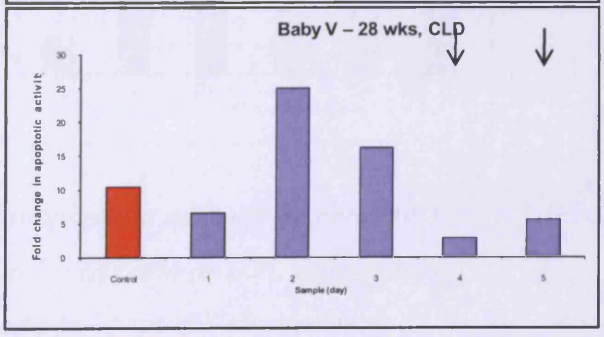

Baby G – 25 wks, CLD
Fold change in apoptotic activit
Sample (day)


Baby R – 29 wks, CLD
Fold change in apoptotic activit
Sample (day)


Baby L – 26 wks, CLD
Fold change in apoptotic activit
Sample (day)


Baby U – 28 wks, CLD
Fold change in apoptotic activit
Control
1
2
3
4
Sample (day)


Baby N – 25 wks, CLD
Fold change in apoptotic activit
Sample (day)


Baby V – 28 wks, CLD
Fold change in apoptotic activit
Control
1
2
3
4
5
Sample (day)

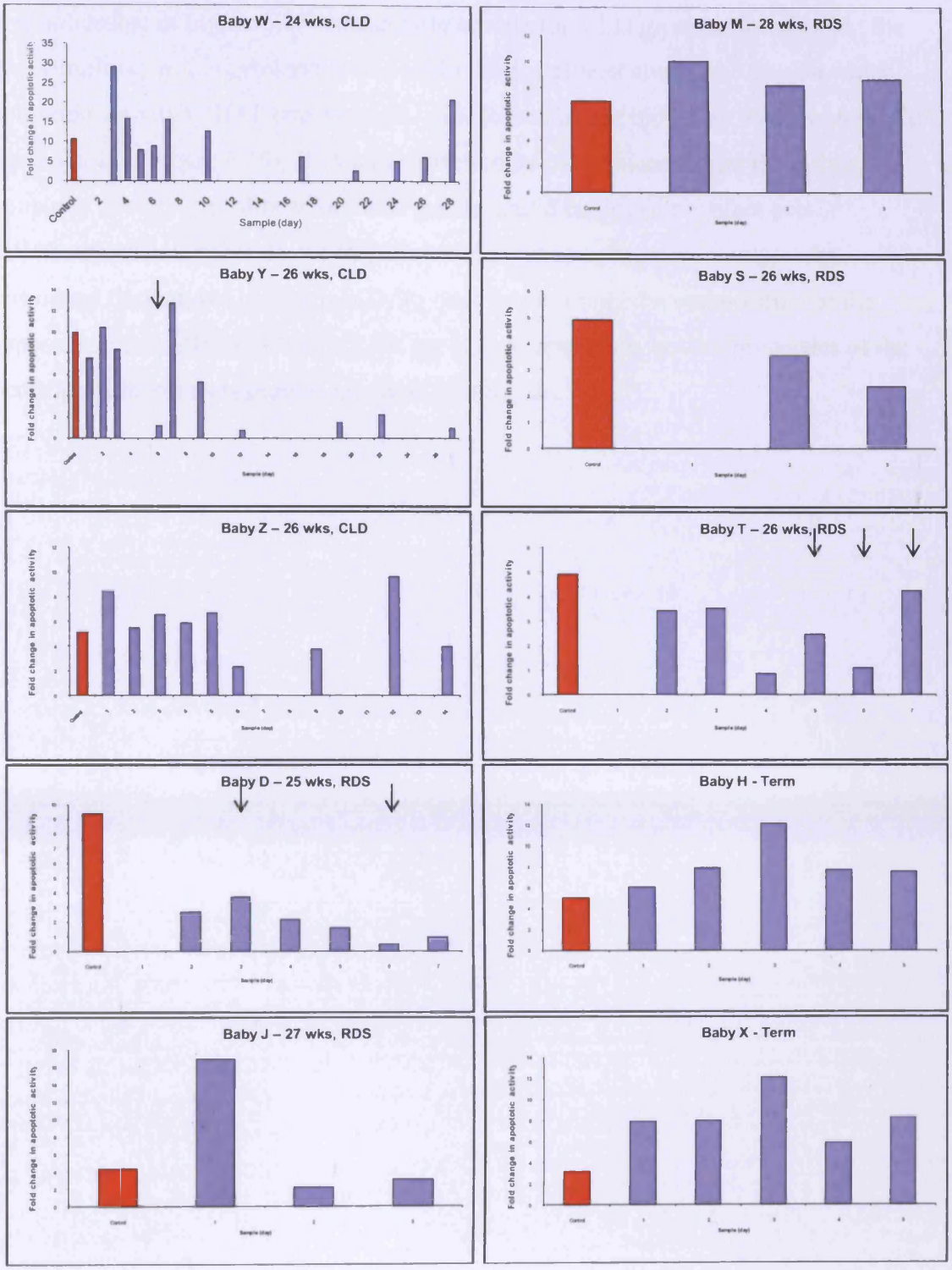
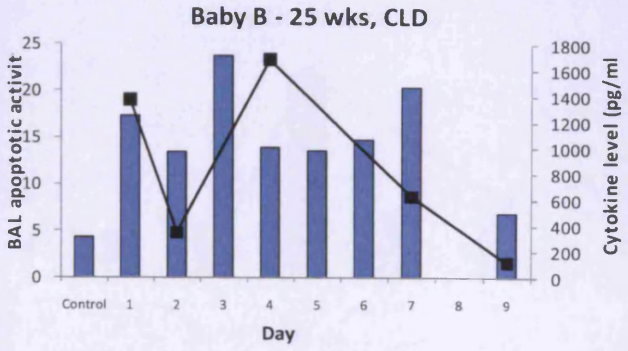
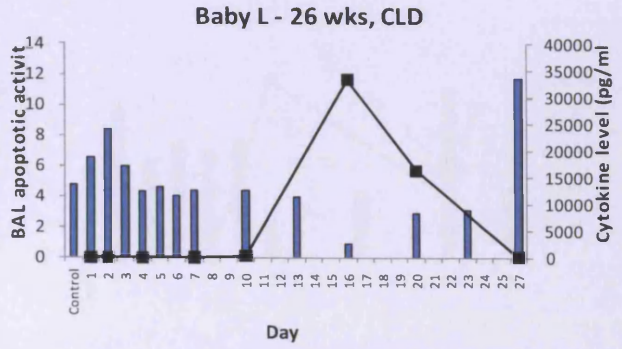
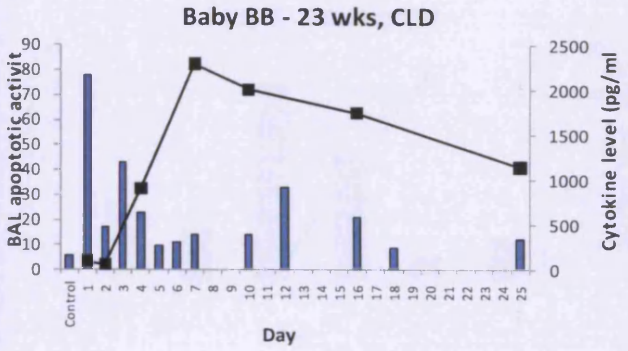


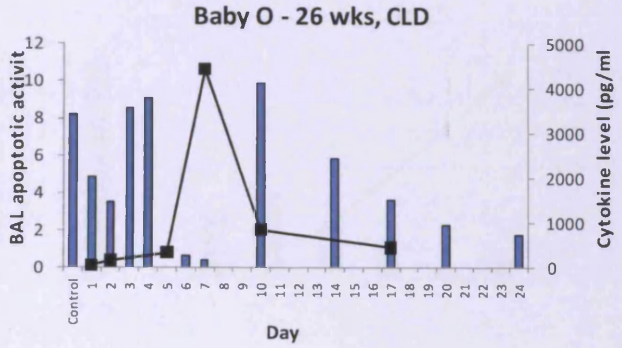
Figure 5.24 Bar graphs showing the fold change in apoptotic neutrophils between 5 and 20 hours of incubation with either saline control (red bars) or BAL supernatants (blue bars). Bars taller than control are considered pro-apoptotic, shorter than control are relatively anti-apoptotic. Vertical arrows indicate that 16s rRNA genes were isolated from that sample.

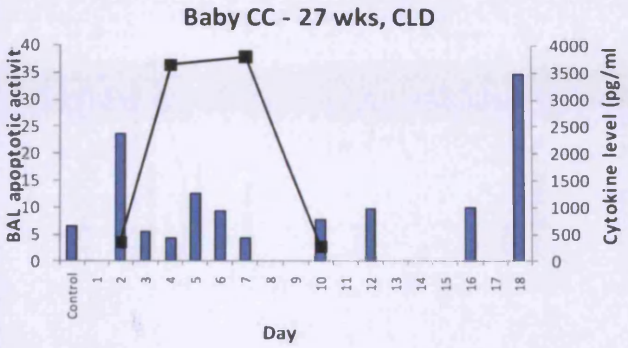
It is noticeable in Figure 5.25, particularly among the CLD group of infants, that the day/sample on which cytokine levels are highest is almost always the day on which apoptotic activity in BAL supernatants, as assessed by the bio-assay, is the lowest. To illustrate this (Figure 5.25), IL-8 was chosen to be plotted against the BAL fluid apoptotic activity, but almost identical graphs could be drawn for other pro-inflammatory mediators. However, this trend is, once again, not a completely consistent finding, raising the possibility that it is not only the composition of the supernatant that affects or controls the process of apoptosis, but the properties of the neutrophils themselves may be inherently important.

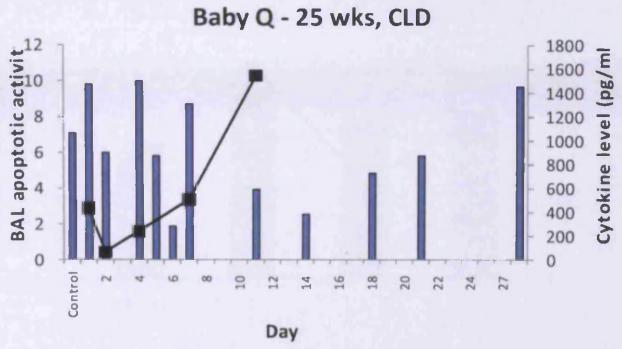

Baby B - 25 wks, CLD
BAL apoptotic activit
Cytokine level (pg/ml
Control 1 2 3 4 5 6 7 8 9
Day

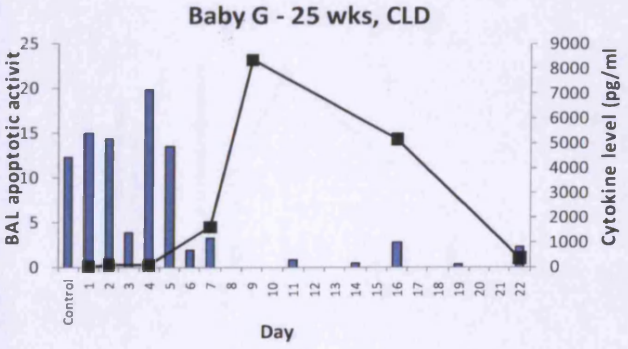

Baby L - 26 wks, CLD
BAL apoptotic activit
Cytokine level (pg/ml
Day

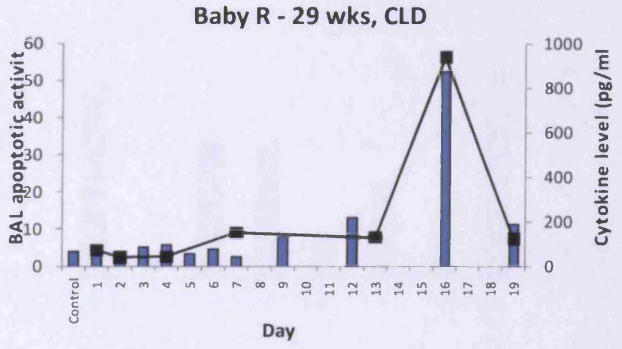

Baby BB - 23 wks, CLD
BAL apoptotic activit
Cytokine level (pg/ml
Day

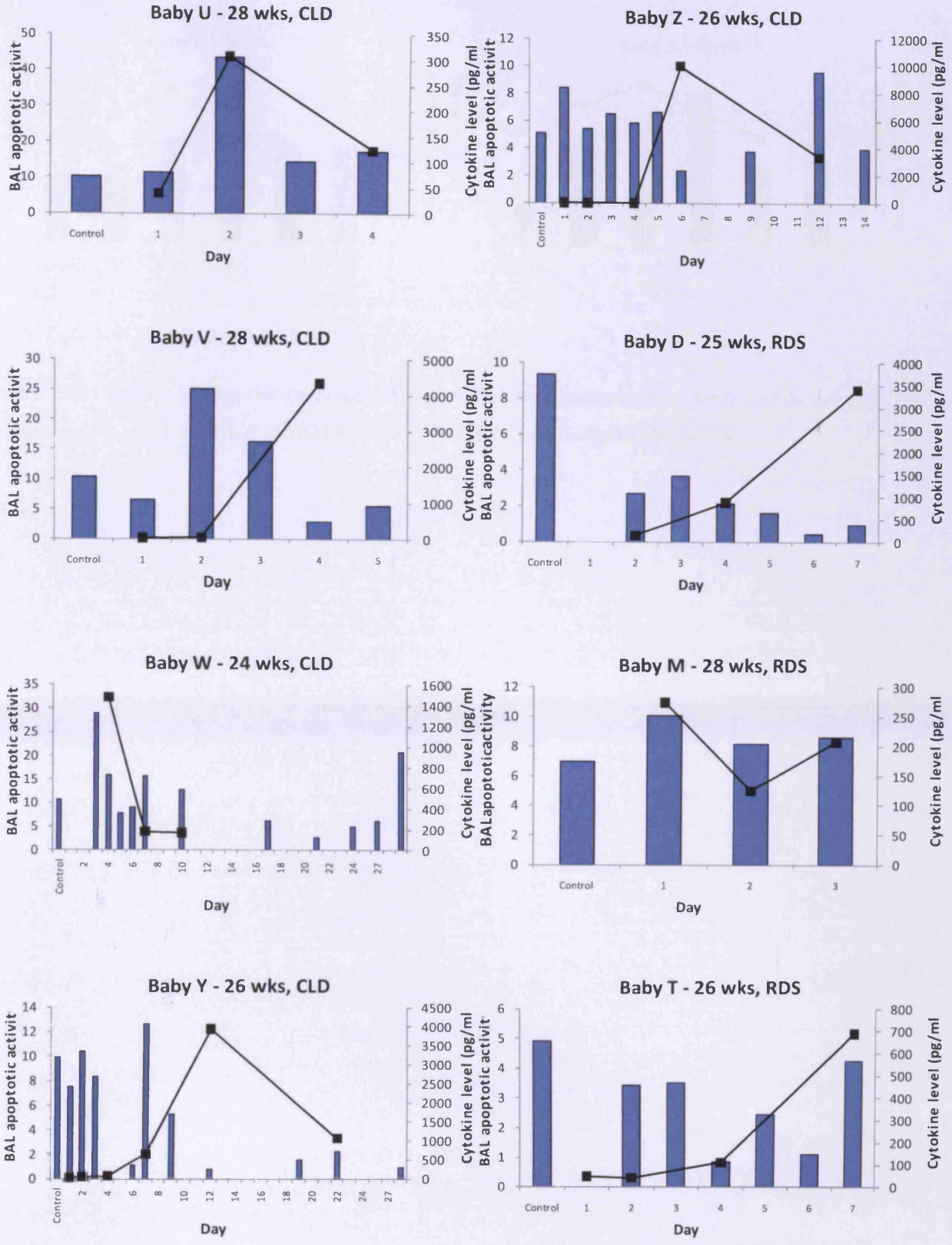

Baby O - 26 wks, CLD
BAL apoptotic activit
Cytokine level (pg/ml
Day


Baby CC - 27 wks, CLD
BAL apoptotic activit
Cytokine level (pg/ml
Day


Baby Q - 25 wks, CLD
BAL apoptotic activit
Cytokine level (pg/ml
Day


Baby G - 25 wks, CLD
BAL apoptotic activit
Cytokine level (pg/ml
Day


Baby R - 29 wks, CLD
BAL apoptotic activit
Cytokine level (pg/ml
Day


Baby U - 28 wks, CLD
BAL apoptotic activit
Cytokine level (pg/ml
Control
Day
Baby Z - 26 wks, CLD
Baby V - 28 wks, CLD
Baby D - 25 wks, RDS
Baby W - 24 wks, CLD
Baby M - 28 wks, RDS
Baby Y - 26 wks, CLD
Baby T - 26 wks, RDS

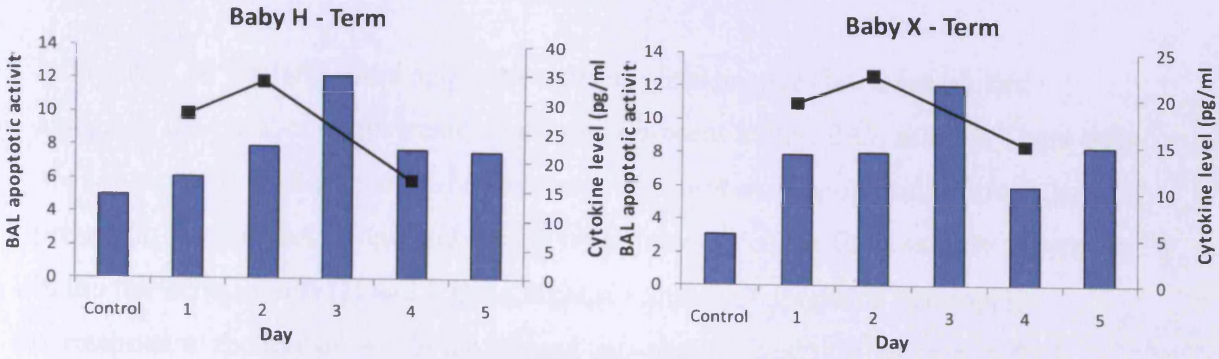
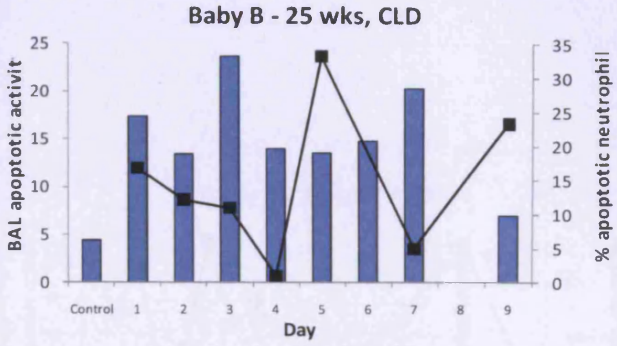
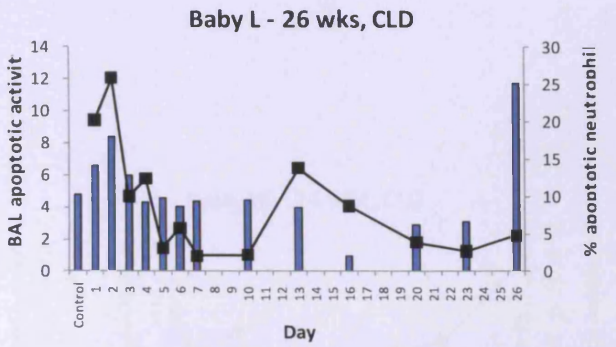


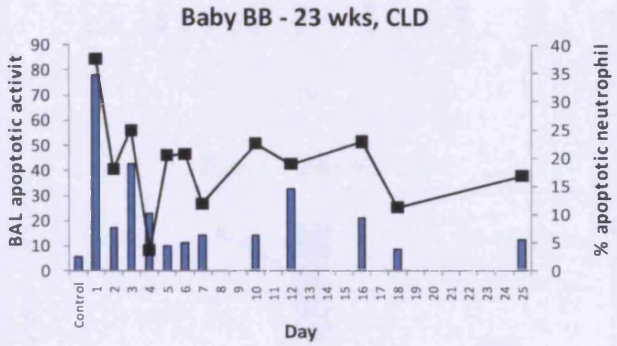
Figure 5.25 Graphs showing the apoptotic activity of BAL supernatants (blue bars) and the level of proinflamatory cytokines (IL-8 shown) measured by CBA in the samples (black lines).

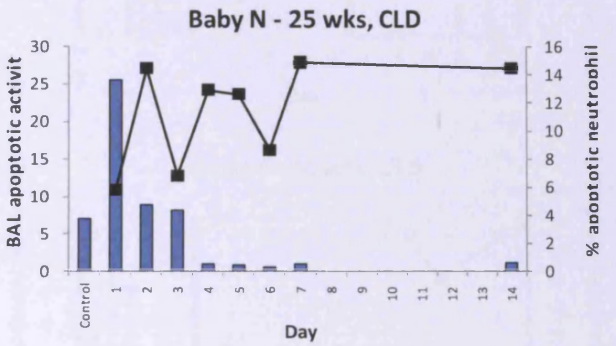
5.4.1 BAL fluid apoptotic activity and apoptotic neutrophil counts

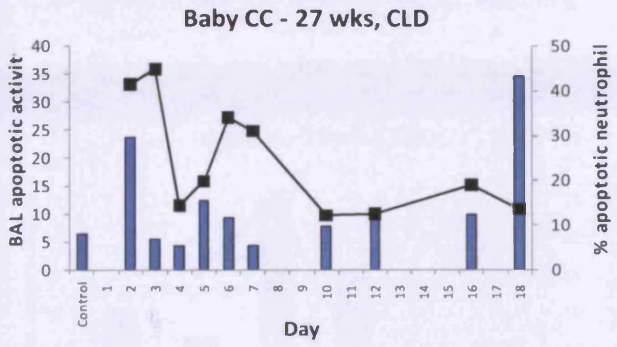
In Figure 5.26 the BAL fluid apoptotic activity (fold change) has been plotted alongside the number of apoptotic neutrophils present in that BAL sample. There does not appear to be a clear relationship between the number of apoptotic neutrophils present in preterm BAL fluid and the apoptotic activity of the BAL sample. However, in the two term infants (H and X), the highest number of apoptotic neutrophils correspond to the day on which the highest pro-apoptotic activity as seen in BAL fluid. This may be a chance finding due to the very small number of term infants which were studied but may also indicate that neutrophils in term infant lungs are able to respond appropriately to pro-apoptotic stimuli in the BAL fluid more effectively than neutrophils in preterm lungs.

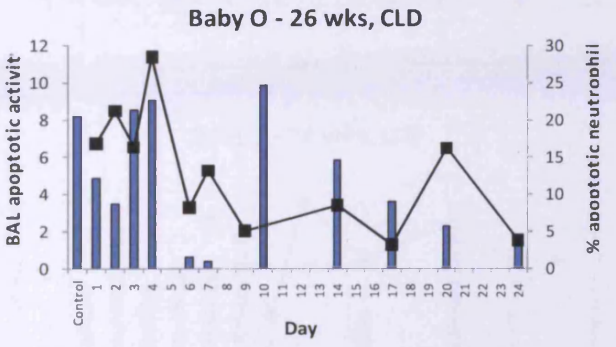

Baby B - 25 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day

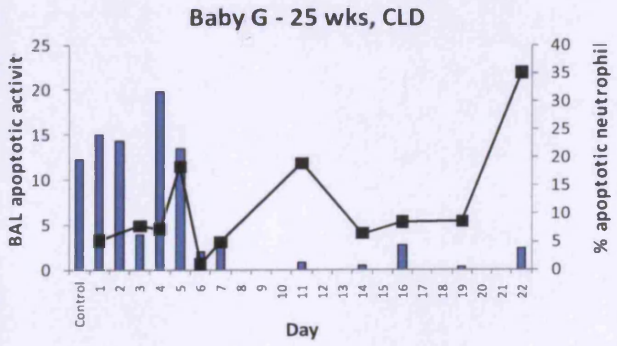

Baby L - 26 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day

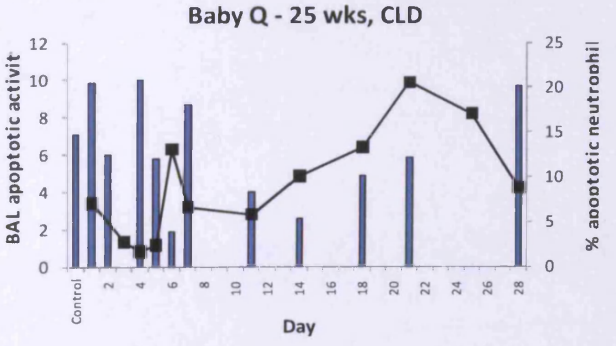

Baby BB - 23 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day

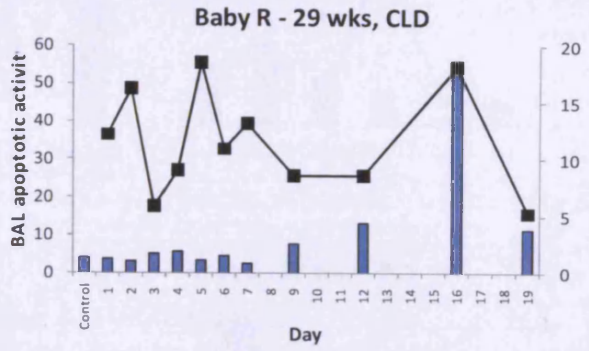

Baby N - 25 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day

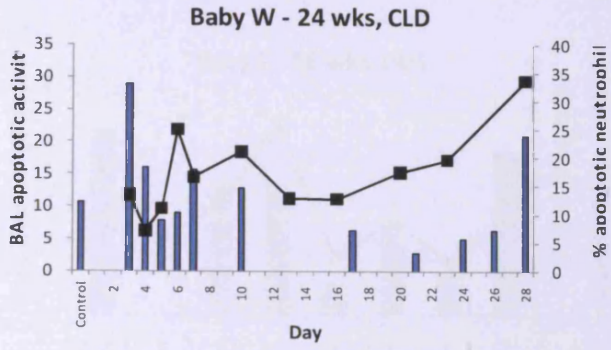

Baby CC - 27 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day

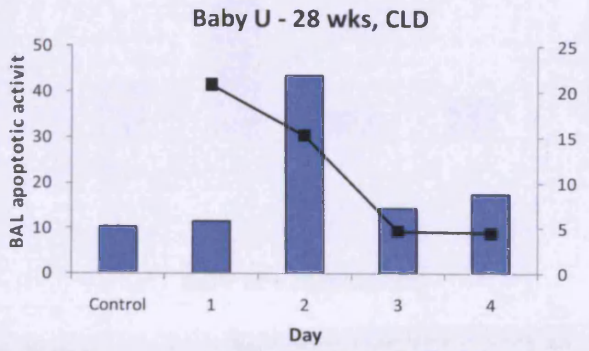

Baby O - 26 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day

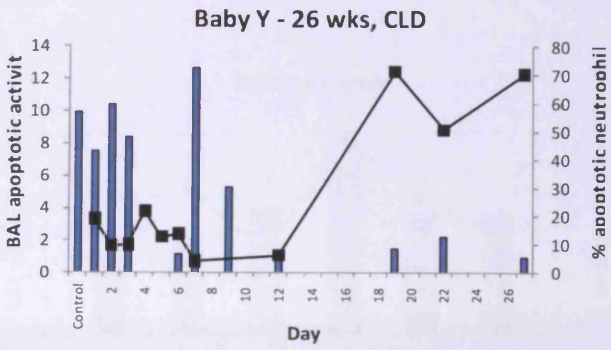

Baby G - 25 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day

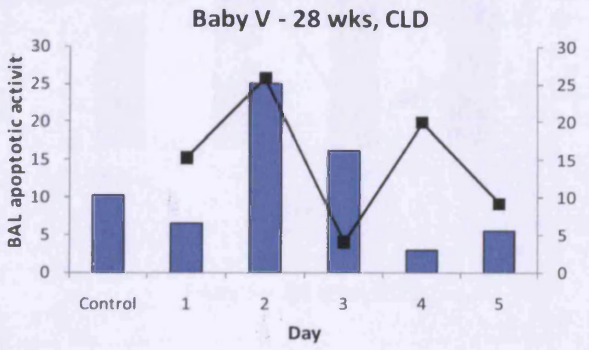

Baby Q - 25 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day

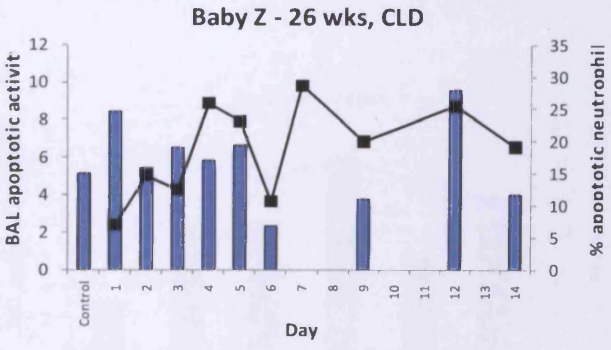

Baby R - 29 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day


Baby W - 24 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day


Baby U - 28 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day


Baby Y - 26 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day


Baby V - 28 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day


Baby Z - 26 wks, CLD
BAL apoptotic activit
% apoptotic neutrophil
Control
Day

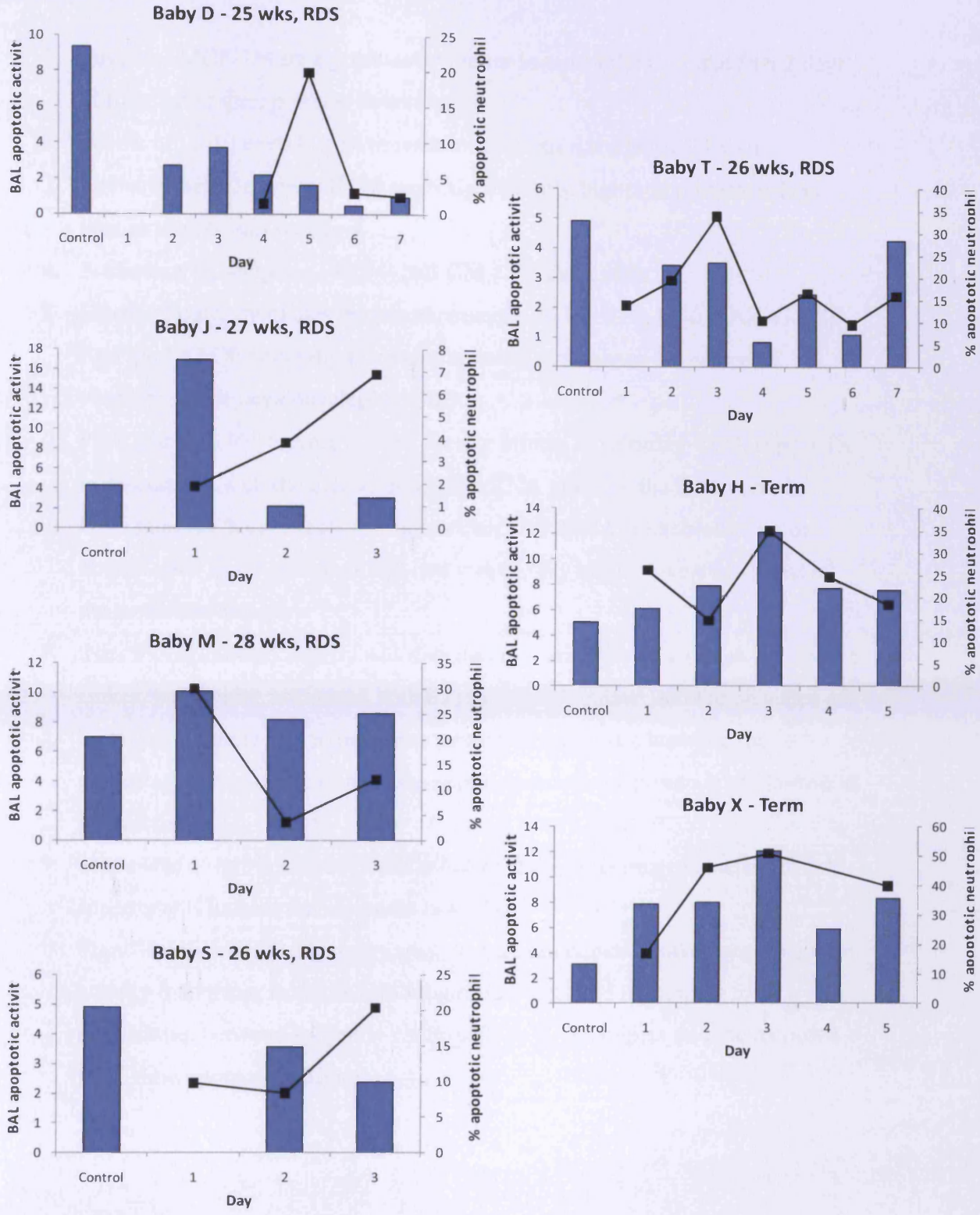


Figure 5.26 Graphs showing BAL fluid apoptotic activity (blue bars) and the percentage of apoptotic neutrophils (black line) in each BAL sample.

5.5 Summary of key findings

1. Levels of MCP-1 were significantly higher in term infants on the first 2 days of life than in their preterm counterparts.
2. Levels of IL-10 were lowest in term infants, but not significantly so.
3. Levels of MIP-1α and MIP-1β were significantly higher in preterm infants than in term babies on day 4.
4. A minority of samples (14/104) had GM-CSF detectable.
5. Levels of other cytokines measured, namely IL-1β, IL-8, IL-6, TNF, G-CSF, FasL and VEGF generally increased steadily throughout the period of ventilation in infants developing CLD.
6. Peak cytokine levels were highest among infants developing CLD, especially in association with the detection of 16S rRNA genes in the BAL sample.
7. BAL samples from infants in whom either 16S rRNA microbial genes or *Ureaplasma spp.* were identified had statistically higher levels of almost all the mediators tested.
8. Neutrophil elastase activity was detected in a minority of samples in episodic spikes, temporally associated with increasing cell counts and the presence of infection but there was a much less obvious relationship between the percentage of apoptotic or necrotic neutrophils and the presence of elastase in BAL fluid.
9. There was a significant relationship between the detection of elastase in BAL supernatants and the development of CLD.
10. Significantly more BAL supernatants from term babies showed pro-apoptotic activity than those from preterm infants, but there did not appear to be a clear relationship between apoptotic neutrophils in BALsamples and the recorded BAL fluid apoptotic activity.

5.6 Discussion

5.6.1 Introduction

The inflammatory environment in the lung is likely to be critical to the development of CLD. Not only do proteins like elastase have a direct effect on lung tissue, but the cytokine balance in the lung will have a marked effect on the composition of the cellular inflammatory response and the behaviour of those cells, thus contributing to the resolution or maintenance of the inflammatory response.

5.6.2 Cytokines

A large number of publications have studied cytokines in the neonatal lung under various conditions and reported generally that, although multiple pro-inflammatory and chemotactic factors are present in the airspaces of ventilated preterm infants, they are present in higher concentrations in those who develop CLD. Infants who develop CLD are also generally felt to be relatively lacking in the anti-inflammatory mediators which should promote resolution of their lung disease by various mechanisms including enhanced neutrophil apoptosis.

In this study, only MCP-1, MIP-1α and MIP-1β yielded statistically significant results for the differences between term and preterm infants. This may be partly as a result of the small number of infants studied as well as the fact that inflammatory mediators were measured only on selected days, raising the possibility of a significant peak in levels having been missed.

Peak levels of all the cytokines measured were significantly higher in infants developing CLD which may be related to the higher incidence of infection and higher cell counts in this group of infants.

Our longitudinal data for MCP-1 showed significantly higher levels of MCP-1 in term infants compared to infants born preterm on the first 2 postnatal days, but there was no difference between babies with RDS and those who go on to develop CLD. MCP-1 was measured in serial TAF samples from 56 preterm newborns by Baier *et al* (Baier

et al., 2001, Baier et al., 2002, Baier et al., 2004) who noted that MCP-1 rose over the first week of life and the highest levels were found in infants who developed CLD. Similarly, I found a marked increase in MCP-1 levels after day 2 and from day 4 onwards; some of the highest levels of MCP-1 were obtained from CLD infants, in concordance with the Baier papers. They showed a marked difference between RDS and CLD groups but their study lacked term controls.

Another study of MCP-1 levels in term infants with congenital diaphragmatic hernia showed a relationship between high levels of MCP-1 and severe pulmonary hypoplasia with persistent pulmonary hypertension but even the highest levels of MCP-1 in this study were of the order of 10^2-10^3 pg/ml rather than 10^4-10^5 pg/ml as in our infants (Okawada et al., 2007). This may be related to the fact that our control group is very small but also that these infants had surgery for their gastroschisis within hours of birth – usually before the first BAL sample was obtained, in keeping with our study protocol. Animal studies show elevated MCP-1 levels following soft tissue injury and surgical trauma (Kobbe et al., 2008, Kotzampassi et al., 2009) and this may be the mechanism of the elevated levels seen in our term infants.

MIP-1α and MIP-1β levels were expected to be elevated in preterm infants, particularly those developing CLD (Baier et al., 2004) and our data does confirm higher levels of both these cytokines on day 4 but there are insufficient data to make further comparisons.

One might expect that levels of pro-inflammatory cytokines might be dramatically increased in infants developing CLD while anti-inflammatory mediators may be reduced in concentration. This was not the case in our cohort and may reflect the very high cell counts in the CLD group compard to term infants – the amount of anti-inflamatory cytokines present may indeed be relatively low compared to pro-inflammatory cytokine levels but still elevated compared to term controls. There may be dysregulation of cytokine production on account of the profuse inflammatory response in the neonatal lung which may cause cells and cytokines to interact in ways that can not or have not yet been reproduced *in vitro*.

It is important to note that levels of inflammatory mediators are affected by a wide variety of other clinical factors, including the administration of antenatal steroids (Kramer, 2008), mode of ventilation (Capoluongo et al., 2005, Lista et al., 2008), oxygen concentration (Bhandari and Elias, 2006), pulmonary haemorrhage (Baier et al., 2002), drugs and medications including azithromycin (Aghai et al., 2007, Ballard et al., 2007), indomethacin (Sirota et al., 2001) and melatonin (Gitto et al., 2005) but may be unaffected by others e.g. nitric oxide (Truog et al., 2007).

The presence or absence of antenatal infection and the presence of postnatal systemic or isolated pulmonary infection may all play an important role in the alteration of cytokine expression in the lung (Kramer, 2008). I have shown a significant elevation in levels of almost all the mediators studied in infants in whom *Ureaplasma* or 16S rRNA genes were detected – this may be a contributory factor to the ongoing inflammatory process and highlight a possible reason for the significant relationship between the presence of *Ureaplasma* or other microbes and the development of CLD.

All of the above mentioned clinical factors may have contributed to the lack of marked difference between RDS and CLD groups of infants in our cohort and the cohort was too small to control for such a large number of possible confounders.

A caveat to all the cytokine experiments and results is that the amount of cytokine detected may not always be clinically relevant as the amount detected may not be proportional to its biological activity due to variable degrees of tissue binding, the availability of receptors and the presence of inhibitors or enhancers of cytokine activity which may be unmeasured or even unknown.

5.6.3 Elastase

There is much evidence to support the role of elastase as part of the complex pathogenesis of CLD. In the pre-surfactant era very high elastase activity was detected in the majority of lung lavage samples from infants with RDS, particularly in those infants who developed CLD (Merritt et al., 1983, Ogden et al., 1984, Watterberg et al., 1994). However more recently, following the introduction of routine use of exogenous surfactant in extremely preterm infants, the pattern of

elastase activity appears to have changed. A number of recent studies have found elastase in only a minority of samples from the lungs of preterm infants and no relationship to the development of CLD in these infants (Speer et al., 1993, Sveger et al., 2002, Sluis et al., 1994, Groneck et al., 1994). This may be as a result of changing neonatal practices such as exogenous surfactant administration and gentler ventilator techniques as well as a changing, more premature, neonatal population and increasingly specific laboratory methods for the detection of neutrophil elastase activity.

Our data appear to agree with this trend for fewer samples exhibiting elastase activity, with only around one quarter of samples and 19 out of 32 infants having detectable elastase activity. There was however a significant relationship between the presence of elastase and the development of CLD in the preterm group.

Previous studies in so-called "new" CLD have only examined elastase activity at single points in time (Sluis et al., 1994, Speer et al., 1993, Griese et al., 1998) or taken up to four samples from individual infants and frequently there were large gaps between time points (Sveger et al., 2002, Groneck et al., 1994).

This component of our study benefits from more frequent BAL sampling, enabling patterns to be evaluated in individual infants over time as well as being more likely to detect the brief increases in elastase which occur. These brief rises may be missed by "one off" sampling or overlooked in studies with large time gaps between samples.

The temporal relationship between cell counts and elastase activity is present in at least half of the infants studied. Elastase peaks are also frequently, but not invariably, associated with the presence of microbial genes in the BAL supernatants. Elastase is a product of neutrophil degranulation and a high neutophil load, possibly provoked by the presence of a pathogen in the respiratory tract, may produce sufficient elastase to overwhelm the natural elastase inhibitors, such as alpha-1-anti-trypsin, which would normally serve to neutralise its tissue damaging effects. Additionally the release of elastase potentiates the inflammatory response and enhances the recruitment of further inflammatory cells (Nakamura et al., 1992).

5.6.4 Apoptosis – the "Sheffield bio-assay"

In the only previous paper to study the effect of neonatal BAL supernatants on apoptosis of purified adult neutrophils, Kotecha *et al* (Kotecha et al., 2003) obtained 134 samples from 45 ventilated term and preterm neonates (in addition to a group of babies undergoing ECMO) and found that BAL fluid from babies whose RDS resolved was pro-apoptotic to adult neutrophils and CLD BAL fluid was not. Our cohort of 182 samples from 32 babies represents more samples per baby on average than the previous study but we found very few samples which were considered pro-apoptotic i.e. resulting in a higher proportion of apoptotic neutrophils than the saline control – only 9/182 samples at 5 hours and 3 at 20 hours. This was despite no obvious changes to the method used in the Kotecha *et al* paper, but subtle differences in patient population, BAL technique, reagents, purification of adult neutrophils or conduct of the assay can not be completely excluded from the list of reasons for differing findings. Neutrophils are highly sensitive cells to a wide variety of stimuli and some aspect of our experiment may have caused a degree of cell activation and thus a failure to show any pro-apoptotic effect of the supernatants.

The use of calculated rates of change in apoptosis between the 5 hour and 20 hour time points in our cohort produces a significant difference between term and preterm infants but no difference between RDS and CLD groups. High levels of cytokines (e.g. IL-8) in the BAL fluid tended to correlate with more anti-apoptotic activity in the BAL supernatant, which may be related to the presence of infection, as samples from which 16S rRNA genes were isolated were among the most anti-apoptotic samples in each individual baby. The relationship between apoptotic neutrophils and BAL supernatant apoptotic activity was more difficult to interpret, although there was the impression of a better response by term neutrophils to the pro-apoptotic nature of the BAL supernatant. Although inconclusive in this study, it is possible that term neutrophils are able to respond better to pro-apoptotic signals in their surroundings than neutrophils from more preterm infants.

The "Sheffield bio-assay" used purified adult neutrophils, however the results of this *in vitro* experiment may not be able to be extrapolated directly to preterm neutrophils, as neutrophils from preterm infants may exhibit some significant differences in their

ability to undergo apoptosis, compared to either adult or term neutrophils. (The differences between term newborn and adult neutrophils will be explored further in chapter 6 and this may be exaggerated in more preterm infants).

This *in vitro* experiment, however, may offer better insights into the response of lung neutrophils *in vivo* than experiments using individual cytokines or chemokines *in vitro*, as BAL supernatant constituents are a mixture of pro- and anti-inflammatory substances which can be quantified by methods such as CBA, used here.

5.6.5 Conclusion

This chapter has focussed on the BAL supernatant which reflects the environment in which the BAL cells are found. Various cytokines as well as neutrophil elastase were measured in the supernatants and the relative apoptotic activity of the supernatants against adult neutrophils was also assessed.

The relationship between cytokines and the development of CLD is complex and influenced by a wide variety of factors, particularly in relation to the clinical management of the infant. Further progress in neonatal intensive care may result in methods of management which further reduce the pro-inflammatory nature of the exudates within the alveoli and lead to a reduction in or amelioration of CLD.

Although elastase seems less important in the pathogenesis of "new" CLD, it is still a mechanism by which lung tissue damage can be mediated. Elastase may also contribute to the overall pro-inflammatory environment within the lung by being chemotactic for inflammatory cells and by regulating the activity of cytokines and chemokines (Nakamura et al., 1992, Pham, 2006).

The relative pro- or anti-apoptotic ability of BAL supernatants was also studied and a temporal relationship between low apoptotic activity and higher cytokine levels and the presence of microbial genes was seen in individual infants. However, this was not a completely consistent finding and raises the possibility that neutrophil apoptosis may not be completely regulated by factors external to the cell.

Overall, the relationship between the studied constituents of BAL supernatants and CLD is complex and reflects the multi-factorial nature of the disease. In order to control for the very wide variety of influences on the content of the BAL supernatant, many *in vitro* studies have been conducted, but these ignore the complex and multi-facetted interactions between components of the BAL supernatant, between the different types of BAL cells and their environment and the impact of clinical variables such as changing modes of ventilation and the administration of drugs whose deliberate effect or unintended side-effect may be pro- or anti-inflammatory.

Chapter 6

Cord blood

Chapter 6
Cord blood

6.1 Introduction

In chapters 3 and 4 I showed that, while large numbers of neutrophils continue to be present in the lungs of preterm infants who progress to CLD, the proportion of the neutrophil population which is undergoing apoptosis is significantly reduced in preterm infants compared to those born at term. Additionally in chapter 5, it was seen that neutrophil apoptosis is not always directly related to the levels of various pro- or anti-apoptotic influences in the supernatant. One possible explanation for this could be that neutrophls in the preterm infant are inherently more resistant to apoptosis, possibly due to a degree of immaturity of one or more parts of the apoptotic pathways.

It has previously been shown that cord blood neutrophils are relatively resistant to apoptosis when compared to adult neutrophils (Molloy et al., 2005, Molloy et al., 2004, Koenig et al., 2005, Luo et al., 2003, Hanna et al., 2005, Allgaier et al., 1998) but there is also a single paper that reports exactly opposite findings (Uguz et al., 2002). Many of these studies had investigated aspects of the role of infection in this process *in vitro* and some have attempted to investigate the possible mechanism for the differences observed. Most investigations have concentrated on factors external to the neutrophil (labour, infection, medications, cytokines, Fas and colony stimulating factors) while two (Luo et al., 2003, Hanna et al., 2005) have looked more closely at the caspases and the Bcl-2 family of proteins.

In this chapter, I have examined the effect of maturity upon the ability of neutrophils to undergo apoptosis by comparing neutrophils isolated from newborn term cord blood and adult blood. In particular I have examined:

- the changes in the rate of apoptosis related to infection, using LPS to mimic some of the effects of infection
- levels of activated caspase 3, the most central workhorse of the caspases in the apoptotic process

- the amount of Bax present, a pro-apoptotic member of the Bcl-2 family of proteins
- amounts of RNA present for both caspases and the Bcl-2 family of proteins using a ribosomal protection assay (RPA)
- the amounts of some of the Bcl-2 proteins in adult and cord neutrophils using reverse transcription and quantitative PCR techniques.

6.2 Results

6.2.1 Patient information/demographics

Cord blood was collected (as detailed in section 2.2) in the first few minutes following delivery of term infants by elective Caesarean section. The most common indication for Caesarean section was in mothers who had had a previous Caesarean and had opted for this mode of delivery in their subsequent pregnancy. None of the women was in labour at the time of operation, since the process of labour may be responsible for activation of neonatal neutrophils (Molloy et al., 2004), nor did they have any identified risk factors for infection, another potential activator of neonatal neutrophils.

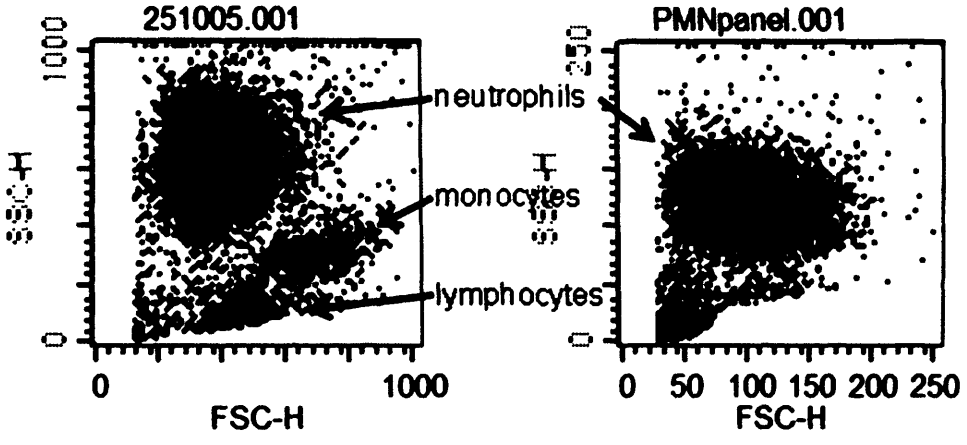
In accordance with ethical guidelines and so as not to delay sample collection, verbal assent was obtained from the mother in advance of the cord blood being collected and written consent was obtained post-operatively, later the same day. Cord blood samples were collected and then processed in parallel with peripheral venous blood samples from adult volunteers as in section 2.2, ensuring cord neutrophils and adult control neutrophils were exposed to identical experimental conditions as far as possible.

6.2.2 Purity of neutrophil preparations

There is considerable debate about whether neutrophil preparations should be completely free from other cell types, particularly monocytes, in experiments of this nature because of the ability of these macrophages to produce cytokines which may activate neutrophils and alter experimental results (Sabroe et al., 2004). I elected to try

to obtain the purest possible neutrophil preparations, without resorting to magnetic bead separation or other processes, such as flow sorting, which required extensive handling/manipulation of the neutrophils and could result in cell activation.

Using the technique detailed in section 2.2.2, it was possible to obtain a population of cells consisting of in excess of 97% neutrophils with negligible activation of the neutrophils. Any contaminating cells were usually eosinophils, very occasional monocytes, or, in the case of cord blood samples, immature (nucleated) red blood cells. There was no significant difference in the percentage of contaminating cells between adult and cord blood samples (Adult mean 0.84%, median 0.80%; cord mean 1.89%, median 1.40%; p=0.329) (Figure 6.1).



A B

Figure 6.1 Flow cytometer (forward scatter vs side scatter) plots showing (A) whole blood and (B) adult or cord neutrophil preparations. The lack of lymphocyte and monocyte populations in the prepared neutrophils can be clearly seen.

6.2.3 Phenotyping of cord blood

Using flow cytometry I compared the surface expression of relevant cell surface markers (CD15, CD16, CD14, CD11b, TLR2 and TLR4) and combinations of these markers (CD14/15, CD15/16, and CD14/16) between 7 paired samples of cord and adult neutrophils in order to assess whether there was any difference in surface marker expression between the two types of sample immediately after neutrophil

separation. No significant differences in the chosen markers were observed (Figure 6.2).

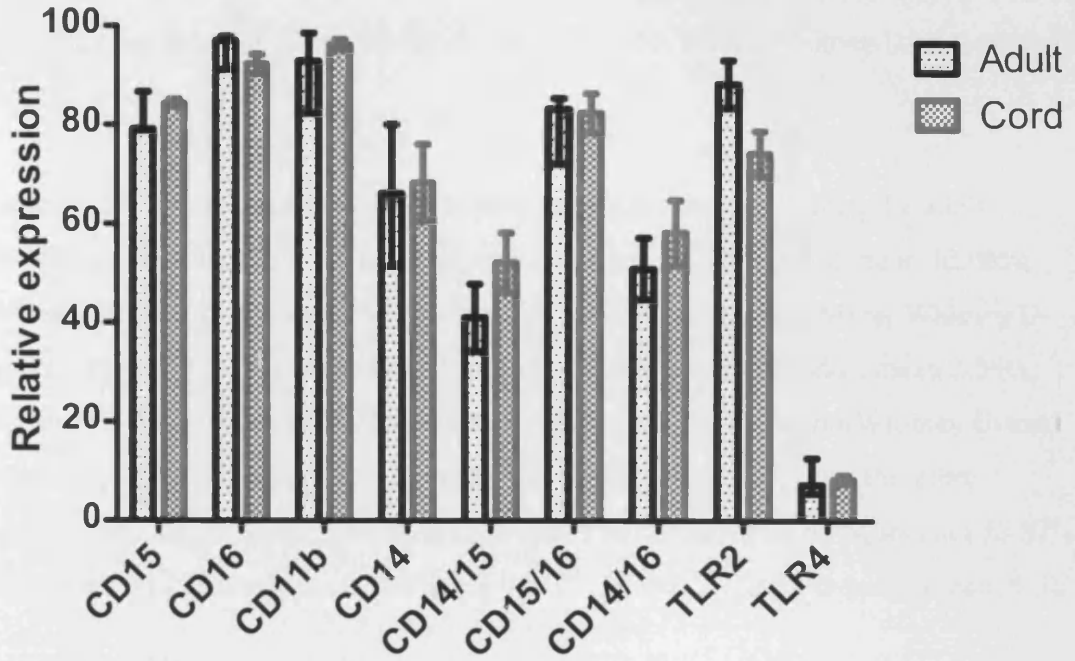


Figure 6.2 Bar graph showing median relative expression of various neutrophil cell surface markers in cord and adult neutrophils. Error bars show interquartile ranges. There are no statistically significant differences between any of the pairs. (n=7 pairs)

6.2.4 Neutrophil apoptosis in cord and adult blood

Neutrophil apoptosis was quantified after neutrophil purification (time 0) and again after 6 and 20 hours in culture, by means of flow cytometry using annexin-V and To-Pro 3 staining (see methods 2.1.4.2 and 2.1.4.3) in 8 paired cord and adult blood samples. The proportion of apoptotic cells was also confirmed by light microscopy of cytospin preparations.

The percentage of apoptotic neutrophils in cord and adult blood immediately after neutrophil isolation was similar in both cord and adult blood (Adult mean 4.90%, median 3.34%, range 2%-12.97%; cord mean 4.62%, median 3.01%, range 0.51%-12.00%, p=0.73, Mann-Whitney U-test).

It was not unexpected therefore that the percentage of necrotic cells and the viable percentage were also not significantly different (necrotic adult mean 2.02%, median 1.45%; cord mean 1.47%, median 1.08%, p=0.95; viable adult mean 90.20%, median 92.77%; cord mean 86.60%, median 92.06%, p=0.80, Mann-Whitney U-test) between the two groups at the outset.

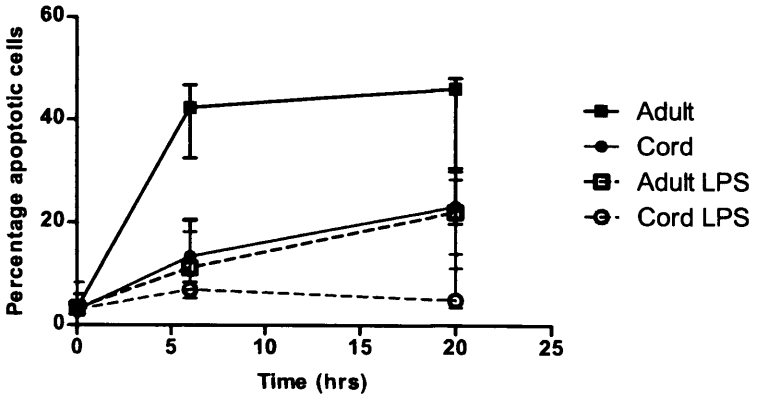
Following 6 hours in culture the percentage of apoptotic cells among the adult neutrophils was significantly higher than in cord neutrophils (adult mean 40.08%, median 42.38%; cord mean 15.58%, median 13.36%; p=0.0013, Mann-Whitney U-test) (Figure 6.3). The percentage of necrotic cells was similar (adult mean 2.39%, median 1.07%; cord mean 3.71%, median 1.66%; p=0.3659, Mann-Whitney U-test) (Figure 6.4) and the percentage of viable neutrophils remaining was therefore significantly higher among the cord neutrophils (adult mean 55.85%, median 53.97%; cord mean 78.77%, median 82.42%; p= 0.0127, Mann-Whitney U-test) (Figure 6.5).

After 20 hours in culture the adult neutrophils continued to have a significantly higher percentage of apoptotic cells (adult mean 41.22%, median 46.24%; cord mean 21.64%, median 23.20%; p=0.020, Mann-Whitney U-test) (Figure 6.3) and a significantly lower percentage of viable cells (adult mean 30.33%, median 29.42%; cord mean 57.18%, median 56.66%; p=0.0027, Mann-Whitney U-test) (Figure 6.4). The percentage of necrotic cells among adult neutrophils was slightly higher than in cord blood (adult mean 23.67%, median 25.24%; cord mean 16.06%, median 15.30%) but this was not statistically significant (p=0.155, Mann-Whitney U-test) (Figure 6.5).

Addition of 50 ng/ml LPS to the culture medium resulted in a statistically significant reduction in the percentage of apoptotic neutrophils in adult samples at both 6 and 20 hours (6 hours - adult mean 40.08%, median 42.38%; adult LPS mean 13.44%, median 11.12%; p=0.0002, Mann-Whitney U-test) (20 hours - adult mean 41.22%, median 46.24%; adult LPS mean 24.29%, median 22.06%; p= 0.0207, Mann-Whitney U-test) (Figure 6.3). In cord blood neutrophils, the same conditions resulted in no significant difference in the percentage of apoptotic cells at 6 hours (cord mean 15.58%, median 13.36%; cord LPS mean 11.31%, median 6.93%; p= 0.180, Mann-Whitney U-test) but there was a statistically significant reduction in the percentage of

apoptotic cells by 20 hours when LPS was added to cord blood neutrophils in culture (Cord mean 21.64%, median 23.20%; Cord LPS mean 7.89%, median 4.96%; p= 0.0260, Mann-Whitney U-test) (Figure 6.3). There was no significant difference in the percentage of necrotic cells present in any of the cultures at either 6 (adult vs adult LPS: adult mean 2.39%, median 1.07%, adult LPS mean 3.35%, median 1.40%; p= 0.792; cord vs cord LPS: cord mean 3.71%, median 1.66%; cord LPS mean 5.11%, median 2.93%; p= 0.423, Mann-Whitney U-test) or 20 hours (adult vs adult LPS: adult mean 23.67%, median 25.24%, adult LPS mean 26.13%, median 22.62%; p= 0.959; cord vs cord LPS: cord mean 16.06%, median 15.30%; cord LPS mean 18.30%, median 17.88%; p= 0.699, Mann-Whitney U-test) (Figure 6.5).

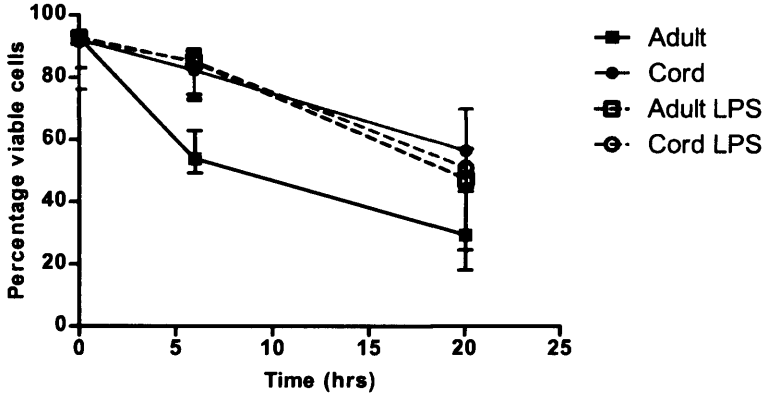
As a result of the large reduction in apoptosis in adult cells after the addition of LPS and the negligible change in apoptotsis in cord blood cells to which LPS had been added, the significant difference seen between adult and cord neutrophil apoptosis at 6 hours of culture was not present when LPS was added to both adult and cord neutrophils (adult mean 13.44, median 11.12; cord mean 11.31, median 6.93; p= 0.345, Mann-Whitney U-test) (Figures 6.3, 6.4 and 6.5) however, the significant difference in apoptosis between the two types of samples was apparent at 20 hours when neutrophils were cultured with LPS (adult LPS mean 24.29, median 22.06; cord LPS mean 7.89, median 4.95; p= 0.0013, Mann-Whitney U-test) (Figure 6.3). There was no significant difference in the percentage of necrotic cells between the two sample types at either time point (6 hours adult LPS vs cord LPS p= 0.0811; 20 hours adult LPS vs cord LPS p= 0.796, Mann-Whitney U-test). The percentage of viable cells was not significantly different at 6 hours (p= 0.846) or 20 hours (p= 0.302).



At 6 hrs, p=0.0013 for adult vs cord and p=0.0002 for adult vs adult LPS.

At 20 hrs, p=0.002 for adult vs cord, p=0.0207 for adult vs adult LPS, p=0.026 for cord vs cord LPS and p=0.0013 for adult LPS vs cord LPS.

Figure 6.3 Graph showing the percentage of apoptotic cells in cultured cord and adult neutrophils at three time points, with and without addition of LPS. (n= 8 pairs)



For adult vs cord, p=0.012 at 6 hrs and p=0.0027 at 20hrs.

Figure 6.4 Graph showing the percentage of viable cells in cultured cord and adult neutrophils at three time points, with and without addition of LPS. (n= 8 pairs)

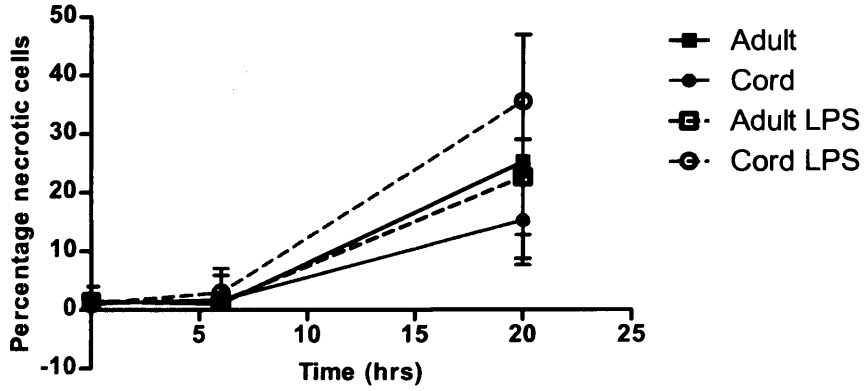


Figure 6.5 Graph showing the percentage of necrotic cells in cultured cord and adult neutrophils at three time points and two conditions (with and without addition of LPS). (n= 8 pairs).

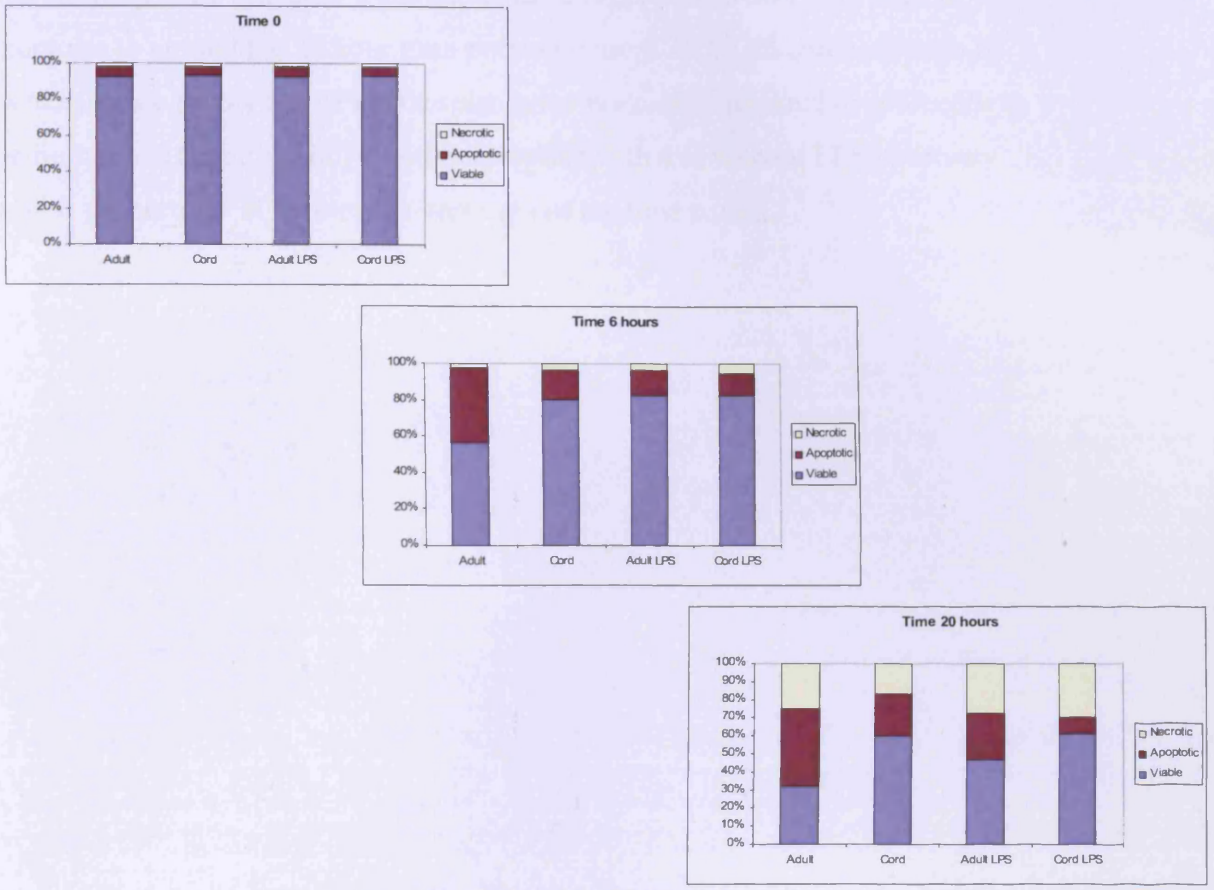
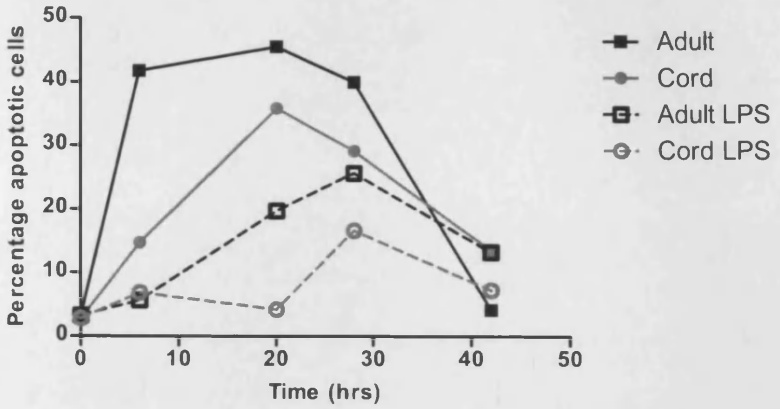


Figure 6.6 Bar graphs showing the relative proportions of apoptotic, necrotic and viable neutrophils in cultured adult and cord cells at (A) time 0, (B) 6 hours and (C) 20 hours. (n= 8 pairs)

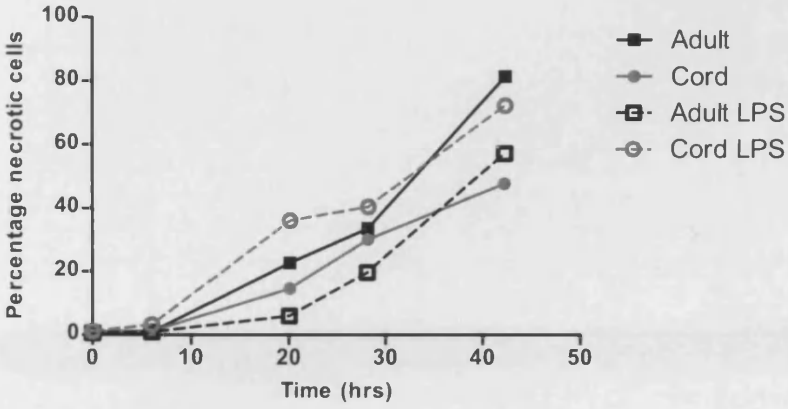
6.2.5 Extended time points

In one adult/cord pair, the neutrophils were continued in culture for 42 hours in order to observe any further changes (Figure 6.7 A, B and C). Although this is only a single pair of samples, in both adult and cord neutrophils incubated with medium alone, the percentage of apoptotic cells reaches a peak at around 20 hours before beginning to fall again as viable cells steadily decrease and the proportion of necrotic cells increases. This probably reflects an increasing number of apoptotic cells undergoing secondary necrosis in the absence of macrophages to remove the apoptotic cells by phagocytosis.

The addition of LPS to both adult and cord cells appears to delay the peak in apoptosis to around the 28 hour time point (Figure 6.7A). LPS can be seen to be increasing the proportion of viable cells quite markedly compared to adult cells in medium alone (Figure 6.7C). Cord neutrophils with and without LPS have very similar proportions of viable cells throughout the time period.



A



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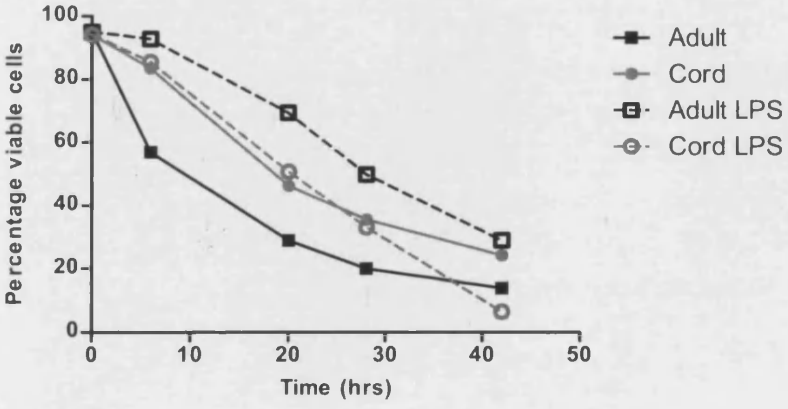


C

Figure 6.7 Graphs showing the percentage of (A) apoptotic, (B) necrotic and (C) viable neutrophils in adult and cord cells with and without the addition of LPS over a period in culture of 42 hours.

6.2.6 Possible mechanisms for delayed apoptosis in cord blood

6.2.6.1 Bax

An early and critical step in the so-called intrinsic apoptosis pathway is the activation of Bax. Bax activation allows Bax to pierce holes in the outer mitochondrial membrane allowing for cytochrome c to be released and combine with Apaf 1. This complex in turn is able to activate caspase 9.

I chose to examine Bax activation in 8 paired samples of purified adult and cord neutrophils by flow cytometry, as described in section 2.2.4.4.

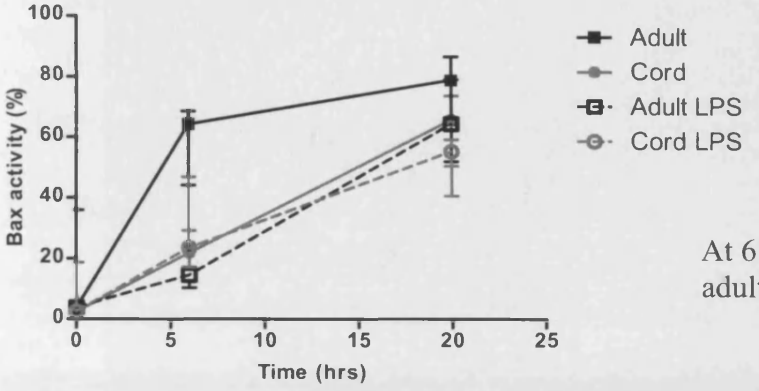
Immediately following neutrophil separation, there was no significant difference in the expression of Bax in adult and cord neutrophils (Adult mean 38.89%, median 5.01%; cord mean 35.92%, median 11.04%; p=1.00).

After 6 and 20 hours in culture with medium alone there was no significant difference between the percentage of cells expressing Bax in adult and cord samples (p=0.06 at 6 hours and p=0.09 at 20 hours), despite the percentage of apoptotic cells in these samples being significantly different (see 6.2.4 above). One might expect a significant increase in the percentage of cells expressing Bax when the percentage of apoptotic cells in the sample is significantly inceased, however this was not seen.

At 6 hours, more adult neutrophils cultured in medium alone expressed Bax than adult neutrophils cultured with LPS. This was a statistically significant difference (p=0.031) consistent with the observation that there are significantly more apoptotic cells among medium cultured adult neutrophils than among those cultured with LPS. After 20 hours there was no significant difference between the 2 conditions (p=0.15), despite a significant difference being observed in the percentage of apoptotic cells present (see 6.2.4 and Figure 6.3 above). This raises a number of questions: is there a delay between Bax activation and the appearance of apoptosis detectable by flow cytometry or does this reflect a maximal activation of Bax within the neutrophil at 20 hours or is there another, anti-apoptotic, factor acting in opposition to the pro-apoptotic effects of Bax?

Cord neutrophils cultured with LPS had similar levels of Bax activity to cord neutrophils cultured in medium only (6 hours p=0.5887; 20 hours p= 0.1797).

These results suggest that a difference in Bax is probably not the reason for the differences observed in rates of apoptosis between cord and adult neutrophils, although the reduction in apoptosis in the presence of LPS, particularly in adult neutrophils at 6 hours, may be related to a reduction in Bax activity, but this is not observed in cord blood.



At 6 hours, p=0.031 for adult vs adult LPS.

Figure 6.8 Graphs showing the percentage of cells with detectable Bax activity at 3 time points, with and without LPS.

6.2.6.2 The Bcl-2 family of apoptotic proteins

The Bcl-2 family of proteins, which includes Bax, is central to the so-called intrinsic pathway of apoptosis. I chose to further investigate this family of molecules in cord and adult neutrophil preparations.

RNA was extracted from purified cord and adult neutrophils (4 pairs) as explained in section 2.2.5. The extracted RNA was frozen and then sent to Sheffield, where it was used in RNase protection assay (RPA) and RT-PCR, performed by Vanessa Singleton and Dr Lynne Prince, in order to further investigate the possible differences in the Bcl-2 family of proteins between the two types of cells. RNA was only extracted from fresh neutrophils, not from cells which had been left to apoptose in culture, because of

the very large number of neutrophils required to obtain sufficient RNA for the assays and in order to assess if any differences exist between adult and cord neutrophils at baseline, rather than once apoptosis has commenced.

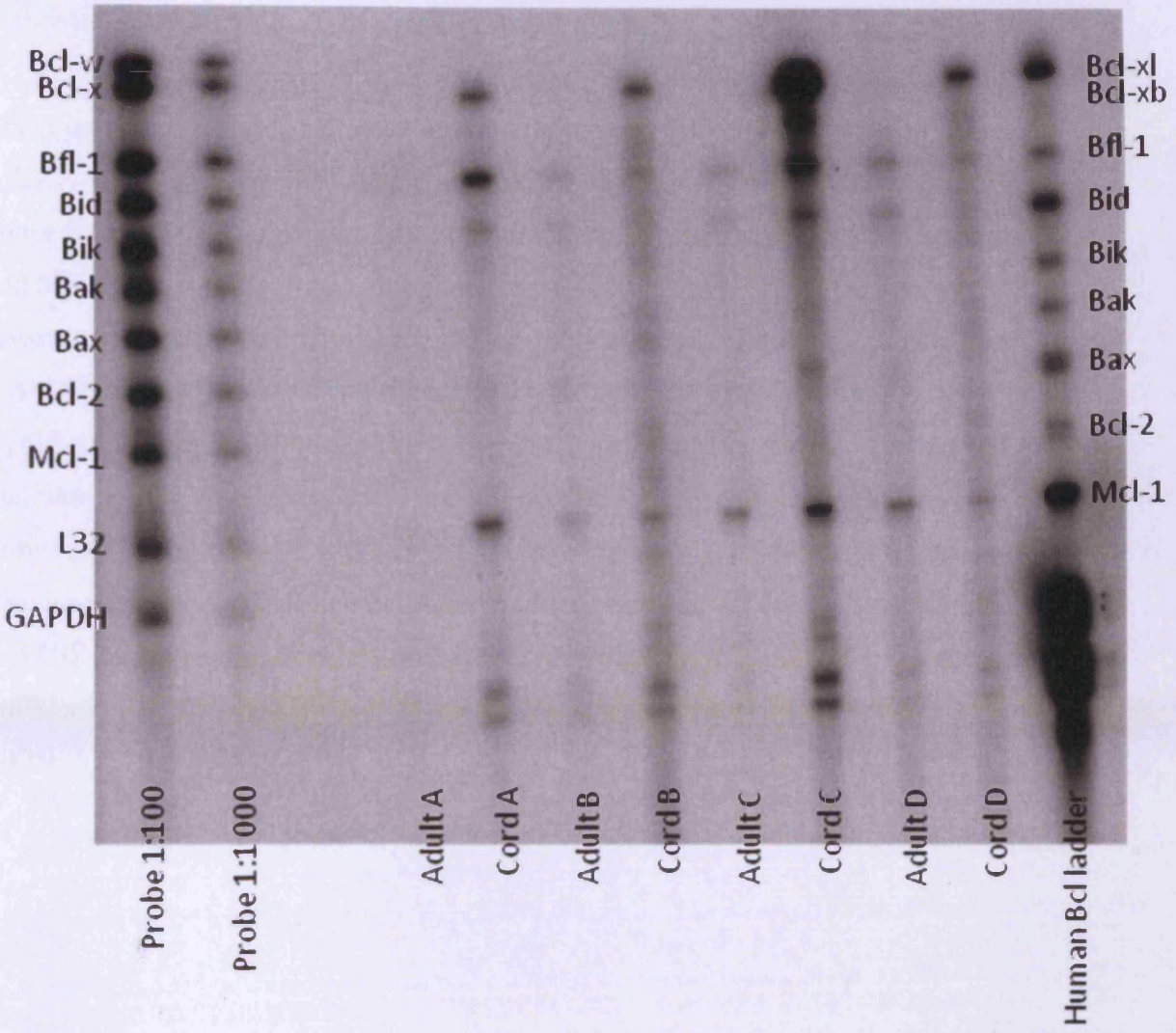


Figure 6.9 Photograph of RPA autoradiograph showing larger amounts of Bcl-xl in cord and Mcl-1 in adult lanes.

An equal load of RNA was placed in each lane of the gel, however it can be seen in Figure 6.9 that "housekeeping" genes such as GAPDH are expressed in greater quantities in cord neutrophils; this may be because cord neutrophils are more transcriptionally active and may have a higher proportion of their RNA present as mRNA, rather than rRNA, explaining the higher global gene expression in the cord samples.

Looking at the four pairs of samples that were subjected to this analysis, and allowing for the relatively heavier staining of the cord lanes, there appear to be larger amounts of both the anti-apoptotic proteins Bcl-xl and A1 (bfl-1) in cord blood neutrophils and higher levels of Mcl-1 in adult neutrophils.

These perceived differences were further analysed by RT-PCR as described in section 2.2.5.3, the results of which are summarised in Figure 6.10. When normalised for either GAPDH or β-actin, Bcl-xl was significantly upregulated in the cord neutrophils and Mcl-1 was significantly more abundant in adult neutrophils. There was no statistically significant difference in A1 or in GAPDH between adult and cord cells. GAPDH and HIF1α are both induced by hypoxia but were not statistically significantly different between the two groups, indicating that the relative hypoxia of the intra-uterine environment was not likely to be the cause of the differences observed. HIF 2α, along with HIF 1α, is also a key regulator of the transcriptional response to hypoxia (Bracken et al., 2005, Scortegagna et al., 2003), however HIF 1α and HIF 2α have unique targets and different biological effects (Patel and Simon, 2008) and it is mainly HIF 1α which regulates neutrophil survival (Walmsley et al., 2005).

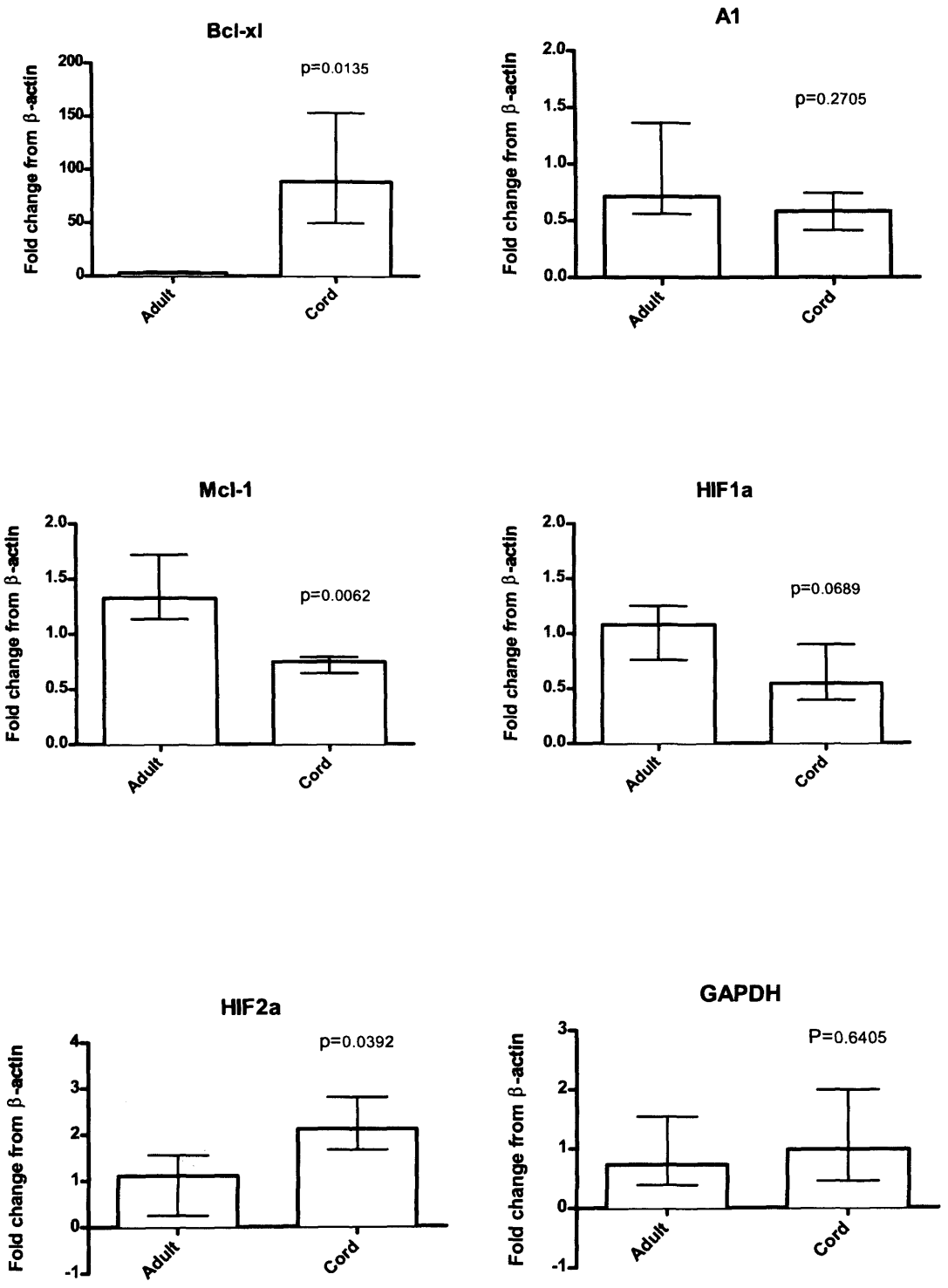


Figure 6.10 Graphs showing median fold change in (A) Bcl-xl, (B) A-1, (C) Mcl-1, (D) HIF-1α, (E) HIF-2α and (F) GAPDH for cord and adult neutrophil RNA subjected to RT-PCR. (n=4)

6.2.6.3 Caspase 3

Caspase 3 is a central executioner of apoptosis which cleaves many key cytoskeletal components at an Asp-Xaa-Xaa-Asp (DXXD) motif (Cohen, 1997). Caspase 3 activation by cleavage of pro-caspase 3 is an early apoptotic event. I chose to study this caspase further due to its probable central role in the "final common pathway" of both intrinsic and extrinsic proposed pathways of apoptosis.

Caspase 3 was measured by flow cytometry at each of the three time points (0, 6 and 20 hours) in neutrophils purified from 8 pairs of cord and adult blood samples using Apo Logix ™ - SR Sulforhodamine Caspase Detection Kit (Peninsula Laboratories Inc, California, USA) as described in chapter 2, section 2.2.4.3.1.
There was no significant difference in MFI or the percentage of cells positive for activated caspase 3 at any of the time points or conditions, despite the significant differences observed in the percentage of apoptotic cells in section 6.2.4 above.

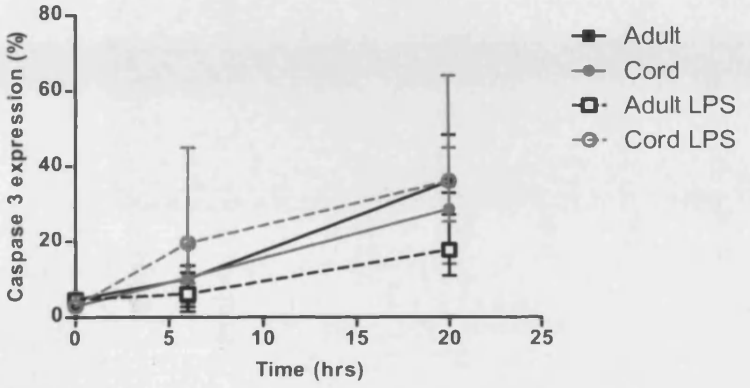


Figure 6.11 Graph showing percentage of cells expressing caspase 3 at 3 time points, with and without LPS.

6.2.6.4. Other caspases

Similar to the Bcl-2 family, the caspases were also analysed by RPA methodology by Vanessa Singleton in Sheffield, using RNA extracted and frozen in Cardiff from neutrophils which were isolated from 4 pairs of cord and adult blood samples. Again, despite equivalent loading of RNA, the cord neutrophils appear to have higher

amounts of the loading controls GAPDH and L32. Caspases 1, 4 and 8 are expressed highly in both cord and adult neutrophils but differences in caspases between cord and adult neutrophils are difficult to elucidate from this assay. These findings have not been further analysed by RT-PCR.

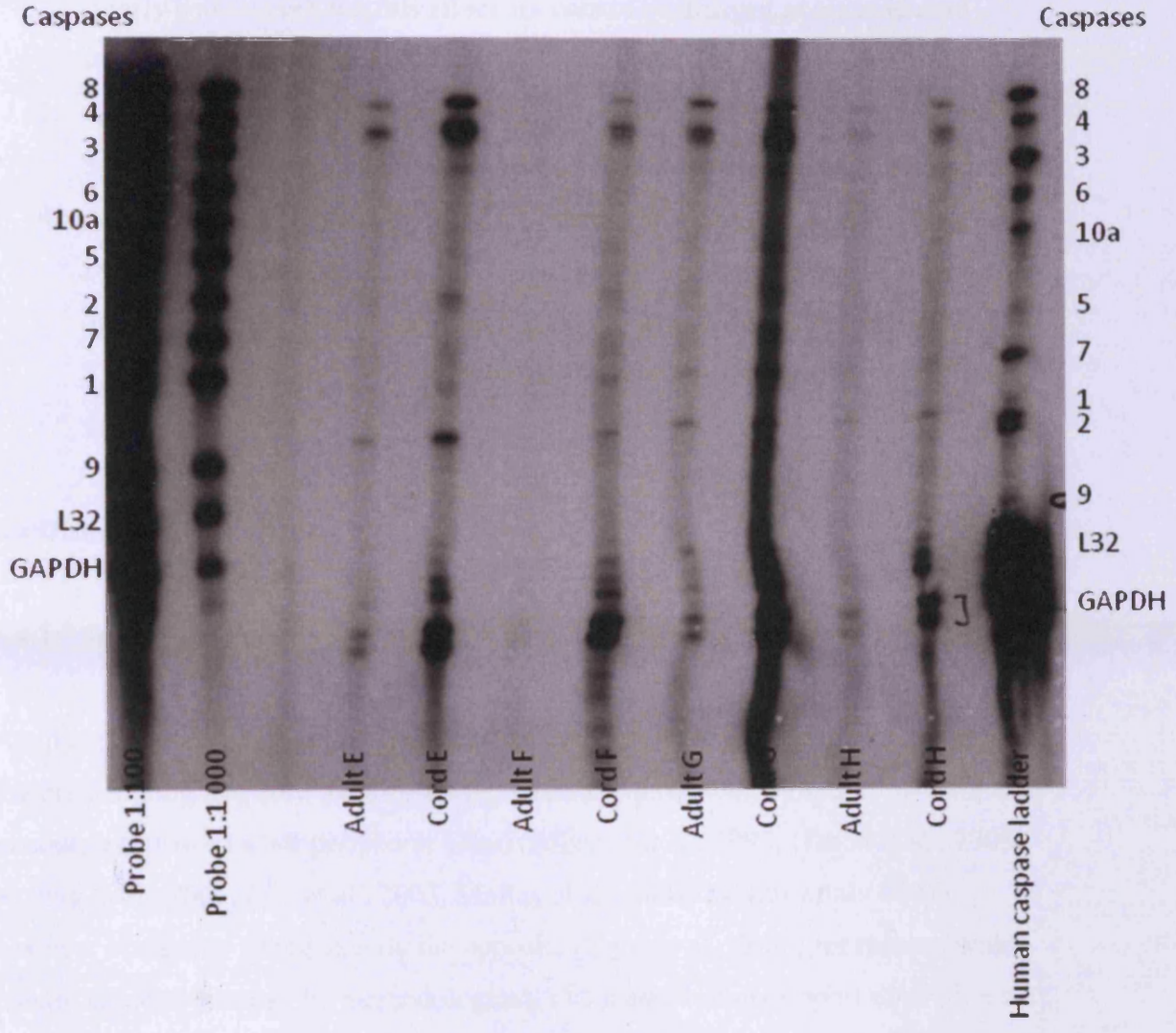


Figure 6.12 Photograph of autoradiograph of RPA for caspases, showing high expression of caspases 1, 4 and 8 in adult and cord lanes.

6.3 Summary of main findings

1. I confirmed that cord blood neutrophils from term infants undergo apoptosis less readily than adult neutrophils.
2. Apoptosis in adult neutrophils can be delayed by the addition of LPS (seen clearly by 6 hours), but this effect appears to be delayed in onset in cord neutrophils (only seen at 20 hours).
3. A similar proportion of both cord and adult neutrophils have Bax and caspase 3 activity detected despite higher levels of apoptosis in adult neutrophils.
4. Adult and cord blood have differing expression of anti-apoptotic proteins of the Bcl-2 family, specifically cord neutrophils have higher levels of the anti-apoptotic protein Bcl-xl and lower levels of the pro-apoptotic protein Mcl-1. These differences cannot be attributed solely to the relative hypoxia of intra-uterine life.

6.4 Discussion

6.4.1 Introduction

Apoptosis in cord blood neutrophils has been studied by a number of groups, with the majority finding that cord neutrophils were relatively resistant to apoptosis compared to neutrophils from adult peripheral blood (Allgaier et al., 1998, Hanna et al., 2005, Koenig et al., 2005, Luo et al., 2003, Molloy et al., 2004, Molloy et al., 2005), however one group found exactly the opposite (Uguz et al., 2002) for reasons which remain unclear, but may be methodological. The method of neutrophil separation may have a profound effect on neutrophil activation and thus the ability of the cells to undergo apoptosis. "Contamination" of neutrophil preparations with monocytes may also produce altered results in apoptosis experiments as monocytes are a source of cytokines and other inflammatory mediators which prolong neutrophil survival (Sabroe et al., 2004). It is possible that these studies differed in their results because of the way in which the neutrophils were purified and handled prior to commencing the apoptosis experiments.

I elected to use umbilical cord blood from infants born at term by elective Caesarean section to healthy mothers for two main reasons:

- cord blood is available in sufficient quantities at delivery without compromising the health or medical care of the mother or baby (the cord and the blood contained therein are normally disposed of following inspection by the delivering midwife) (range <5 – 60 ml per cord, average 20 – 25 ml per cord in experienced hands).
- babies delivered by Caesarean section prior to the onset of labour are the least likely to have neutrophils which have been primed or activated either by infection or the process of labour.

6.4.2 Neutrophil apoptosis

I confirmed the findings of published studies by using flow cytometry to assess neutrophil apoptosis in preparations of cord and adult neutrophils which were effectively free of monocyte contamination, which may have altered rates of apoptosis of the neutrophils in culture (Sabroe et al., 2004).

Cord blood neutrophils did indeed undergo apoptosis less readily that those from adult peripheral blood and also appeared less able to respond to the addition of LPS. This hyporesponsiveness of neonatal neutrophils to LPS has previously been described by Molloy *et al* (Molloy et al., 2008) in a paper published shortly before our experiments had been completed. They attributed this relative lack of response to LPS to the failure of neonatal neutrophils to upregulate TLR 4 in response to sepsis (Molloy et al., 2006, Viemann et al., 2005). I found a similar expression of TLR 2 and 4 and CD14 on cord and adult neutrophils, suggesting that the poor response to LPS that was witnessed in cord neutrophils is mediated by intracellular mechanisms, rather than the neutrophils being unable to respond to the stimulus.

6.4.3 Mechanisms for the delay in neonatal neutrophil apoptosis

Luo *et al* (Luo et al., 2003) used RT-PCR and enzymatic assays to describe a reduction in caspase 3 mRNA and functional activity as a possible cause for the delay in Fas mediated neutrophil apoptosis in cord neutrophils. Hanna *et al* (Hanna et al.,

2005) described reduced expression of the Fas receptor in neonatal neutrophils as well as reduced expression of caspase 3 and the pro-apoptotic proteins Bax, Bad, and Bak by flow cytometry.

I found no significant difference in caspase 3 activation between adult and cord samples, either in fresh neutrophils or those allowed to undergo apoptosis in culture. The results of the RPA appeared to agree with this finding, but suggest that further investigation of other caspases may yield more useful results.

The Bcl-2 family comprises both pro- and anti-apoptotic member proteins. Bax is a pro-apoptotic protein which, following activation by Bid, makes holes in mitochondrial membrane. Our data show that levels of Bax activation are remarkably similar in cord and adult neutrophils, especially when the delay in apoptosis in cord neutrophils is considered. This raises the question of whether there is another, anti-apoptotic, factor acting in cord blood to produce the delay in apoptosis which I observed.

The delay in cord neutrophil apoptosis may be partly explained by the significant increase in Bcl-xl which I observed in cord neutrophils. Bcl-xL appears to be the dominant regulator of apoptosis and has been called the "survival protein" because of its cell death repressor activity. Bcl-xl has been shown to compete with Bax and prevent the binding of Bax to the mitochondrial membrane (Billen et al., 2008), thus delaying or preventing apoptosis. This may be the mechanism by which cord blood neutrophils experience a delay in apoptosis and still have the similar levels of Bax activation which I have described.

Bax and Bcl-xl have been studied in adult and neonatal rat cardiac myocytes, where neonatal cells have high levels of both Bcl-xl and Bax but with age, Bcl-xl is maintained but Bax levels are barely detectable in adult rat hearts (Cook et al., 1999). In neuronal tissue Bcl-xl is not downregulated in the adult rat although Bax levels are 20- to 140-fold lower in adult compared to neonatal rat tissues (Vekrellis et al., 1997). Adult peripheral blood neutrophils have previously been shown to express very low levels of both Bcl-xl and Bax, as well as Bcl-2, Bik and caspase 2 (Santos-Beneit and Mollinedo, 2000), although an earlier study found only Bax and no other Bcl-2 family

members in adult neutrophils (Ohta et al., 1995). Apart from the Hanna *et al* (Hanna et al., 2005) paper, there have been no published comparisons, to our knowledge, looking in detail at the Bcl-2 proteins in adult and neonatal neutrophils.

Additionally, I have shown levels of Mcl-1 protein are significantly increased in adult neutrophils when compared to cord cells. Mcl-1 is generally an anti-apoptotic member of the Bcl-2 family (Michels et al., 2005) and had been described as being essential for the survival of neutrophils (Dzhagalov et al., 2007). It promotes cell survival by interfering in the cascade of events leading to release of cytochrome c from mitochondria. However, Mcl-1 can also be cleaved by caspases during apoptosis to produce a cell death promoting molecule (Michels et al., 2005) and this may also be significant in explaining the differences observed between adult and cord neutrophil apoptosis. The cell death promoting activity of Mcl-1 may be partly responsible for adult neutrophils undergoing apoptosis more readily than cord neutrophils.

Hypoxia inducible factor (HIF) is an oxygen-sensitive transcription factor which regulates cell responses to hypoxia and is upregulated under conditions of low oxygen tension (Walmsley et al., 2008). HIF-1α promotes neutrophil survival but the mechanism of this has yet to be fully elucidated (Mecklenburgh et al., 2002, Walmsley et al., 2005a, Walmsley et al., 2005b). The fact that HIF-1α is not upregulated in cord neutrophils indicates that the significantly prolonged survival of cord neutrophils is unlikely to be due to the relative hypoxia of the intra-uterine environment. A study of guinea pig fetuses failed to show increased expression of Bcl-xl following hypoxia, although Bax expression was increased by hypoxic conditions (Abedin et al., 2005). HIF-2α shows upregulation in cord neutrophils but it appears to be less important in neutrophils than HIF-1α (Mecklenburgh et al., 2002, Walmsley et al., 2005a, Walmsley et al., 2005b).

A1 (bfl-1) has been previously studied and found in similar amounts in adult and cord neutrophils (Hanna et al., 2005). I initially tried to study this protein by flow cytometry but no effective antibody was available for this purpose. Levels of A-1 are not significantly different between adult and cord blood in our study when the results of the RT-PCR are reviewed.

6.4.4 Conclusion

This chapter aimed to examine the effect of maturity (term newborn vs adult) on the ability of neutrophils to undergo apoptosis, in light of the significantly lower percentage of the neutrophil population that was apoptotic in BAL samples from preterm infants.

Neonatal neutrophils displayed a delay in apoptosis when compared to adult neutrophils and were also hyporesponsive to the pro-survival effects of LPS.

The differences in the Bcl-2 family members between adult and cord neutrophils may be the most significant of the factors investigated in explaining the delay in apoptosis in term cord neutrophils. It is interesting to observe the differential use of the Bcl-2 family of proteins in promoting neutrophil survival between adults and newborn infants. It would be a useful addition to this study to investigate the same Bcl-2 family members in cord blood taken from preterm infants to assess whether a decrease in gestation would further alter the regulation of apoptosis in preterm infants compared to term babies.

Chapter 7

Final Discussion

Chapter 7
Final Discussion

7.1 Overview

In this thesis, I have sought to examine the inflammatory process in the lungs of ventilated newborn infants. I have described both the cellular and supernatant components of bronchoalveolar lavage samples from term and preterm infants and focused on the relationship of various components of the pulmonary inflammatory response to the pathogenesis of CLD. I have paid particular attention to the role of infection in lung disease of preterm infants and tried to identify a predictive factor for the development of CLD. I have focused more specifically on the role of neutrophil apoptosis in CLD and have sought to understand the mechanism for the delay in neutrophil apoptosis in newborn infants and how this may impact on lung disease. I used flow cytometry, a technique not previously reported in the study of neonatal BAL, to accurately describe the cellular component of BAL samples and to study neutrophil and macrophage surface markers in BAL samples as well as neutrophil apoptosis in BAL samples. I have also attempted to study apoptosis in neonatal neutrophils in the *in vitro* setting to discover reasons for their delayed apoptosis.

7.2 Initiation of the inflammatory response

Initially I confirmed previously published findings (Merritt et al., 1983, Kotecha et al., 2003, Arnon et al., 1993) which showed that the total cellular load, as well as the number of both neutrophils and macrophages, in the lungs of ventilated preterm infants is much greater than their term counterparts but I failed to show a significant difference between preterm infants whose RDS resolves and those who go on to develop CLD. This lack of a significant result may be due to the small numbers in each patient group in our cohort. However, our study has the advantage of daily sampling in the first week of life which may have detected changes in cell influx that would have been missed by less the frequent sampling protocols used in other studies (Kotecha et al., 2003). Many of the studies of the cellular components of BAL samples mentioned above date from 5-10 years ago and have a cohort of infants with

RDS that are ventilated for longer periods than in this study – this may imply subtle differences in the studied population or in the care offered to these infants in different intensive care units at different times. Also this study cohort was slightly atypical, even for the Welsh Regional Neonatal Unit, as a significant proportion of the enrolled infants had not received a full course of antenatal steroids, which may have had an impact on the initiation of the pulmonary inflammatory response in these infants.

RDS is a neutrophil-dominant pathology (Arnon et al., 1993) and it is likely that it is the persistence of neutrophils and their impact on enhancing and maintaining the ongoing inflammatory process that allows for the development of the lung injury that characterises CLD (Haslett, 1999, Serhan and Savill, 2005). I showed that significantly fewer neutrophils from the preterm lung have surface expression of TLR 2 and TLR 4 which are important in mediating the recognition of pathogens in the lung so that possibly despite increased neutrophil numbers the response of the preterm infant to an invading micro-organism may be impaired and thus allow an infection to become established rather than rapidly cleared. Although in freshly isolated cord blood neutrophils there were no significant differences in TLR 2 and 4 expression when compared to freshly isolated adult neutrophils there appear to be differences in expression in neutrophils found in term and preterm lungs. It would therefore be useful to compare preterm and term cord blood neutrophils to assess whether the difference observed in BAL neutrophils is related to gestation and maturation or occurs as a result of failure of the preterm neutrophils to upregulate TLR 2 and 4 expression in response to the environment in the lung.

Part of the strength of this study is the repeated samples taken from babies, particularly in the first week of life which enabled me to look more closely at individual infants and identify sudden increases in cell count which often coincided with the presence of microbial DNA in the BAL fluid. I was also able to show that the peak in total, neutrophil and macrophage counts was delayed in infants who developed CLD, more than either those babies with RDS or those born at term. This may be because cell influx continues over a longer period in CLD infants, to reach a higher peak, and the inflammatory process continues long after this process has received a signal to begin resolution in the other groups of babies. This prolongation of cell influx is likely to be contributed to by the presence of infection or colonisation

with micro-organisms. The presence of an endotracheal tube in the ventilated neonate provides a portal of entry for infection as well as an indwelling foreign body for maintenance of infection or colonisation (in addition to the multiple venous access devices frequently present in these infants). Infants developing CLD were ventilated for a longer period, allowing increased opportunity for colonisation or infection with microbes and therefore may have required ongoing ventilation, thus worsening CLD, which required ongoing intubation in a self-perpetuating cycle.

7.3 Maintenance of the inflammatory response

I found a significant association between the presence of microbial DNA in BAL samples and CLD, particularly when microbial DNA was detected beyond the first 3 days of life. This implies an important role for hospital-acquired, late-onset neonatal infection in the development of CLD, probably related to the persistence of the endotracheal tube in these long term ventilated patients. Episodes of microbial infection or colonisation were temporally associated with further increases in total, neutrophil and macrophage counts, exaggerating the inflammatory response and lung injury in these babies.

Similarly the presence of *Ureaplasma spp.* in BAL samples was also associated with CLD. This may be partly as a result of *Ureaplasma* being a well recognised cause of preterm labour (Kirchner et al., 2007), but antenatal infection or colonisation with this organism may start the inflammatory cascade before birth in the most immature lungs and this is then further exaggerated by the routine activities of clinical neonatology, such as ventilation and the provision of supplemental oxygen, which are known to be pro-inflammatory (Davis et al., 1991, Jobe and Bancalari, 2001).

Integral to the maintenance of the inflammatory response are various cellular messengers including cytokines and growth factors. I found significantly elevated peak levels of numerous pro-inflammatory mediators, but also of anti-inflammatory cytokines, like IL-10 in preterm infants who developed CLD. I believe this reflects complete dysregulation of the inflammatory process and cells may be unable to respond to anti-inflammatory stimuli because of the sheer size of the ongoing pro-

inflammatory reaction. The presence of microbial DNA in BAL samples was associated with further increases in cytokine levels

7.4 Tissue damage

I studied only one major mediator of tissue damage in BAL samples, namely elastase. Neutrophil elastase activity was detected in a minority of samples in episodic spikes, temporally associated with the presence of infection but there was a significant relationship between the detection of elastase in BAL supernatants and the development of CLD. There was also a noticeable link between the presence of microbial DNA from species other than *S. epidermidis* and the highest elastase levels, adding a further possible mechanism for the association seen between infection and CLD. Historically, elastase was considered one of the most important mediators of lung injury in preterm ventilated infants (Merritt et al., 1983, Ogden et al., 1984, Watterberg et al., 1994), however, more recently the role of elastase in CLD in the era of antenatal steroid and postnatal surfactant treatment has become less prominent (Speer et al., 1993, Sveger et al., 2002, Sluis et al., 1994). The uncontrolled release of neutrophil elastase into lung tissue is still a cause of lung tissue injury and the association of peaks in elastase with the presence of microbial DNA confirms the importance of microbial infection or colonisation in the pathogenesis of CLD.

The reason for uncontrolled neutrophil elastase release in the lung may be speculated to be due to large numbers of neutrophils present in the lung, particularly neutrophils dying by necrosis. This was not clearly shown by data from this cohort.

A commonly held view of the pathogenesis of CLD is that it is a process of aberrant lung development (Jobe and Bancalari, 2001) contributed to by numerous pro- and – anti-inflammatory mediators and growth factors present in the preterm lung. My findings of elevated peak levels of a number of cytokines in infants developing CLD would support this view. Additionally the significantly higher cytokine levels demonstrated in BAL samples where 16S rRNA or *Ureaplasma* were isolated, along with the higher neutrophil counts associated temporally with infection, may allow aberrant repair of elastase damaged tissues due to abnormally high levels of cytokines

present or lack of resolution because of ongoing neutrophil recruitment in response to high levels of, particularly pro-inflammatory, cytokines.

7.5 Resolution of inflammation

The reason for this ongoing response in what are frequently the smallest and most immature infants may be related in part to my findings among the monocyte/macrophage population. I showed that the macrophages have a more immature phenotype in preterm infants and that term infants have a higher proportion of mature alveolar macrophages from as early as the first day of life. In fact, this was one of the only differences between the BAL samples of term and preterm infants at this early stage, although probably not clinically useful as a prognostic indicator of the future development of CLD as no differences were shown between RDS and CLD infants. The presence of mature macrophages in the lung may allow the process of resolution of pulmonary inflammation to begin very early in the ventilator course in term infants, by allowing phagocytosis of apoptotic neutrophils and production of anti-inflammatory cytokines. This process of resolution is likely to be reflected as an improvement in the infant's clinical status and facilitates rapid extubation and thus further reduction in the pro-inflammatory stimuli associated with prolonged ventilation, which include barotrauma, hyperoxia and secondary infection.

I hypothesise that the more immature monocyte-like cells may also be able to produce an exaggerated cytokine response (Maus et al., 2002b, Maus et al., 2001, Rosseau et al., 2000a, Rosseau et al., 2000b) and be less able to phagocytose apoptotic neutrophils (Hallwirth et al., 2004), thus less able to provide an anti-apoptotic signal to promote resolution of inflammation (Haslett, 1999). This was not apparent from this study, but nevertheless may merit further investigation by *in vitro* studies of the ability of immature macrophages to interact with apoptotic neutrophils from cord blood of both term, initially, and then preterm infants.

A recent study (Kevill et al., 2008) has shown that macrophage migration inhibitory factor may be an important protective factor in the development of CLD in both a mouse model and in human infants. This may serve to support my hypothesis that the presence of immature macrophages in the newborn lung is a critical component of the

pathogenesis of CLD. G-CSF and GM-CSF are known to promote macrophage maturation, however their presence in the lung may also make a considerable contribution to the accumulation of neutrophils, thus negating any positive effect from these mediators (Papoff et al., 2001).

The clearance of neutrophils from the site of inflammation is vital to the resolution of the inflammatory process. Part of this process is the death of neutrophils by apoptosis and their subsequent phagocytosis by macrophages. I hypothesised that there may be a disruption or dysregulation of neutrophil apoptosis in infants who develop CLD (Kotecha et al., 2003) as a result of factors inherent in the neutrophil, dysfunctional macrophage recognition or phagocytosis of apoptotic neutrophils or factors related to the inflammatory environment in which the neutrophils are found.

I showed that a significantly higher percentage of the neutrophil population was apoptotic in term infants compared to preterm babies. Having more of the neutrophil population undergoing apoptosis renders fewer neutrophils available to actively contribute to the inflammatory process and tissue injury (Whyte et al., 1993, Dransfield et al., 1995). This, in combination with a large number of mature macrophages available to remove these effete neutrophils before they become secondarily necrotic (Matute-Bello et al., 1997), allows for the resolution of inflammation to begin.

Preterm infants had higher peak neutrophil counts and lower percentages of apoptotic neutrophils, which would effectively leave higher numbers of viable neutrophils present in the airways. These neutrophils are however inefficient in responding to microbial pathogens (Anderson et al., 1990, Levy et al., 1999), for reasons including their cell surface receptor expression (particularly TLRs), but also other factors not studied in this thesis e.g. adherence, chemotaxis, phagocytosis and non-oxygen dependent bactericidal activity (Anderson et al., 1990, Levy et al., 1999). The neutrophils are also delayed in entering the process of apoptosis. These factors allow neutrophil numbers to accumulate and potentially release their toxic contents causing a further escalation in the pro-inflammatory environment and further lung injury. These large numbers of neutrophils remain active at the site of inflamation (Liles et al., 1996, Leavey et al., 1998, Kasahara et al., 1997) until they eventually undergo

apoptosis but then may not be able to be effectively taken up by the immature mononuclear phagocytes so then undergo secondary necrosis, further exaggerating the inflammatory problem. I showed that preterm infants do indeed have higher numbers of necrotic neutrophils present in BAL samples.

There appears to be a dysregulated relationship between apoptotic neutrophils, the mononuclear phagocyte population and the presence of infection, possibly because the system is completely overwhelmed by the numbers of neutrophils present and its regulation may be far more complex than originally thought.

I showed that the proportion of apoptotic neutrophils in preterm BAL samples reached a peak later than in term infants. This appears to be consistent with previously published findings that apoptosis is delayed in neonatal neutrophils from umbilical cord blood *in vitro* when compared to adult cells (Allgaier et al., 1998, Hanna et al., 2005, Luo et al., 2003, Molloy et al., 2004). I was able to confirm these *in vitro* findings and went on to investigate possible reasons for this delay.

I found that the Bcl-2 family of proteins, particularly the anti-apoptotic proteins Bcl-xl and Mcl-1, are differentially expressed in term cord blood and adult neutrophils. The action of Bcl-xl in preventing permeablisation of the mitochondrial membrane by activated Bax may be critical in the delay in neutrophil apoptosis seen in cord neutrophils. This difference in Bcl-2 expression between cord and adult blood had not previously been described.

The addition of LPS to *in vitro* experiments with cord and adult neutrophils to mimic some of the effects of infection showed a very small further delay in apoptosis in cord neutrophils. This highlighted the hyporesponsiveness of infant neutrophils to infective stimuli and the likelihood that infection could further contribute to delayed neutrophil apoptosis.

The environment within the lung may also exert a significant influence on the resolution of pulmonary inflammation. I studied the BAL supernatants to determine their relative pro- or anti-apoptotic activity and found that significantly more supernatants had pro-apoptotic activity among the term population. It therefore

appears that resolution of the inflammatory response in the preterm lung is hampered not only by an inherent delay in neutrophil apoptosis within the neutrophil itself but also that the cell finds itself in an environment which supplies anti-apoptotic stimuli external to the neutrophil as well.

In summary, it would seem that the inflammatory process in the neonatal lung is characterised by a series of interconnecting circles of failure of resolution of an inflammatory process causing, and in turn caused by, an increase in pro-inflammatory stimuli.

7.6 Difficulties and limitations

No discussion would be complete without a review of some of the difficulties experienced in the conduct of this piece of work.

The collection of BAL samples, particularly on a daily basis in the first week of life and then the process of preparing the samples for FACS analysis, is necessarily time consuming and labour intensive, frequently being undertaken outside of normal working hours, which may make this technique not well suited for routine clinical use. In my view these daily samples were an important element of this study and provided useful information particularly in the term and RDS group of infants. Term and RDS babies were only ventilated for very short periods. Perhaps this cohort are not a true representation of the duration of ventilation in RDS because of the small sample size, or perhaps ongoing improvements in neonatal care are truly having an impact in this group of infants, but in view of this short ventilation period, if sampling had taken place less frequently, there would have been very little data on the term or RDS babies at all.

Neonatal BAL is frequently performed on the smallest, sickest infants from whom a sample of any type will be small and often difficult to obtain and ethical issues around consent for this sort of study will always be raised. Flow cytometry is not a technique that has been used previously in the analysis of preterm neonatal lung lavages, although it has been used to some extent in adult sputum studies. It took some time to establish appropriate processing techniques, including the use of DTT, and FACS

staining and settings for this somewhat unusual sample type, particularly in view of the very tiny sample volumes obtained. The use of multi-colour (3-colour in this study) flow cytometry techniques enabled multiple antigens to be detected simultaneously, thus making good use of small samples. This was complicated by the high level of auto-fluorescence seen in both neutrophils and macrophages in these samples and fluorophores must be chosen with care in order to minimise the amount of FACS compensation required.

7.7 Future studies

This thesis has raised a number of questions which I believe could be further investigated:

I have identified a relative immaturity in lung macrophage populations in preterm infants. Unfortunately because of small sample sizes there were very few babies in whom these cells could be fully characterised. It would be interesting to fully characterise lung macrophages, using a large panel of potentially significant antigens/antibodies eg. CD14, CD36 and HLA-DR, along with CD31, CD44 and the "PS-receptor" among others, in a population of ventilated infants of various gestational ages to ascertain whether increasing prematurity increases macrophage immaturity. Exposure to antenatal infection has been reported to precipitate migration of macrophages to the lung in the fetus (Alenghat and Esterly, 1984) and characterisation of macrophage maturity in the first few days of life in infected and uninfected premature infants might show marked differences and help to explain later responses to the inflammatory stimuli of routine preterm neonatal care.

The relationship between macrophages and neutrophil apoptosis involves numerous factors, including properties of both the neutrophil and the macrophage, their interaction and their environment (Vandivier et al., 2006). The interactions between apoptotic neutrophils and the macrophages which should engulf them are known to be complex (Vandivier et al., 2006) and are perhaps best studied in an *in vitro* setting where some of the many interacting factors in the process can be controlled or eliminated for study purposes. There may be further neutrophil dependent factors, such as altered surface antigen expression which, after the delay in apoptosis, prevent

effective recognition and therefore removal of the neutrophils by phagocytes. Observation of neutrophil-macrophage interactions in term and preterm blood cells would appear to be a logical extension of this work.

I have noted a relationship between the presence of *Ureaplasma* and the development of CLD. This remains a controversial area of neonatal practice. The natural history of untreated *Ureaplasma* colonisation is unclear. One of our studied infants, who developed CLD, had no treatment for *Ureaplasma* but was noted to have cleared the organism from his pulmonary secretions by 112 days of age in a sample taken for other clinical purposes. While this lack of clarity over the role of *Ureaplasma* in neonatal lung disease exists, it could still be ethically acceptable to observe the natural history of *Ureaplasma* colonisation in affected infants. There is little evidence (Mabanta et al., 2003) that treatment for *Ureaplasma* is useful in preventing the development of CLD but this is based on only 37 colonised patients from 2 studies where treatment protocols differed too much for the infants' data to be analysed together as part of the review. A well-designed clinical trial of treatment of this organism in the preterm neonatal population is warranted, although the effects of *Ureaplasma* in the preterm lung may begin antenatally and treatment, even from birth before *Ureaplasma* colonisation status is known, may be too late to prevent CLD.

All our *in vitro* studies of neutrophil apoptosis were conducted using cord blood from term infants collected following elective Caesarean section. I would like to assess the impact of decreasing gestational age on the delay in neutrophil apoptosis and its causes, particularly the Bcl-2 proteins. Working with preterm cord blood will be more challenging than in term infants for a number of reasons:-

- Significantly preterm infants are not often delivered by elective Caesarean section and those that are, are often in situations of severe fetal compromise or maternal ill health, so samples might not be easily obtained from this group.
- An extremely preterm or severely compromised fetus frequently has a very small, thin umbilical cord which will yield only a very small blood sample. This may drive the development of methods of separating neutrophils from and working with very small amounts of cord blood. This small sample methodology might then be useful in working with blood samples from infants and children – currently prevented by the need for large blood samples.

- Infants who are born spontaneously preterm often do so because of antenatal infection or inflammation which may be clinically undiagnosed (Goldenberg et al., 2000). Methods of identifying these infants and/or their mothers will be important in differentiating the potential anti-apoptotic effect of infection from a gestational delay in apoptosis.

I would also like to be able to observe the "maturation" of neutrophil apoptosis in infants and children to determine at what point it reaches adult rates. Again, this would require the ability to work with very small blood samples for clinical and ethical reasons.

The therapeutic use of cytokine analogues or antagonists has been effective in inflammatory diseases of adults e.g. rheumatoid arthrititis (Drynda et al., 2002). It has been suggested that similar interventions may be beneficial in the neonate. The effects of such drugs in reducing the inflammatory response may be beneficial but this may have to be traded against a further reduction in the ability of the infant to combat pathogens. The administration of substances which will act externally to the neutrophil to attempt to enhance apoptosis (e.g. FasL) may still be unable to overcome the anti-apoptotic effect of proteins like Bcl-xl which are having their effect at a mitochondrial membrane level.

7.8 Final summary

In this thesis I have sought to understand aspects of the inflammatory process in the neonatal lung by analysis of bronchoalveolar lavage fluid and cells.

I have performed detailed longitudinal analysis of cells types and counts in the airways of ventilated neonates using flow cytometry – a technique not previously used for analysis of neonatal BAL cell phenotypes. I have established a reliable method for the use of flow cytometry in the very small samples obtained from neonatal BAL and shown that the use of DTT as a mucolytic in these samples has no significant effect on the detection of specific cell surface markers by flow cytometry. I have sought and found relationships between the presence of either 16S rRNA genes or *Ureaplasma* in BAL samples and the development of CLD.

I have found significantly elevated peak levels of a number of different cytokines in BAL supernatants from preterm infants and more pro-apoptotic activity against adult neutrophils in BAL supernatants from term infants.

I have focused on the role and process of neutrophil apoptosis in infants at risk of developing CLD and found a delay in apoptosis in lung neutrophils in preterm infants and confirmed a delay in neutrophil apoptosis in blood neutrophils of term infants compared to adults. I have shown that this delay might be due to differential expression of anti-apoptotic proteins Bcl-xl and Mcl-1 between cord and adult neutrophils, something which has not previously been described.

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Appendix 1

Parent/patient information leaflets and consent forms

GIG CYMRU NHS WALES
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Cardiff and Vale University Health Board

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Minicom 029 2074 3632

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Caerdydd, CF14 4XW
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Minicom 029 2074 3632

Eich cyf/Your ref
Ein cyf/Our ref
Welsh Health Telephone Network 1872
Direct Line/Llinell uniongychol

INFORMATION SHEET FOR PARENTS/GUARDIANS Ver 3

Principle Investigators: Professor Sailesh Kotecha, Consultant Neonatologist
Contact Details: Neonatal Unit, 029 20 74 3374

1. **Study Title**
Is chronic lung disease of prematurity a consequence of immature regulation of neutrophil apoptosis in preterm infants?

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

Thank you for reading this leaflet.

3. What is the purpose of the study?
Chronic lung disease (CLD), a common disease of premature babies, is characterised by prolonged oxygen dependency. Cells of inflammation, called neutrophils, are increased in the lungs of babies who develop CLD. Neutrophils are removed from the lungs by a process called apoptosis. We recently showed that the removal of neutrophils by this process of apoptosis is increased in babies who recover from their lung disease but not in those who develop CLD. In addition, cells called macrophages, which remove the apoptotic neutrophils, were increased in babies who recovered from their breathing difficulties but not if they developed CLD. We wish to determine whether CLD develops due to immaturity of factors that normally regulate this process called apoptosis. In particular, we wish to determine if apoptosis of neutrophils is related to the prematurity of the baby.

Using blood and lung washings from babies who do and do not recover from their lung disease, we will identify whether any factors important in neutrophil apoptosis are deficient or the macrophages are immature in babies who develop CLD. The results will help us understand why some babies progress to develop CLD whilst others recover and may help us develop therapies which can enhance apoptosis.

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

We wish to obtain samples from two groups of babies:

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

(b) babies who need help with their breathing with breathing machines because of non-breathing problems e.g. surgery.

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

5. Does my baby have to take part?

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

6. What will happen if my baby takes part?

(a) If at all possible, we would like to obtain blood (about a teaspoon) from the umbilical cord when the baby is born.

(b) We would also like to obtain 0.5 – 1.0 ml of blood (5 ml is a teaspoon) if and only if the baby is having blood taken for other routine blood tests. We would like to obtain blood daily for the first week and twice weekly thereafter for the first month.

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

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

7. Will this affect my or my baby's treatment?
The medical care of you or your baby will not be affected by this study. The information from this study will not be used to diagnose or treat you or your baby.

8. What will happen to the samples collected?
We will first separate the cells and fluid from the samples collected. We will determine the proportion of different cells types and also how many neutrophils are apoptotic by using modern techniques which can look at the small number of cells obtained from small babies. We will use the fluid to measure or identify proteins which promote or inhibit apoptosis. Some fluid and cell samples will be sent to Professor Moira Whyte's group in Sheffield as they have specific expertise in apoptosis of neutrophils. They will work together with us in identifying any particular factors which may be increased or decreased in babies who develop CLD.

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

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

11. Will my baby's taking part be kept confidential?
All information which is collected about your baby during the course of the research will be kept strictly confidential. Any information about your baby which leaves the hospital will have your and the baby's name and address removed so that you cannot be recognised from it. We will assign a number to each baby and use this to label the samples obtained for the study.

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

13. Who is paying for the study?
The study is funded by the Wellcome Trust which is a major medical charity in the UK.

14. Who had reviewed the study?
This study has been reviewed by the South East Wales Research Ethics Committee and also by the Wellcome Trust who have funded the study.

15. Who can I contact for further information?
You may contact Professor Sailesh Kotecha by asking one of the staff on the neonatal unit or by telephoning 029 20 74 3374 or by email (KotechaS@Cardiff.ac.uk) or by mail to: Professor Sailesh Kotecha, Neonatal Unit, Heath Hospital, Heath Park, Cardiff CF14 4XN.

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

Dr Nicola Maxwell
Clinical Research Fellow

Professor Sailesh Kotecha
Consultant in Neonatal Medicine

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Caerdydd, CF14 4XW
Ffôn 029 2074 7747
Ffacs 029 20743838
Minicom 029 2074 3632

Eich cyf/Your ref
Ein cyf/Our ref
Welsh Health Telephone Network 1872
Direct Line/Llinell uniongychol

Patient Identification Number for this study:

CONSENT FORM

Is chronic lung disease of prematurity a consequence of immature regulation of neutrophil apoptosis in preterm infants?

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

Please initial box

1. I confirm that I have read and understand the information sheet dated 6th September 2005 (version 3) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my baby's participation is voluntary and that I am free to withdraw my baby at any time, without giving any reason, without my baby's medical care or legal rights being affected. ☐
3. I understand that sections of any of my baby's medical notes may be looked at by the research individuals I give permission for these individuals to have access to my baby's records. ☐
4. I agree for my baby to take part in the above study. ☐

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

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

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Eich cyf/Your ref
Ein cyf/Our ref
Welsh Health Telephone Network 1872
Direct Line/Llinell uniongychol

INFORMATION SHEET FOR PARENTS/GUARDIANS Version 1 (6th December 2005) –
NORMAL TERM CONTROLS

Principle Investigators: Professor Sailesh Kotecha, Consultant Neonatologist
Contact Details: Neonatal Unit, 029 20 74 3374

3. **Study Title**
Is chronic lung disease of prematurity a consequence of immature regulation of neutrophil apoptosis in preterm infants?

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

Thank you for reading this leaflet.

3. What is the purpose of the study?
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Using blood and lung washings from babies who do and do not recover from their lung disease and comparing these to umbilical cord blood from healthy babies born at term, we will identify whether any factors important in neutrophil apoptosis are deficient or the macrophages are immature in babies who develop CLD. The results will help us understand why some babies progress to develop CLD whilst others recover and may help us develop therapies which can enhance apoptosis.

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

We wish to obtain samples from three groups of babies:

(c) babies who are born at or less than 32 weeks gestation and who need help with their breathing with breathing machines because of under-developed lungs, or

(d) babies who need help with their breathing with breathing machines because of non-breathing problems e.g. surgery.

(e) babies born at term (more than 37 weeks gestation) who are healthy

We would like to ask you to join our study as you have had a normal pregnancy and use the blood from the umbilical cord as controls (group c) to compare the results from premature babies (groups a and b).

5. Does my baby have to take part?

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

What will happen if my baby takes part?

We would like to obtain blood (about a teaspoon) from the umbilical cord when the baby is born.

7. Will this affect my or my baby's treatment?

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

8. What will happen to the samples collected?

We will first separate the cells and fluid from the blood samples collected. We will determine the proportion of different cell types and also how many neutrophils are apoptotic by using modern techniques which can look at the small number of cells obtained from babies. We will use the fluid to measure or identify proteins which promote or inhibit apoptosis. Some fluid and cell samples will be sent to Professor Moira Whyte's group in Sheffield as they have specific expertise in apoptosis of neutrophils. They will work together with us in identifying any particular factors which may be increased or decreased in babies who develop CLD.

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

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

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

11. Will my baby's taking part be kept confidential?
All information which is collected about your baby during the course of the research will be kept strictly confidential. Any information about your baby which leaves the hospital will have your and the baby's name and address removed so that you cannot be recognised from it. We will assign a number to each baby and use this to label the samples obtained for the study.

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

13. Who is paying for the study?
The study is funded by the Wellcome Trust which is a major medical charity in the UK.

14. Who had reviewed the study?
This study has been reviewed by the South East Wales Research Ethics Committee and also by the Wellcome Trust who have funded the study.

15. Who can I contact for further information?
You may contact Professor Sailesh Kotecha by asking one of the staff on the delivery suite or by telephoning 029 20 74 3374 or by email (KotechaS@Cardiff.ac.uk) or by mail to: Professor Sailesh Kotecha, Neonatal Unit, Heath Hospital, Heath Park, Cardiff CF14 4XN.

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

Dr Nicola Maxwell
Clinical Research Fellow

Professor Sailesh Kotecha
Consultant in Neonatal Medicine

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Welsh Health Telephone Network 1872
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Patient Identification Number for this study:

CONSENT FORM - Controls

Is chronic lung disease of prematurity a consequence of immature regulation of neutrophil apoptosis in preterm infants?

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

Please initial box

1. I confirm that I have read and understand the information sheet dated 6th December 2005 (version 1) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my baby's participation is voluntary and that I am free to withdraw my baby at any time, without giving any reason, without my baby's medical care or legal rights being affected. ☐
3. I understand that sections of any of my baby's medical notes may be looked at by the research individuals I give permission for these individuals to have access to my baby's records. ☐
4. I agree for my baby to take part in the above study. ☐

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

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

Appendix 2

Flow cytometry staining templates

Full panel (>1.1 million cells)

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	IgG1-BIO	IgG2a-BIO	-	-	CD11b-BIO	CD11b-BIO	CD11b-BIO	CD11b-BIO
	-	IgM-APC	IgG2a-APC	IgG1-PE	PE-Cy5.5	PE-Cy5.5	CD15-APC	CD16-PE	PE-Cy5.5	CD15-APC CD16-PE PE-Cy5.5	HLA-DR-APC CD16-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5
B	CD11c-BIO	CD11c-BIO	CD14-BIO	CD14-BIO	CD95L-BIO	TNF' -BIO	TRAIL-BIO	TLR2-BIO	TLR4-BIO			
	HLA-DR-APC CD16-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD16-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	CD15-APC CD36-PE PE-Cy5.5	CD15-APC CD36-PE PE-Cy5.5			
C	-	-	-	-								
	AnnexinV-PE/EDTA	AnnexinV-PE	To-Pro3	AnnexinV-PE/ To-Pro3								

(2 million cells = approx 75 000/well)
(For 1.1 million cells - controls reduced to 25 000/well and other wells at 50 000/well)

Reduced panel (0.8 - 1million cells)

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	IgG1-BIO	IgG2a-BIO	-	-	CD11b-BIO	CD11b-BIO	CD11b-BIO	CD14-BIO
	-	IgM-APC	IgG2a-APC	IgG1-PE	PE-Cy5.5	PE-Cy5.5	CD15-APC	CD16-PE	PE-Cy5.5	CD15-APC CD16-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD16-PE PE-Cy5.5
B	CD14-BIO	CD95L-BIO	TNF' -BIO	TRAIL-BIO	TLR2-BIO	TLR4-BIO						
	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	CD15-APC CD36-PE PE-Cy5.5	CD15-APC CD36-PE PE-Cy5.5						
C	-	-	-	-								
	AnnexinV-PE/EDTA	AnnexinV-PE	To-Pro3	AnnexinV-PE/ To-Pro3								

(For 0.8 million cells, controls use 25 000/well and remaining 12 wells at approx. 50 000/well)

Minimum panel (Around 0.5million cells)

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	IgG1-BIO	-	-	CD11b-BIO	CD11b-BIO	CD11b-BIO	CD14-BIO	
	-	IgM-APC	IgG2a-APC	IgG1-PE	PE-Cy5.5	CD15-APC	CD16-PE	PE-Cy5.5	CD15-APC CD16-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	
B	-	-	-	-								
	AnnexinV-PE/EDTA	AnnexinV-PE	To-Pro3	AnnexinV-PE/ To-Pro3								

(For 0.5 million cells, controls use 25 000/well and remaining 8 wells at approx. 40 000/well)

Cord blood panel

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	IgG1-BIO	IgG2a-BIO	-	-	CD11b-BIO	CD11b-BIO	CD11b-BIO	CD11b-BIO
	-	IgM-APC	IgG2a-APC	IgG1-PE	PE-Cy5.5	PE-Cy5.5	CD15-APC	CD16-PE	PE-Cy5.5	CD15-APC CD16-PE PE-Cy5.5	HLA-DR-APC CD16-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5
B	CD11c-BIO	CD11c-BIO	CD14-BIO	CD14-BIO	CD95L-BIO	TNF' -BIO	TRAIL-BIO	TLR2-BIO	TLR4-BIO			
	HLA-DR-APC CD16-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD16-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	HLA-DR-APC CD36-PE PE-Cy5.5	CD15-APC CD36-PE PE-Cy5.5	CD15-APC CD36-PE PE-Cy5.5			
C	-	-	-	-	Casp3/ Inhib.	Casp3	Casp3/ ToPro	IgG1-PE	Bax-PE	-		
	AnnexinV-PE/EDTA	AnnexinV-PE	To-Pro3	AnnexinV-PE/ To-Pro3								

Appendix 3

Abstracts and publications from this work

Poster - European Respiratory Society – Stockholm 2007

Delayed Neutrophil Apoptosis may predispose to Lung Injury in Chronic Lung Disease of Prematurity

Nicola C. Maxwell, Philip L. Davies, O. Brad Spiller, Eamon P. McGreal, Sailesh Kotecha
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Introduction:
The pathogenesis of Chronic Lung Disease of prematurity (CLD) involves interactions between numerous factors. Neonatal Respiratory Distress Syndrome (RDS) is characterised by alveolar neutrophil (PMN) infiltration, which disappears as RDS resolves (1). Persistence of PMN infiltration is strongly associated with the development of CLD (2,3). PMN apoptosis is important in regulation and resolution of inflammation. Apoptosis causes functional downregulation of PMN. Recognition and clearance of PMN by macrophages further downregulates the inflammatory response. Developmental immaturity of the neonatal immune system is well recognised (4). Several recent studies have shown reduced apoptosis, both spontaneous and induced, in neonatal compared to adult neutrophils (5,6,7,8). The reasons for this are unclear. There is currently no published data regarding rates of apoptosis in preterm neutrophils.

Hypotheses:
There is increasing dysregulation of neutrophil apoptosis with decreasing gestational age.
Dysregulation of neutrophil apoptosis in preterm infants contributes to development of CLD.

Aim:
We compared rates of apoptosis of PMN between adult peripheral blood and cord blood from term infants and tried to elucidate underlying mechanisms for the differences noted.

Method:
PMN were isolated from adult peripheral blood and from umbilical cord blood from term infants delivered by elective caesarean section (n= 6 adult/cord pairs). PMN were cultured in the presence or absence of lipopolysaccharide (LPS)(50ng/ml) and assessed at baseline and then 6, 12 and 20 hours from commencement of culture for apoptosis by flow cytometry, using Annexin-V-PE (MBL International) and To-Pro-3 (Invitrogen). Cells positive for Annexin-V and To-Pro-3 were considered necrotic and those positive for Annexin-V alone were apoptotic. Activity of the apoptotic effector, caspase 3 (Apo-Logix®, Peninsula Laboratories) and anti-apoptotic regulator, Bax (monoclonal phycoerythrin-conjugated mouse anti human Bax (2D2), Santa Cruz Biotechnology), were similarly assessed.

Results:
PMN from cord blood underwent apoptosis at a significantly slower rate than adult PMN ($p<0.02$ at 6, 12 and 20 hours) although there was no significant difference in the number of apoptotic cells in the fresh samples (p=NS). The addition of LPS reduced adult PMN apoptosis significantly by 12 hours ($p=0.04$) but did not reduce cord PMN apoptosis. Caspase 3 is a key executioner of apoptosis and its activation is an early apoptotic event. It is able to activate other caspases as well as cleave cytoskeletal components. Caspase 3 activation reaches similar levels in cord and adult neutrophils undergoing apoptosis in culture, both with and without LPS. However,

despite similar levels of caspase 3 activation, neonatal neutrophils remain resistant to apoptosis. Possible mechanisms for this are neonatal caspase 3 is inefficient or a molecule or pathway necessary for apoptosis but distal to caspase 3 activation is deficient or inhibited. Bax is a pro-apoptotic member of the Bcl2 group of apoptotic regulators. Within the cell, mitochondria sequester pro-apoptotic proteins e.g. cytochrome c which are released under the influence of Bax. Bax activity, similar to adult levels is seen in cord blood neutrophils in culture, despite levels of apoptosis in cord neutrophils being suppressed. The significance of this result has yet to be assessed.

Conclusion:

Term infant PMN undergo apoptosis at a slower rate than adult PMN. These differences were not due to caspase 3 or Bax activity suggesting an alternative mechanism for the differences in apoptosis we have noted. This may be exaggerated with decreasing gestation, hence contributing to the development of CLD.

Future aims/questions:

Determine the impact of gestational age on the rate of neutrophil apoptosis (spontaneous and induced). We will compare neutrophil apoptosis in preterm infants born following spontaneous preterm labour, where subclinical infection is a leading cause of preterm delivery, with neutrophil apoptosis in infants born electively preterm for a maternal indication or foetal growth restriction.

Further investigate possible mechanisms for alterations in the rate of neutrophil apoptosis in infants. Term cord blood neutrophils undergo apoptosis more slowly than adult neutrophils but levels of caspase 3 activation appear remarkably similar. This initial data implies that there may be a defect in the apoptotic pathway distal to caspase 3. We plan to investigate other caspases as well as other pro-(Bak, Bad) and anti-apoptotic (A1, BclXL) Bcl2 family members to possibly explain the mechanism of reduced apoptosis in cord neutrophils.

Dr Maxwell is supported by the Wellcome Trust and Dr Davies by Arriva Pharmaceuticals.

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Poster - European Respiratory Society – Stockholm 2007

The Role of Proteases in the Development of Chronic Lung Disease of Prematurity

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Introduction

Proteases are enzymes that hydrolyse proteins. They are produced by a number of cell types within the lung, including inflammatory cells such as neutrophils, where they play a key role in host defence and airway remodelling. An imbalance of neutrophil proteases (elastase and matrix metalloproteinases, MMP-9) and anti-proteases (alpha 1-antitrypsin and tissue inhibitors of matrix metalloproteinases, TIMPs) has been implicated in the development of classical Chronic Lung Disease of prematurity (CLD). "New" CLD affects extremely preterm infants and is thought to represent an aberration of lung growth.

Aims

To assess the proteolytic balance in infants with new CLD and to examine relationships between proteolytic changes.

Methods

Ventilated preterm infants (<32 weeks gestation) and term controls underwent serial bronchoalveolar lavages (BAL) performed until extubation. Elastase activity assays were performed using a specific chromogenic substrate (Bachem, St Helens, UK) against a standard curve of neutrophil elastase (Athens Research and Technology, GA, USA). Total MMP-9 and MMP-9/TIMP-1 ELISAs (R&D Systems, Minneapolis, USA) were performed on all samples .

Results

48 infants were recruited. 20 developed CLD, 17 had respiratory distress syndrome (RDS) that resolved, 5 died and 6 were term controls. The demographic profile of these infants is shown on table 1. Peak total MMP-9 was significantly greater in infants who developed CLD (median 763ng/ml) compared to infants with resolved RDS (median 47ng/ml), p=0.003, figure 1. Peak MMP-9/TIMP1 complex was also significantly greater in CLD infants (median 29.9ng/ml) compared to resolved RDS infants (median 1.5ng/ml), p=0.004. Elastase activity was only detected in a minority of samples but was present in at least one sample from more CLD infants (10/20) than resolved RDS infants (2/17), p=0.013.

Peaks of MMP-9, MMP-9/TIMP-1 complex and elastase tended to coincide and frequently occurred late figure 2. There was a strong correlation between log MMP-9 and log MMP-9/TIMP-1 R=0.89 and a moderate correlation between log elastase activity and log MMP-9 R=0.63.

Conclusions

Peak MMP-9, MMP-9/TIMP-1 complex and elastase were increased in CLD infants although protease levels were generally less than in classical CLD. Episodic peaks in MMP-9 concentrations occurred for individual infants who underwent longitudinal sampling and these were temporally related to concentrations of MMP-9/TIMP-1 complex and if present neutrophil elastase activity. It remains to be seen if these smaller increases in proteases adversely affect lung growth that occurs in "new" CLD.

Speculation

The underlying cause of the episodic increases in proteolytic activity continues to be investigated, however, infection remains a likely factor. Proteases are key to the neutrophil's bactericidal activity and we speculate that infection triggers proteolytic release that may cause lung injury.

Poster - European Respiratory Society – Stockholm 2007

Comparison of Neutrophil Elastase in Adults and Cord Blood from Term Infants

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Introduction

Neutrophil elastase (HNE) is important in the antimicrobial defence of the lung and may also be important in the development of chronic lung disease of prematurity (CLD). Infants are at greater risk of lung infection than adults and infant maturity is important in the changing pattern of CLD.

Aims

To assess differences in newborn and adult neutrophil elastase that may account for differences in lung pathology.

Methods

Neutrophils were isolated from newborn cord and adult blood using histopaque-1077 (Sigma Aldrich, Irvine,UK) and the cells resuspended to a million cells/ml in Hanks buffered saline. Cells were stimulated with the following inflammatory stimulants: N-formyl-methionyl-leucyl-phenylalanine (fMLP), cytochalasin B, cytochalasin B and fMLP, cytochalasin B and lipopolysaccharide (LPS) or cytochalasin B and interleukin-8 (IL-8) at 1:1000 for 10 minutes or they received no stimulation. Stimulants were chosen as they represented a spectrum of potency in their ability to activate neutrophils. The solution was centrifuged at 13000rpm for 10 minutes and the supernatant removed. The cell pellet was then dissolved using distilled water and Triton X for two hours. Elastase activity was determined using a specific chromogenic substrate, Suc – Ala – Ala – Pro – Val - pNA (Bachem, St Helens, UK) on the supernatant and cellular fractions.

Results

Ten full term cord blood samples and ten adult blood samples were analysed. The total elastase present in unstimulated neutrophils was significantly lower from cord blood (mean 15.2 +/-5.2nM) compared to adult blood (25.2 +/-8.4nM), p=0.007. Cytochalasin B in combination with fMLP proved to be the most effective stimulus. The proportion of neutrophil elastase released by cord blood and adult blood was not significantly different for any of the stimuli. (fMLP15.9% cord vs 12.2% adult; cytochalasin B 12.6% vs 8.9%; cytochalasin B and fMLP 66.3% vs 72.7%; cytochalasin B and LPS 14.0% vs 10.9% and cytochalasin B and IL-8 21.3% vs 20.9%).

Conclusions

Adult neutrophils contain significantly greater total elastase than cord blood neutrophils, although similar proportions of elastase are released by the different inflammatory stimulants.

Reduced elastase content of neutrophils with reduced maturity may be important in explaining differences in lung pathology observed in the neonatal lung.

The study was sponsored by Arriva Pharmaceuticals and a grant by the Wellcome Trust.

Poster - European Respiratory Society – Berlin 2008

Ureaplasma in Chronic Lung Disease of Prematurity – Strains, Resistance and Treatment

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Background:
The role of Ureaplasma in chronic lung disease of prematurity (CLD) remains controversial. In 2005, a meta-analysis (2216 babies, 23 studies) showed an odds ratio of 2.83 (2.29-3.51) for the relationship between the presence of Ureaplasma and CLD at 28 days of life (1).
Less is known about the role of antimicrobial treatment for the prevention of CLD. Two randomised controlled trials (2,3) of erythromycin used to eradicate Ureaplasma and prevent CLD have been performed. Together the studies include only 37 colonised patients. There was no significant reduction in CLD with treatment in either study – disparate study designs prevented the results from being combined in the meta-analysis (4).

Aim:
To further elucidate the role of Ureaplasma in CLD by examining different strains and the relationship between its treatment and clinical outcome.

Method:
Serial bronchoalveolar lavage (BAL) was performed on 26 infants <32 weeks gestation. BAL fluid was cultured for presence of Ureaplasma and confirmed by polymerase chain reaction (PCR). Further PCR was used to determine species, either Ureaplasma parvum (Up) or Ureaplasma urealyticum (Uu). All isolates were tested for antibiotic resistance using a novel microbroth dilution method.

Results:
Of 26 infants, 10 had Ureaplasma isolated from BAL. Moderate or severe CLD developed in 8/9 infants with Ureaplasma and 7/16 infants without ($p=0.027$). Sequencing showed 7/10 were Up and 2/10 Uu (1 not identifiable). Five of the infants received treatment for Ureaplasma, at the discretion of the infant's consultant neonatologist, from day 5 of life at the earliest. Ureaplasma was successfully eradicated following treatment in 2 babies (both Up, one treated with erythromycin and the other with clarithromycin) and it recurred after treatment in a third (Uu). In one infant a highly erythromycin resistant strain of Up was isolated. One infant died. All surviving treated infants developed CLD. Up appeared to clear spontaneously from a sixth infant. The mutation thought to be responsible for eruthromycin resistance was characterised and a 6 base pair (corresponding to 2 amino acids – Arg and Gln) deletion, was characterised.

Conclusion:
Up is the more frequently isolated Ureaplasma in preterm infants. Erythromycin resistance was noted in one infant and the molecular mechanism characterised.

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Dr Maxwell is supported by the Wellcome Trust.

Poster – Perinatal Medicine 2008– Harrogate, UK

The Relationship between Chronic Lung Disease of Prematurity and *Ureaplasma spp.*: A Survey of Senior Neonatologists

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Background:

A 1995 meta-analysis (1479 babies, 17 studies) (1) found a significant association between chronic lung disease of prematurity (CLD) at 28 days of life and *Ureaplasma urealyticum* colonisation. In 2005, a further meta-analysis (2216 babies, 23 studies) showed an odds ratio of 2.83 (2.29-3.51) for the relationship between the presence of *Ureaplasma* and CLD at 28 days of life (2). Two randomised controlled trials (3,4) were included in a Cochrane review of erythromycin used to eradicate *Ureaplasma* and prevent CLD (5). Together the studies included only 37 colonised patients and there was no significant reduction in CLD with treatment in either study – disparate study designs prevented the results from being combined in the meta-analysis.

Aim:

To seek the views of senior UK neonatologists on whether *Ureaplasma spp.* is important in the development of chronic lung disease of prematurity and to gauge the need for a randomised control trial to determine if CLD can be prevented by treatment of *Ureaplasma.*

Method:

A structured questionnaire consisting of 18 questions was sent to 300 consultant neonatologists and paediatricians with a special interest in neonatology working in UK neonatal units.

Results:

Fifty seven percent (172/300) of questionnaires were returned. Of the 172 respondents, 137 were consultant neonatologists while the remainder were paediatricians with a special interest in neonatal medicine. One hundred and twenty five worked in level 3 intensive care units.

Most respondents felt that there was neither evidence in favour of *Ureaplasma spp.* causing CLD nor any evidence to show that it did not. The respiratory colonisation rate of preterm infants with *Ureaplasma spp.* was unclear as few units tested for the organism regularly. Of the 102 respondents who said they ever tested for *Ureaplasma*, the samples sent varied widely in type and timing. Endotracheal secretions were the most frequent sample sent for testing, with many units sending more than one sample type. The most common time to send a sample for *Ureaplasma* was in relation to the patient's clinical condition rather than samples being sent routinely. Only 49/172 (28%) consultants were able to estimate the number of infections seen each year, with most saying they were unable to answer the question due to lack of testing. Fifty nine percent (59%) said that they would be interested in an affordable test to identify *Ureaplasma spp.*. There was a very strong call (68%) for a randomised trial involving infants born between 23 – 28 weeks gestation to address this controversy. A majority of consultants favoured erythromycin as a treatment for *Ureaplasma* with the duration of treatment chosen as 7-14 days (range 3-28 days, mode 14 days). Just under half were concerned about possible adverse effects of drug treatment to eradicate *Ureaplasma spp.* in this group of patients, with most respondents concerned about gastrointestinal effects of erythromycin, antibiotic resistance, alterations to normal

microbial flora and phlebitis causing difficulties with venous access in the smallest preterm infants.
Within a trial, a majority (71/117) were in favour of delaying the start of treatment until results of testing for *Ureaplasma* were available, however opinion was divided (Yes 48, No 45, Don't know 24) as to whether such a delay in starting treatment would mean that it would be too late to be of benefit.

Conclusion:

There is no clear view among neonatologists regarding the role of *Ureaplasma spp.* in the development of CLD. However, there was a clear call for a randomised controlled trial to determine if eradication of *Ureaplasma spp.* decreases the development of CLD.

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Dr Maxwell is supported by the Wellcome Trust.

Appendix 4

Table of data

Clinical information

Baby	Gestation	Weight (kg)	Diagnosis	Vent. Days	Oxygen days	pPROM	Antenatal infection suspected	Delivery
A	Term	2.8	Term	2	2	no	no	Vaginal
B	25+5	0.74	CLD	22	44	yes	yes	Vaginal
C	31+2	1.92	RDS	1	0	yes	yes	Vaginal
D	25+4	0.84	RDS	12	12	no	no	Vaginal
E	28	0.91	CLD	3	20	no	no	Caesarean
F	31	1.69	RDS	2	3	yes	yes	Caesarean
G	25+3	1.04	CLD	31	63	no	no	Vaginal
H	Term	3	Term	9	30	no	no	Caesarean
I	26+6	1	RDS	18	47	no	no	Vaginal
J	27	0.7	RDS	18	32	yes	yes	Caesarean
K	Term	2.81	Term	3	3	no	no	Caesarean
L	27	0.85	CLD	35	36	no	no	Caesarean
M	28+4	1.31	RDS	5	2	yes	yes	Vaginal
N	25+3	0.79	CLD	29	67	no	yes	Vaginal
O	26	0.74	CLD	58	101	yes	yes	Vaginal
P	26+5	0.87	Died	5	5	no	yes	Vaginal
Q	25+4	0.76	CLD	43	107	no	no	Vaginal
R	29+3	1.02	CLD	44	107	no	no	Caesarean
S	26+4	0.89	RDS	10	18	no	yes	Caesarean
T	26+4	1.04	RDS	13	18	no	yes	Caesarean
U	28+3	1.18	CLD	17	75	no	yes	Caesarean
V	28+3	1.08	CLD	12	75	no	yes	Caesarean
W	24+4	0.69	CLD	49	89	no	no	Vaginal
X	Term	2.63	Term	5	2	no	no	Vaginal
Y	26+2	0.9	CLD	88	154	no	yes	Vaginal
Z	26+2	1.08	CLD	28	69	no	yes	Vaginal
AA	Term	2.57	Term	4	1	no	no	Caesarean
BB	23+4	0.56	CLD	45	86	no	no	Vaginal
CC	27+1	1.23	CLD	36	109	no	no	Caesarean
DD	28	0.91	RDS	16	44	no	no	Caesarean
EE	24+2	0.67	CLD	39	45	no	no	Vaginal
FF	27+1	1.18	RDS	1	1	no	no	Vaginal

Baby	Day	Gest	Wt	Dx	Cell count	Abs CD14	Abs CD36	Abs HLADR	PMN%	MNC%	% debris	CD14hiMNC	Abs PMN	Abs MNC	Abs mono	PMN:MNC	Elastase	16s	Uu
A	1	38	2.8	Term	1.04	0.1401		0.053664									0	0	0
AA	1	38.428	2.57	Term	0.6857	0.0644	0.0445705	0.07083281	9.06	10.33	80.61	1.49	0.0621244	0.0708328	0.0102169	2.23703704	0	1	0
	2	38.428			0.7182	0.1366	0.05307498	0.1217349	26.48	16.95	56.57	1.62	0.1901794	0.1217349	0.0116348	5.13178295	0	0	0
B	1	25.714	0.74	CLD	4.66	0.1154		0.270746									0	1	0
	2	25.714			9.99	0.2804		0.403596									0.295	1	0
	3	25.714			2.4	0.3333		0.20064									5.5	0	0
	4	25.714			3.24	0.2828		0.308124									58.1	0	0
	5	25.714			5.88	1.059		0.944328									20.655		0
	6	25.714															6.44	0	0
	7	25.714			1.22	1.0325		0.179096									0		0
	9	25.714			1.34	1.2726		0.230212									0	0	0
BB	1	23.57	0.56	CLD	2.35	0.1859	0.13583	0.120085	24.62	5.89	69.49	4.85	0.57857	0.138415	0.113975	4.17996604	0	0	0
	2	23.57			0.9429	0.8766	0.05035086	0.0452592	41.73	7.64	50.63	8.74	0.3934722	0.0720376	0.0824095	5.46204188	0		0
	3	23.57			2.5667	1.2912	0.23921644	0.16503881	33.3	9.32	57.38	9.26	0.8547111	0.2392164	0.2376764	4.3134715	0		0
	4	23.57			4.1625	1.2672	0.39585375	0.46953	40.81	11.28	47.91	11.24	1.6987163	0.46953	0.467865	4.32767762	0	0	0
	5	23.57			9.675	3.0495	2.145915	1.124235	59.97	25.84	14.19	21.69	5.8020975	2.50002	2.0985075	2.32082043	0.19	1	0
	6	23.57			5.9	1.1526	1.14932	1.4278	5.5	39.12	55.38	16.26	0.3245	2.30808	0.95934	0.14059305	0	1	0
	7	23.57			4.44	2.0517	1.044732	1.070484	22.13	61.64	16.23	10.34	0.982572	2.736816	0.459096	0.35902012	0	1	0
	9	23.57			4.5	2.9673	1.18935	1.0701	40.68	26.43	32.89		1.8306	1.18935		1.62915499	0	1	0
	13	23.57			2.08	3.8064	0.625872	0.622128	13.89	30.09	56.02	27.56	0.288912	0.625872	0.573248	0.50380849	0	1	0
	16	23.57			1.5	4.192	0.3255	0.3681	40.56	24.54	34.9	20.78	0.6084	0.3681	0.3117	1.69140951	0	0	0
	20	23.57			3.75	5.628	0.75	0.823875	45.41	29.87	24.72	25.82	1.702875	1.120125	0.96825	1.52025444	0	0	0
	23	23.57			0.7	6.0237	0.1785	0.16723	28.26	25.63	46.11	23.83	0.19782	0.17941	0.16681	1.10261412	0	0	0
C	1	31.857	1.92	RDS	1.14	0.0769		0.026562									0	0	0
CC	2	27.143	1.23	CLD	0.9667	0.4822	0.04205145	0.02494086	17.64	5.63	76.73	5.34	0.1705259	0.0544252	0.0516218	3.13321492	0	1	0
	3	27.143			0.8462	0.4593	0.03494806	0.03697894	11.54	4.48	83.98	4.49	0.0976515	0.0379098	0.0379944	2.57589286	0	1	0
	4	27.143			0.71	0.4492	0.063545	0.037204	10.34	8.95	80.71	4.98	0.073414	0.063545	0.035358	2.20940171	0.245	1	0
	5	27.143			1.54	0.3255	0.165704	0.141218	12.64	13.66	73.7	2.19	0.194656	0.210364	0.033726	0.92532943	0.53	1	0
	6	27.143			1.42	0.6204	0.169406	0.30317	11.44	21.35	67.21	2.82	0.162448	0.30317	0.040044	1.10960233	0	1	0
	7	27.143			1.8923	0.4235	0.26094817	0.30712029	1.86	16.23	81.91	1.46	0.0351968	0.3071203	0.0276276	0.54705882	0	1	0
	9	27.143			1.5938	0.9513	0.26887406	0.6526611	12.62	40.95	46.43		0.2011376	0.6526611		1.20534862	0		0
	13	27.143			0.7538	2.3166	0.12558308	0.23450718	6.67	31.11	62.22	11.25	0.0502785	0.2345072	0.0848025	0.27493817	0	0	0
	16	27.143			1.8789	4.3984	0.50899401	0.79007745	15.42	42.05	42.53	24.61	0.2897264	0.7900775	0.4623973	0.38588589	0	1	0
	20	27.143			0.8867	5.738	0.20163558	0.3795076	28.37	42.8	28.83	23.94	0.2515568	0.3795076	0.212276	0.93016393	0	0	0
D	2	25.57	0.84	RDS	2	0.1572		0.1442		7.21	92.79			0.1442			0	0	0
	3	25.57			1.46	0.2148	0.046574	0.1898	1.91	19.93	78.16		0.027886	0.290978		0.09583542	0	0	0
	4	25.57			3.2	0.196	0.1792	0.28128	11.76	18.06	70.18		0.37632	0.57792		0.65116279	1.59	1	0
	5	25.57			3.14	0.578	0.2198	0.20567	28.5	15.14	56.36		0.8949	0.475396		1.88243065	2.12	0	0
	6	25.57			2.04	0.7926	0.073032	0.04386	58.39	16.9	24.71		1.191156	0.34476		3.45502959	18.69	0	0
	7	25.57			3.33	0.6265	0.177156	0.113886	35.21	12.36	52.43		1.172493	0.411588		2.8487055	0	1	0
DD	1	28	0.91	RDS	0.5333		0.04042414	0.03050476	2.57	7.58	89.85		0.0137058	0.0404241		2.35779817	0	0	0
	2	28			0.7	0.2152	0.05908	0.01932	5.06	8.44	86.5	1.96	0.03542	0.05908	0.01372	2.28959276	0	0	0
E	1	28	0.91	RDS	4.4	0.0684		0.30448						0			0	0	0

Baby	Day	Gest	Wt	Dx	Cell count	Abs CD14	Abs CD36	Abs HLADR	PMN%	MNC%	% debris	CD14hiMNC	Abs PMN	Abs MNC	Abs mono	PMN:MNC	Elastase	16s	Uu
EE	3	24.286	0.67	CLD	2.65	0.4704	0.292825	0.26765	48.54	11.52	39.94	10.19	1.28631	0.30528	0.270035	4.21354167	0	0	1
	4	24.286			1.625	0.4852	0.11635	0.1285375	31.37	7.91	60.72	7.57	0.5097625	0.1285375	0.1230125	4.29726027	0	0	1
	5	24.286			2.8	0.8745	0.37184	0.2954	55.52	13.28	31.2	9.33	1.55456	0.37184	0.26124	4.28395062	0	0	1
	6	24.286			15	1.5282	3.036	2.5875	74.13	21.19	4.68	20.76	11.1195	3.1785	3.114	3.49834828	0.2	0	1
	7	24.286			5.0857	1.4532	0.7781121	0.75726073	72.09	17.43	10.48	14.41	3.6662811	0.8864375	0.7328494	4.13597246	0	0	1
	9	24.286			4.0875	2.6622	0.92254875	0.9344025	58.19	30.03	11.78	25.85	2.3785163	1.2274763	1.0566188	1.93772894	0.275	0	1
	13	24.286			0.6875	1.6913	0.09851875	0.15021875	14.6	21.85	63.55	8.59	0.100375	0.1502188	0.0590563	1.04885057	0	0	0
	16	24.286			0.7333	1.8016	0.17210551	0.16880566	28.33	23.47	48.2	6.24	0.2077439	0.1721055	0.0457579	3.62740077	0	0	0
	20	24.286			0.864	3.372	0.1616544	0.0870912	43.75	20.46	35.79	15.5	0.378	0.1767744	0.13392	2.13831867	0	0	0
	23	24.286							64.42	27.28	8.3	21.5					42.565	1	0
	27	24.286			0.324	5.5161	0.086022	0.1290492	3.99	39.83	56.18	13.98	0.0129276	0.1290492	0.0452952	0.175	0	0	0
F	1	31	1.69	RDS	1.76	0.0084	0.015312	0.0038896	3.7	2.56	93.74		0.06512	0.045056		1.4453125	0	0	0
	2	31			1.84	0.0318	0.021896	0.009936	10.46	3.12	86.42		0.192464	0.057408		3.3525641	0	0	0
FF	1	27.14	1.18	RDS	1.26				5.7	4.23	90.07		0.07182	0.053298		1.34751773			0
G	1	25.428	1.04	CLD	2.85	0.0126	0.03021	0.00114	4.25	3.03	92.72		0.121125	0.086355		1.40264026	0	0	0
	2	25.428			0.93				6.8	3.22	89.98		0.06324	0.029946		2.11180124	0	0	0
	3	25.428			1.81	0.684	0.102265	0.430418	14.64	28.67	56.69		0.264984	0.518927		0.5106383	0		0
	4	25.428			1.88	2.2288	0.5593	1.231588	9.35	67.36	23.29		0.17578	1.266368		0.13880641	0	0	0
	5	25.428			1.45	2.2815	0.415425	1.065605	7.84	73.49	18.67		0.11368	1.065605		21.1891892	0	0	0
	6	25.428			2.5	1.7178	0.404175	1.0255	11.14	41.02	47.84		0.2785	1.0255		0.39239169	0	1	0
	7	25.428			2.8	2.4829	0.62468	1.14604	29.64	40.93	29.43		0.82992	1.14604		0.84252416	0	1	0
	9	25.428			6.06	2.1897	1.084134	0.417534	62.52	30.04	7.44		3.788712	1.820424		2.08122503	0	0	0.5
	13	25.428			3.93	1.3364	0.289248	0.368634	36.1	14.84	49.06		1.41873	0.583212		2.43261456	1.4	1	1
	16	25.428			2.29	1.36	0.154346	0.21526	47.02	16.62	36.36		1.076758	0.380598		2.82912154	2.73	1	0
	20	25.428			1.78	2.014	0.244928	0.433964	44.59	29.88	25.53		0.793702	0.531864		1.49230254	0	0	0
	23	25.428			1.43	1.7503	0.124982	0.218361	18.23	15.27	66.5		0.260689	0.218361		1.87938144	0	0	0
H	1	38	3	Term	1.28	0.063	0.099712	0.09408	5.57	9.63	84.8		0.071296	0.123264		0.57840083	0	0	0
	2	38			1.21	0.0448	0.03751	0.028556	2.1	3.1	94.8		0.02541	0.03751		0.67741935	0	0	0
	3	38			1.11	0.1263	0.037851	0.045399	2.82	4.09	93.09		0.031302	0.045399		1.29953917	0		0
	4	38			0.44	0.28	0.028204	0.046024	3.57	10.46	85.97		0.015708	0.046024		0.40022422	0	0	0
	5	38			0.44	0.6535	0.034716	0.030008	9.83	10.41	79.76		0.043252	0.045804		0.94428434	0	0	0
I	1	26.857	1	RDS	3.06	0.0756	0.028458	0.012852	25.07	8.49	66.44		0.767142	0.259794		2.95288575	0	0	1
	2	26.857			6.29	0.1292	0.279905		30.41	8.1	61.49		1.912789	0.50949		3.75432099	0	0	1
J	1	27	0.7	RDS	1.95	0.0709	0.202605	0.23205	51.31	19.06	29.63		1.000545	0.37167		2.69202518	0		0
	2	27			1.35	0.0962	0.08208	0.08532	45.65	6.32	48.03		0.616275	0.08532		66.1594203	0	0	0
	3	27			1.45	0.183	0.107445	0.08932	13.16	7.41	79.43		0.19082	0.107445		3.52815013	0	0	0
K	1	38.428	2.81	Term	1.7	0.0398	0.12019	0.12767	7.2	7.51	85.29	0.87	0.1224	0.12767	0.01479	2.80155642	0	0	0
	2	38.428			0.74	0.027	0.042402	0.04514	42.66	6.1	51.24	1.52	0.315684	0.04514	0.011248		0	0	0
L	1	26.857	0.85	CLD	0.7		0.05852	0.0476	5.71	8.36	85.93		0.03997	0.05852		1.94880546	0		0
	2	26.857			0.8571	0.1998	0.03728385	0.02965566	9.75	5.28	84.97		0.0835673	0.0452549		1.84659091	0	0	0
	3	26.857			0.2		0.0281	0.02024	19.57	14.05	66.38		0.03914	0.0281		2.12486428	0	0	0
	4	26.857			0				14.15	40.12	45.73		0				0		0
	5	26.857			0.8		0.20936	0.48216	1.69	60.27	38.04		0.01352	0.48216		0.3359841	0	0	0
	6	26.857			2.1	0.2346	0.2331	0.29799	5.27	14.19	80.54	3	0.11067	0.29799	0.063		0	0	0
	7	26.857			1		0.1203	0.1463	7.07	14.63	78.3		0.0707	0.1463		0.87717122	0	0	0
	9	26.857			1.3333	0.6678	0.16946243	0.12359691	20.19	12.71	67.1	2.27	0.2691933	0.1694624	0.0302659	1.75108413	0	0	0
	13	26.857			0.5333		0.12356561	0.17134929	5.04	32.13	62.83		0.0268783	0.1713493		0.23863636	0	0	0
	16	26.857			2.875	1.2528	0.6816625	0.4568375	16.81	23.71	59.48	9.23	0.4832875	0.6816625	0.2653625	0.85113924	89.94	1	0
	20	26.857			3	0.63	0.3129	0.2325	47.79	10.43	41.78	6.74	1.4337	0.3129	0.2022	5.70286396	242.235	1	0
	23	26.857			2.3	1.7457	0.47311	0.52854	41.75	22.98	35.27	12.55	0.96025	0.52854	0.28865	2.18357741	0	1	0
	27	26.857			2.1818	2.6298	0.12174444	0.15468962	15.38	7.09	77.53	3.43	0.3355608	0.1546896	0.0748357	2.19400856	0	0	0

Baby	Day	Gest	Wt	Dx	Cell count	Abs CD14	Abs CD36	Abs HLADR	PMN%	MNC%	% debris	CD14hiMNC	Abs PMN	Abs MNC	Abs mono	PMN:MNC	Elastase	16s	Uu
M	1	28.57	1.31	RDS	9	0.4216	2.6901	3.7953	40.11	44.44	15.45	33.24	3.6099	3.9996	2.9916	0.90256526	0	0	0
	2	28.57			1.6222	0.4662	0.14908018	0.20212612	55.21	25.52	19.27	10.99	0.8956166	0.4139854	0.1782798	2.16340125	0	0	0
	3	28.57			1.12	0.7701	0.10864	0.224	52.62	49.69		15.56	0.589344	0.556528	0.174272	1.05896559	0.745	0	0
N	1	25.428	0.79	CLD	2.6364	0.0969	0.85498452	0.48694308	36.8	36	27.2	5.7	0.9701952	0.949104	0.1502748	1.02222222	0	0	1
	2	25.428			0.3429		0.0037719	0.01676781	10.53	10.06	79.41		0.0361074	0.0344957		1.04671968	0	0	1
	3	25.428			2.3091	0.4257	0.03117285	0.09767493	18.57	4.23	77.2	1.28	0.4287999	0.0976749	0.0295565	10.5511364	0	0	1
	4	25.428			10.9091	0.278	0.72763697	0.981819	59.27	9	31.73	4.91	6.4658236	0.981819	0.5356368	10.2720971	15.13	1	1
	5	25.428			7.0077	0.442	0.45409896	0.26348952	46.56	19.27	34.17	6.72	3.2627851	1.3503838	0.4709174	2.41619097	28.765	1	1
	6	25.428			5.8	0.63	0.4031	0.47966	60.78	8.27	30.95	5.67	3.52524	0.47966	0.32886	7.81233933	17.1	1	1
	7	25.428			7.8	0.5411	0.5343	0.20592	42.42	6.85	50.73	4.72	3.30876	0.5343	0.36816	6.98846787	21.045	0	1
	13	25.428			3.6667	0.7605	0.83564093	0.97680888	50.47	31.2	18.33	16.34	1.8505835	1.1440104	0.5991388	1.61762821	43.095	0	0
O	1	26	0.74	CLD	0.3636	0.0395	0.00785376	0.00705384	7.41	2.7	89.89		0.0269428	0.0098172		2.74444444	0	1	1
	2	26			2.65	0.2618	0.20405	0.249895	33.79	21.32	44.89		0.895435	0.56498		1.58489681	0	0	1
	3	26			2.9846	0.3078	0.28413392	0.20802662	34.61	9.52	55.87		1.0329701	0.2841339		4.89533239	0	0	1
	5	26			0.6667												0	0	0.5
	6	26			1.7143	0.3042	0.06325767	0.06754342	25.67	11.34	62.99		0.4400608	0.1944016		2.26366843	3.11	0	0.5
	7	26			10.1	1.4798	0.59893	0.15756	69.15	17.47	13.38		6.98415	1.76447		3.95821408	0	1	1
	9	26			5.3571	0.6813	0.32731881	0.32946165	58.2	9.92	31.88		3.1178322	0.5314243		5.86693548	6.73	0	1
	13	26			1.3143	2.509	0.14207583	0.07162935	58.98	29.36	11.66		0.7751741	0.3858785		2.00885559	0	0	1
	16	26			2	4.9104	0.391	0.6344	27.09	31.72	41.19		0.5418	0.6344		0.91028226	0	0	1
	20	26			0.6667	10.706	0.17634215	0.35248429	1.11	61.84	37.05		0.0074004	0.4122873		0.01794955	0		0
	23	26			1.2333	1.1799	0.05907507	0.04045224	4.21	6.47	89.32		0.0519219	0.0797945		0.65069552	0	0	0.5
	27	26			0.1833				57.64	14.62	27.74			0.0267985			0	0	0.5
	31	26								21.92	78.08								
P	1	26.714	0.87	Died	2.3182	0.035	0.02040016	0.02735476	3.9	2.44	93.66		0.0904098	0.0565641		1.59836066	0.865	0	0
	2	26.714			3.825	0.0736	0.055845	0.0677025	3.69	1.77	94.54		0.1411425	0.0677025		2.14534884	0.975	0	1
	3	26.714			5.3333	0.3717	0.43679727	1.50772391	1.84	28.27	69.89		0.0981327	1.5077239		0.12707182	0.815	0	1
	5	26.714							12.3	26.93	60.77						0.83	0	1
Q	1	25.57	0.76	CLD	1.4571	0.2226	0.04050738	0.08626032	1.1	14.73	84.17		0.0160281	0.2146308		0.07467753	0	0	1
	2	25.57			2.16	0.3074	0.26028	0.150768	2.8	13.33	83.87		0.06048	0.287928		0.21005251	0	0	1
	3	25.57			1.44	0.7851	0.286848	0.398736	0.75	36.91	62.34		0.0108	0.531504		0.0203197	0	0	1
	4	25.57			0.775	0.208	0.0501425	0.0712225		9.19	90.81			0.0712225			0	0	1
	5	25.57			0.7429	0.304	0.04249388	0.09033664	4.76	12.16	83.08		0.035362	0.0903366		0.86861314	0	0	1
	6	25.57			0.8	1.8912	0.19264	0.41368	3.87	51.71	44.42		0.03096	0.41368		0.11292676	0	0	0.5
	7	25.57			0.35				44.19	17.23	38.58		0.154665	0.060305		2.56471271	0	0	1
	9	25.57			2.6286	3.6153	0.96233046	1.42838124	14.01	54.34	31.65		0.3682669	1.4283812		0.31307263	0	0	1
	13	25.57			0.4133	4.1925	0.16403877	0.20850985	8.98	50.45	40.57		0.0371143	0.2085099		0.23538663	0	0	0
	16	25.57			0.3	3.7888	0.06198	0.06339	27.31	23.49	49.2		0.08193	0.07047		1.16262239	0	0	0
	20	25.57			0.08												0	0	0
	23	25.57			0.1333												0	0	0
	27	25.57			0.7143	7.5168	0.30229176	0.32036355	15.32	49.64	35.04		0.1094308	0.3545785		0.30862208	0	0	0

Baby	Day	Gest	Wt	Dx	Cell count	Abs CD14	Abs CD36	Abs HLADR	PMN%	MNC%	% debris	CD14hiMNC	Abs PMN	Abs MNC	Abs mono	PMN:MNC	Elastase	16s	Uu
R	1	29.428	1.02	CLD	1.8267	0.0323	0.11398608	0.10211253	13.49	6.24	80.27		0.2464218	0.1139861		5.29019608	0	0	0
	2	29.428			0.3733	0.0514	0.01224424	0.00627144	3	3.28	93.72		0.011199	0.0122442		1.50753769	0	0	0
	3	29.428			1.0625	0.1089	0.051	0.0204	3.63	4.8	91.57		0.0385688	0.051		1.87113402	0	0	0
	4	29.428			0.3538	0.4128	0.02614582	0.0263581	20.46	7.45	72.09		0.0723875	0.0263581		4.15010142	0	0	0
	5	29.428			0.6	1.332	0.06768	0.06684	41.03	14.64	44.33		0.24618	0.08784		2.80259563	0	0	0
	6	29.428			1.04	1.4736	0.111384	0.042224	45.09	15.92	38.99		0.468936	0.165568		2.83228643	0	0	0
	7	29.428			0.36	1.3461	0.044316	0.03888	17.11	18.11	64.78		0.061596	0.065196		0.94478189	0	0	0
	9	29.428			0.3857	1.0827	0.05087383	0.05226235	12.4	13.55	74.05		0.0478268	0.0522624		2.80542986	0	0	0
	13	29.428			1.0909	1.6575	0.12959892	0.13570796	40.98	12.44	46.58		0.4470508	0.135708		3.77348066	1.91	0	0
	16	29.428			0.9231	10.0672	0.32926977	0.10144869	41.73	35.67	22.6		0.3852096	0.3292698		1.27497709	25.53	0	0
	20	29.428			1.875	5.026	0.4344375	0.1725	43.31	23.17	33.52		0.8120625	0.4344375		2.8739217	48.7	0	0
S	1	26.57	0.89	RDS	7.725	0.1992	1.259175	1.1672475	42.74	18.11	39.15		3.301665	1.3989975		2.36002209	0	0	0
	2	26.57			3.3	0.3808	0.30789	0.33891	25.09	10.27	64.64		0.82797	0.33891			0	0	0
	3	26.57			3.575	0.5628	0.3557125	0.452595	52.09	12.66	35.25		1.8622175	0.452595		6.20858164	2.25	0	0
T	1	26.57	1.04	RDS	0.5385	0.1865	0.0609582	0.03225615	12.06	11.32	76.62		0.0649431	0.0609582		1.75036284	0	0	0
	2	26.57			1.3	0.1534	0.078	0.0832	6.62	6.4	86.98		0.08606	0.0832		2.35587189	0	0	0
	3	26.57			0.6909	0.1908	0.02922507	0.03551226	11.39	6.22	82.39		0.0786935	0.042974		1.83118971	0	0	0
	4	26.57			0.4867	0.372	0.04755059	0.07013347	22.04	14.41	63.55		0.1072687	0.0701335		1.94871795	0	0	0
	5	26.57			2.93	0.7345	0.359511	0.352772	69.55	12.27	18.18		2.037815	0.359511		6.11697449	1.375	1	0
	6	26.57			2.1	1.0992	0.42315	0.35091	40.28	22.3	37.42		0.84588	0.4683		1.80627803	1.225	1	0
	7	26.57			0.6286	0.609	0.07568344	0.08900976	30.44	14.16	55.4		0.1913458	0.0890098		3.66305656	0	1	0
U	1	28.428	1.18	CLD	0.6	0.0886	0.05118	0.02598	3.44	8.53	88.03	3.44	0.02064	0.05118	0.02064	0.88431877	0	0	0
	2	28.428			1.05	0.1168	0.028875	0.05565	5.59	5.3	89.11	0.7	0.058695	0.05565	0.00735	2.07037037	0	0	0
	3	28.428			0.4429	0.2094	0.02449237	0.02493527	4.55	5.63	89.82	0.81	0.020152	0.0249353	0.0035875	3.20422535	0	0	0
	4	28.428			0.6	0.2916	0.0231	0.04194	2.89	8	89.11	3.87	0.01734	0.048	0.02322	0.36125	0	1	0
V	1	28.428	1.04	CLD	0.6154	0.0601	0.01396958	0.0230775	2.24	3.75	94.01	2.6	0.013785	0.0230775	0.0160004	1.19148936	0	0	0
	2	28.428			0.5385	0.2034	0.02600955	0.0341409	7.42	10.32	82.26	8.87	0.0399567	0.0555732	0.047765	0.71899225	0	0	0
	3	28.428			0.3333	0.3657	0.01246542		0.27	11.34	88.39	2.48	0.0008999	0.0377962	0.0082658	0.02380952	0	0	0
	4	28.428			2.3714	0.4732	0.14536682	0.22030306	35.46	14.77	49.77	8.78	0.8408984	0.3502558	0.2082089	2.40081246	41.44	1	0
	5	28.428			1.6667	0.397	0.02866724	0.16150323	4.05	12.34	83.61	5.41	0.0675014	0.2056708	0.0901685	0.32820097	0	1	0
W	3	24.57	0.69	CLD	0.633	0.6372	0.1137501	0.1998381	4.25	31.57	64.18		0.0269025	0.1998381		0.15103056	0	0	0
	4	24.57			1.45	0.3384	0.04234	0.061625	4.17	22.21	73.62	7.58	0.060465	0.322045	0.10991	0.18775326	0.135	0	0
	5	24.57			1.6167	0.6895	0.21211104	0.17767533	39.78	20.51	39.71	5.34	0.6431233	0.3315852	0.0863318	1.93954169	0.07	0	0
	6	24.57			3.8636	1.0254	0.24765676	0.164203	67.66	66.47	-34.13	12.73	2.6141118	2.5681349	0.4918363	1.01790281	2.345	0	1
	7	24.57			1.525	1.134	0.0666425	0.0468175	23.31	57.37	19.32	54.48	0.3554775	0.8748925	0.83082	0.40630992	0	0	1
	9	24.57			1.1333	1.8027	0.09576385	0.033999	19.53	26.26	54.21	23.62	0.2213335	0.2976046	0.2676855	0.74371668	0	0	0
	13	24.57							1.97	52.2	45.83	16.88					0	0	0
	16	24.57			0.5	0.4672	0.0481	0.0249	0.36	9.62	90.02		0.0018	0.0481		0.06282723	0	0	0
	20	24.57			2.1667	2.766	0.25003718	0.33735519	56.85	15.57	27.58	4.57	1.231769	0.3373552	0.0990182	5.69639279	7.83	0	1
	23	24.57			2.625	8.7906	0.4704	0.5200125	21.89	19.81	58.3	14.22	0.5746125	0.5200125	0.373275	1.39338001	0.295	0	1
	27	24.57			1.57	20.8332	0.585767	0.557664	37.48	41.52	21		0.588436	0.651864		0.9026975	0		1

Baby	Day	Gest	Wt	Dx	Cell count	Abs CD14	Abs CD36	Abs HLADR	PMN%	MNC%	% debris	CD14hiMNC	Abs PMN	Abs MNC	Abs mono	PMN:MNC	Elastase	16s	Uu
X	1	38	2.65	Term	0.4273	0.0643	0.0029911	0.14720485	5.66	34.45	59.89	25.76	0.0241852	0.1472049	0.1100725	0.21603053	0	0	0
	2	38			0.3333	0.2062	0.01279872	0.05772756	7.04	17.32	75.64	1.99	0.0234643	0.0577276	0.0066327	2.5323741	0	0	0
	3	38			0.2258	0.2313	0.00704496	0.03269584	9.26	14.48	76.26	5.68	0.0209091	0.0326958	0.0128254	1.29691877	0	0	0
	4	38			0.0714	0.7088	0.00957474	0.00588336	7.95	13.41	78.64		0.0056763	0.0095747		1	0	0	1
	5	38			0.2941	2.1185	0.01196987	0.04396795	35.29	14.95	49.76	5.05	0.1037879	0.043968	0.0148521	5.82343234	1.37	0	1
Y	1	26.286	0.91	CLD	1.2154	0.052	0.06709008	0.0474006	1.39	5.52	93.09	0.99	0.0168941	0.0670901	0.0120325	1.26363636	0	0	0
	2	26.286			1.0667	0.0948	0.02858756	0.02485411	1.52	2.68	95.8	1.32	0.0162138	0.0285876	0.0140804	0.88888889	0	0	0
	3	26.286			1.4154	0.3993	0.20070372	0.36078546	0.77	25.49	73.74	17.58	0.0108986	0.3607855	0.2488273	0.04333146	0	0	0
	4	26.286			1.4	0.4004	0.23674	0.5614	7.56	40.1	52.34	28.12	0.10584	0.5614	0.39368	0.21129122	0	0	0
	5	26.286			0.6643	0.7255	0.1155882	0.22991423	13.37	34.61	52.02	29.69	0.0888169	0.2299142	0.1972307	0.42566062	0		1
	6	26.286			1.4231	0.888	0.35506345	0.35136339	9.47	24.95	65.58	9.35	0.1347676	0.3550635	0.1330599	0.48663926	0	1	1
	7	26.286			1	0.4165	0.095	0.0351	2.67	9.5	87.83	0.89	0.0267	0.095	0.0089	2.1023622	0	0	1
	9	26.286			0.1818						100						0	0	1
	13	26.286			2.51	1.3676	0.225649	0.270076	15.38	10.76	73.86	4.83	0.386038	0.270076	0.121233	2.32326284	0.805	0	1
	20	26.286			5.3333	8.726	1.24372556	0.97386058	65.64	23.32	11.04	21.2	3.5007781	1.2437256	1.1306596	5.14823529	42.21	0	1
	23	26.286			2.2909	14.49	1.23387874	1.42745979	31.7	62.31	5.99	52.27	0.7262153	1.4274598	1.1974534	0.54920305	0.585	0	0.5
	27	26.286			9.1667	25.137	1.59958915	0.77825283	79.7	32.49		17.37	7.3058599	2.9782608	1.5922558	2.45306248	44.665		1
Z	1	26.286	1.08	CLD	1.1455	0.0719	0.06930275	0.0368851	6.84	6.05	87.11	1.55	0.0783522	0.0693028	0.0177553	3.48979592	0	0	0
	2	26.286			0.24	0.1808	0.016704	0.043368	10.11	18.07	71.82		0.024264	0.043368		1.93307839	0	0	0
	3	26.286			0.5857	0.5283	0.10794451	0.17301578	1.84	29.54	68.62	13.51	0.0107769	0.1730158	0.0791281	0.09823812	0	0	0
	4	26.286			0.5875	0.8828	0.14634625	0.216435	4.52	36.84	58.64	34.46	0.026555	0.216435	0.2024525	0.12932761	0		0
	5	26.286			0.2917	1.153	0.06405732	0.13520295	1.96	46.35	51.69		0.0057173	0.135203		0.05172869	0	0	1
	6	26.286			0.5278	1.1886	0.09284002	0.18847738	7.77	35.71	56.52	10.34	0.0410101	0.1884774	0.0545745	0.42762796	0	0	1
	7	26.286			5.4	4.0404	1.72314	1.01736	56.17	35.89	7.94	21.98	3.03318	1.93806	1.18692	1.56505991	0.905	0	1
	9	26.286			2.1875	2.4399	0.343	0.1771875	37.33	15.68	46.99	8.59	0.8165938	0.343	0.1879063	3.90073145	0	0	1
	13	26.286			0.7667	3.2968	0.14889314	0.16277041	49.91	21.23	28.86	17.91	0.38266	0.1627704	0.137316	2.35091851	0	0	1
	16	26.286			1.8182	1.3552	0.29891208	0.2981848	55.28	16.44	28.28	3.21	1.005101	0.2989121	0.0583642	3.89021816	2.925	0	0.5

Baby	Day	IL-1b	IL-8	MCP-1	IL-6	IL-10	GCSF	GMCSF	MIP1a	MIP1b	TNF	FasL	VEGF	Apopt.	Viable	Necr.	Trypan blue %
A	1	70.3453	5791.58	9357.713	726.3317	1.28464	287.3604	0	73.53495		12.967	21.545	22.2832	16.11	57.59	26.3	
AA	1	67.2839	10360.1	30647.22	3120.22	0	999.6245	0	67.03882	410.1023	0	15.896	0	18.57	56.95	24.48	60
	2	131.912	17922.9	56478.3	2187.09	0	432.5354	0	156.7855	667.0431	0	15.141	0	36.74	46.86	16.4	13
B	1	1369.23	35459.7	39885.23	18899.85	513.643	17313.54	0	1944.285	29416.92	174.02	28.484	19.0176	16.77	4.35	78.87	
	2	347.935	14407.6	67580.27	4094.246	315.355	2609.948	0	1346.411	34843.9	65.707	70.357	61.5526	12.12	45.53	42.35	
	3													10.92	63.49	25.6	
	4	1680.52	79409.7	83389.4	3105.888	81.1012	7499.903	0	3973.557	11465.47	129.1	88.974	680.654	1.04	51.08	47.89	
	5													33.15	37.02	29.82	
	6																
	7	626.473	26324.7	19828.29	2085.096	18.3552	2452.839	0	1433.041	7657.434	97.006	36.301	554.879	4.96	14.15	80.89	
	9	118.502	21817.1	20113.5	731.0233	20.1529	1365.972	0	966.874	6689.456	19.931	44.943	948.17	23.32	34.62	39.06	
BB	1	99.015	24492.8	15422.08	10793.76	162.178	5739.828	0	279.1318	2719.13	26.58	38.865	1140.33	37.56	50.41	12.03	20
	2	63.3337	8623.21	5718.847	3249.34	10.0621	663.9006	0	117.7689	1021.48	0	14.914	898.749	18.01	57.05	24.94	10
	3													24.92	49.51	25.57	7
	4	906.752	65683.2	66179.76	9303.98	200.41	13165.38	0	3609.863	40248.5	96.671	63.383	1881.72	3.69	75.43	20.88	10
	5													20.46	40.61	38.93	21
	6													20.74	61.05	18.21	5
	7	2288.26	132500	78821.06	11333.99	246.783	9921.275	0	931.0261	11719.34	61.677	57.452	2140.82	11.91	62.76	25.33	1
	9	2005.72	73942	86587.49	5663.87	16.0848	7418.799	0	1040.887	8696.06	22.692	67.615	1336.62	22.57	59.34	18.09	7
	13													18.98	55.44	25.57	5
	16	1745.54	23763	54218.06	2482.1	16.6085	2506.602	0	462.8169	2023.44	51.521	32.821	1268.35	22.89	64.47	12.63	20
	20													11.28	66.02	22.7	5
	23	1142.64	17904.5	38582.13	1200.23	0	2050.711	0	335.0952	1116.11	56.254	33.765	1334.43	16.87	68.69	14.44	
C	1	13.5776				1.83212	115.714	0	0		6.2873	12.007	74.8955	15.82	45.76	38.42	
CC	2	356.817	19123.7	10652.95	4219.38	0	3160.365	0	920.2394	7567.88	18.702	4.2836	383.207	41.47	51.07	7.45	16
	3													44.87	48.5	6.64	19
	4	3639.54	92231	29267	18924.49	0	19140.1	0	4577.63	49289.11	35.482	9.0961	684.304	14.47	64.74	20.59	10
	5													19.88	57.54	22.58	13
	6													34.02	43.82	22.15	24
	7	3788.77	131949	78085.17	25819.98	0	35034.01	42.28798	4335.386	42457.32	284.33	28.439	572.267	31.06	52.38	16.55	26
	9	273.926	51668.3	83775.25	2240.68	0	3820.884	14.42969	339.0553	7063.16	9.2881	28.459	460.231	12.2	49.78	38.02	22
	13													12.56	44.09	43.35	26
	16													18.97	48.66	32.37	6
	20													13.56	57.17	29.27	8
D	2	150.951	44122.5	63909.42	1749.58	0	714.3317	0	224.6558	2522.99	12.255	20.656	493.055				
	3																
	4	882.921	30049.3	46875.42	2057.29	21.679	9927.382	0	756.5183	7329.29	255.78	24.736	254.104	1.44	78.29	20.27	
	5													19.81	66.93	13.25	
	6													2.91	82.96	14.13	
	7	3399.44	185004	86587.49	12349.06	59.0532	17456.6	4.244753	6013.286	49363.74	413.61	24.736	1694.84	2.37	66	31.63	
DD	1	38.8554	317.286	980.2672	784.37	0	253.4785	0	0	215.1048	0	6.3762	0	75.96	19.08	4.96	
	2	63.5926	587.193	3319.148	88.57	0	443.6557	0	0	238.0457	0	5.3184	0	24.89	71.97	3.15	1
E	1																1

Baby	Day	IL-1b	IL-8	MCP-1	IL-6	IL-10	GCSF	GMCSF	MIP1a	MIP1b	TNF	FasL	VEGF	Apopt.	Viable	Necr.	Trypan blue %
EE	3													3.09	61.87	35.04	
	4													6.88	76.06	17.05	33
	5													9.61	63.45	26.95	4
	6													9.87	57.39	32.74	29
	7													5.55	74.54	19.91	10
	9													1.02	74.07	24.91	8
	13													6.5	71.03	22.47	12
	16													11.19	75.02	13.79	20
	20													7.06	63.46	29.48	10
	23													9.88	75.47	14.65	14
	27													4.47	48.65	46.88	
F	1	338.579				86.1295			986.205		61.191	50.377	71.3227	4.64	59.84	35.52	
	2	63.4184				6.50981			71.12916		37.482	13.804	8.8505	9.93	55.26	34.81	1
G	1	22.2318	3105.11	9488.682	5475.228	3.11689	474.3641	0	25.90698	541.2549	8.042	15.13	1.15808	5.22	48.19	46.59	10
	2	87.5256	8941.62	7887.289	2081.767	1.41099	938.1691	0	227.9487	1634.322	19.542	15.067	96.9955				
	3													7.78	67.69	24.53	
	4	77.1358	12683.6	54668.43	687.8007	5.43472	349.0121	0	125.2857	603.366	9.0222	30.007	676.237	7.19	80.73	12.09	
	5													18.33	56.54	25.13	
	6													1.01	81.64	17.35	
	7	1615.28	38019.6	81261.92	3108.139	8.93513	4518.812	1.050105	923.9442	4596.657	57.301	41.781	295.252	4.91	83.2	11.89	
	9	8332.43	95571.6	37011.96	13466.67	106.251	10636.46		7769.511	53658.43	3235.6	23.067	237.445	18.9	54.75	26.35	
	13													6.39	82.69	10.92	
	16	5148.59	56646.7	19292.82	7723.785	10.771	2581.205	0.743459	2642.343	6152.206	121.91	29.817	635.785	8.47	71.48	20.05	
	20													8.57	77.95	13.48	
	23	364.984	15251.6	10686.56	701.5599	4.61282	473.7879		330.775	1746.757	43.261	25.884	1207.41	35.01	1.49	63.51	
H	1	28.2494	12107.1	84659.09	1262.209	2.25331	195.2502		111.7811	27.60369	10.39	22.463	15.6269	26.08	46.56	27.36	7
	2	33.8466	8478.34	87071.26	749.5756	1.55839	370.0079		103.0809	26.19826	8.5206	26.739	53.4908	14.84	77.14	8.02	
	3													35	47.75	17.25	
	4	16.8804	930.68	34320.52		0	159.3419		27.25434	18.67242	6.7658	20.976	296.374	24.67	60.06	15.28	
	5													18.44	60.88	20.67	
I	1	254.798	24345.1	18707.41	9499.261	56.067	4412.863		592.5209	8447.685	14.838	18.983	153.498	12.96	56.61	30.43	
	2	501.337	35145.4	36755.46	6112.386	38.8139	4042.045		987.2346	10510.16	25.233	21.292	1119.84	4.06	64.82	31.12	0
J	1	685.144	31984.4	8740.148	6965.2	60.0532	5672.353		740.4218	9866.704	59.784	20.656	335.067	1.86	77.56	20.58	3
	2	110.866	11111.6	20010.33	1710.67	0	601.4726		654.5011	4475.305	8.3672	37.581	893.059	3.81	31.59	64.6	
	3													6.89	52.64	40.47	
K	1	62.4272	5794.9	40311.03	3146.57	0	5449.13		21.62116	342.4276		19.372		6.41	60.67	32.92	
	2	141.302	13529.2	60755.49	2774.52	0	1018.798		138.602	468.6029		9.0961		8.76	54.15	37.1	
L	1	11.7697	821.906	9565.874	2290.855	1.91635	255.5058		8.026792	402.5254	5.0575	8.0747		20.23	63.41	16.36	40
	2	31.6056	2076.36	5493.408	395.7495	1.28464	227.8807		39.26208	461.9162	8.6346	13.268	47.2863	25.88	55.21	18.91	
	3													10.06	64.34	25.61	
	4	24.4007	1331.34	19096.65		1.81107	134.6008		24.21571	237.5009	6.2873	16.519	106.199	12.38	64.96	22.65	
	5													3.09	45.45	51.47	14
	6													5.84	82.33	11.84	10
	7	41.5838	4329.26	30993.81	67.44605	1.53731	266.1144		104.8643	466.4498	6.424	17.561	288.848	2.11	85.99	11.91	10
	9	337.905	31398.4	27346.43	3219.753	6.72065	893.0032		352.5429	2004.27	138.44	27.913	579.846	2.22	70.69	27.1	
	13													13.81	41.76	44.43	6
	16	33336	192968	37778.13	28622.4	116.938	7244.606		5000.025	21441.41	1157.3	32.421	669.309	8.74	56.72	34.54	25
	20	16318.5	193191	38796.64	16959.23	172.928	3031.001		13361.6	14429.79	1956.1	29.055	1019.44	3.92	69.7	26.38	21
	23													2.73	46.9	50.37	6
	27	160.507	28144.5		2125.917	10.1589	3685.525		475.3381	6045.653	26.125	27.057	777.191	4.82	42.07	53.11	12

Baby	Day	IL-1b	IL-8	MCP-1	IL-6	IL-10	GCSF	GMCSF	MIP1a	MIP1b	TNF	FasL	VEGF	Apopt.	Viable	Necr.	Trypan blue %
M	1	273.926	16401.8	5223.152	415.567	130.993	1835.002		346.6835	6727.049	35.482	28.439		29.97	29.17	40.86	15
	2	124.594	6919.68	9232.669	5167.14		1981.244		143.7725	1349.538		17.483		3.63	76.75	19.62	
	3	207.614	10714.1	26778.24	4303.66		793.8428		305.0347	4110.534		28.439		12.07	44.13	43.8	
N	1	392.057	37093.7	7496.631	17969.13	118.781	8208.728		240.4603	2286.719	43.606	34.17	52.7077	5.85	52.55	41.6	
	2	26.852	2131.1	2618.888	393.87	2.4218	311.7069		39.35374	408.8727	12.602	16.393	19.6392	14.48	75.45	10.06	
	3													6.84	73.06	20.1	
	4		193191	43676.73	12198.34	60.4695	6088.746		11967.36	20293.35	2897.2	28.77	193.845	12.94	65.7	21.37	24
	5													12.63	69.79	17.58	22
	6													8.68	75.3	16.02	26
	7		189526	63984.86	14608.9	138.04	6600.545		4360.481	20922.27	1204.6	32.358	137.958	14.89	64.5	20.61	10
	13													14.48	63.69	21.83	23
O	1	58.4122	5281.37	6167.101	1238.81		241.5797		43.01135	1060.48		9.0961	124.127	16.83	49.63	33.53	
	2	159.175	9765.82	23200.36	1915.4	10.8715	344.5804		155.2199	2120.36		29.723	607.278	21.2	37.8	41	0
	3													16.38	54.74	28.89	24
	5	341.922	4062.26	9068.367	351.31	19.2985	416.292		387.0964	2230.48	22.027	8.265	193.711	28.46	42.72	28.83	20
	6													8.23	62.83	28.94	0
	7	4442.42	185004	69454.22	30890.39	264.828	17911.85		7053.178	92613.4	2169.2	92.435	1224.8	13.21	58.2	28.59	10
	9	856.757	56335.5	34978.82	8885.19	21.8695	2338.501		1244.092	17512.58	73.7	73.546	1597.68	5.07	59.49	35.45	24
	13													8.5	47.26	44.25	15
	16	457.193	41024.9	60069.54	6724.83	12.4902	4068.202	2391.824	2391.824	18908.7	23.767	105.92	2144.54	3.22	44.97	51.81	4
	20													16.21	42.11	41.68	10
	23													3.78	89.06	7.16	10
	27													31.61	43.45	24.94	8
	31													20.57	50	29.43	10
P	1	41.8342	3429.2	7987.335	1561.2		1797.43		3.281953	352.751		9.0961	12.0928	5.86	87.67	6.47	0
	2	665.263	97812.1	33085.38	7657.78		3340.301		892.6255	8206.822	102.96	16.727	423.907	7.05	73.41	19.54	15
	3													15.7	56.37	27.93	23
	5													13.42	61.99	24.6	40
Q	1	435.304	23076.5	1314.637	15873.79	137.325	9372.803		328.9038	2743.22	279.62	63.572	114.061	7.09	62.96	29.95	5
	2	68.7086	9484.19	1879.586	599.88	6.25326	523.3198		131.3102	1722.33	37.938	64.706	161.763				33
	3													2.78	50.85	46.37	25
	4	240.77	12845.2	12335.42	404.22		616.0955		868.9397	3223.84	22.385	86.466	236.161	1.68	67.33	30.99	33
	5													2.39	75.76	21.85	30
	6													13.09	39.12	47.79	30
	7	506.021	12276.6	14324.4	1000.8		1673.41		488.3682	2269.48	87.871	9.0961	12.0928	6.64	71.83	21.53	10
	9	1544.71	185004	86587.49	19336.03	244.307	4591.594		2158.619	36653.75	206.97	47.101	577.519	5.8	40.61	53.6	10
	13													10.06	38.36	51.58	5
	16													13.28	72.49	14.23	9
	20													20.55	40.45	39	10
	23													17.05	47.85	35.11	12
	27													8.78	54.59	36.64	0

Baby	Day	IL-1b	IL-8	MCP-1	IL-6	IL-10	GCSF	GMCSF	MIP1a	MIP1b	TNF	FasL	VEGF	Apopt.	Viable	Necr.	Trypan blue %
R	1	72.4645	18067.1	14745.81	48815.75		10585.01		116.2804	1651.79	8.3672	16.69	61.9828	12.14	79.35	8.51	1
	2	39.6001	3521.59	6076.826	3155.34		731.1819			369.3827		9.8517	119.75	16.19	79.72	4.09	13
	3													5.88	88.44	5.69	2
	4	43.5503	5004.91	4778.048	983.77		347.1425		54.40025	640.0856		9.2473	277.038	9.01	70.31	20.68	7
	5													18.5	62.15	19.36	4
	6													10.91	70.56	18.53	5
	7	150.627	8931.41	11781.22	1742.27		498.9029		36.46294	425.5881		10.003	371.391	13.14	74.35	12.52	6
	9													8.57	74.75	16.68	14
	13	128.836	25723.7	18066.92	678.19		865.8677		276.3569	1591.569		7.4717	389.116	8.57	82.25	9.18	20
	16	937.448	54547.8		4411.24	30.8679	2349.823		476.5146	5796.178	301.52	6.6028	354.98	18.11	49.25	32.64	5
	20	122.587	12728.7	8938.113	234.75		854.4819		425.7794	3050.638	18.369	10.041	590.648	5.25	66.52	28.23	31
S	1	1142.61	66768.2	2448.245	79746.64	1142.94	8059.041		917.1643	15350.11	129.06	52.919	105.308	9.75	37.85	52.4	9
	2	207.614	20091.7	3194.109	13818.05	106.616	432.5354		456.1475	5055.731	35.482	28.439	12.0928	8.36	59.86	31.77	30
	3													20.33	51.05	28.63	22
T	1	47.2738	1839.36	2533.188	3033.93		471.899			251.81		11.212	58.9195	13.74	76.33	9.93	7
	2	41.8342	5108.11	3960.932	1422.33		237.3306	26.36942	26.36942	476.63		9.0961	12.0928	19.38	71.1	9.52	0
	3													33.84	52.29	13.87	24
	4	110.801	17502.7	34483.25	2672.97		1155.091	504.6747	504.6747	1533.07		18.918	243.601	10.46	64.95	24.6	20
	5													16.55	47.31	36.14	20
	6													9.52	69.06	21.42	27
	7	688.382	42143.7	25784.96	3440		2625.433	1126.475	1126.475	3649.41	48.58	9.0961	1468.57	15.99	50.5	33.51	31
U	1	39.1144	2868.02	8881.734	3790.36		155.8703			302.2801		13.252	261.982	20.78	75.41	3.81	38
	2	307.083	38019.2	28663.16	12819.41		6028.411		691.1722	3197.463	22.385	9.0961	1356.53	15.19	80.82	3.99	90
	3													4.68	72.85	22.46	2
	4	123.105	15569.2	75536.47	1891.91		432.5354		226.3265	2430.072	11.335	25.039	506.183	4.48	64.57	30.95	10
V	1	36.8479	852.298	1965.794	681.51		150.7863			264.4277		8.4162	49.7291	15.25	80.94	3.82	10
	2	46.5616	2785.93	6788.126	396.43		263.6821		5.384719	347.0156		8.1895	128.065	25.78	54.79	19.44	5
	3													4.06	40.74	55.2	2
	4	4338.46	96904	86587.49	29753.72	20.6792	16959.57	8.323102	3865.137	11852.41	1596.3	23.527	849.296	19.89	54.37	25.74	20
	5													9.15	72.47	18.37	10
W	3													13.5	63.38	23.12	8
	4	1478.2	76891.9	69786.89	11096.57	23.0121	5195.561		2556.513	29768.62	48.784	36.523	440.538	7.15	71.52	21.33	2
	5													11.12	67.05	21.84	15
	6													24.99	41.87	33.15	10
	7	180.934	21607	79751.89	6731.88	51.1499	10557.16		1053.521	15901.91	54.003	26.776	1678.21	16.64	40	43.37	20
	9	169.342	29281.5	32059.77	7432.92		5595.771		1081.252	7579.35	291.49	14.687	669.423	21.13	51.4	27.48	15
	13													12.92	46.29	40.79	18
	16													12.78	44.76	42.46	
	20													17.52	47.34	35.14	
	23													19.67	62.94	17.39	44
	27													33.61	41.16	25.22	25

Baby	Day	IL-1b	IL-8	MCP-1	IL-6	IL-10	GCSF	GMCSF	MIP1a	MIP1b	TNF	FasL	VEGF	Apopt.	Viable	Necr.	Trypan blue %
X	1	19.6008	691.022	13079.19		1.53733	120.1563			158.1623	8.703	24.965	385.614	16.97	32.74	50.3	8
	2	22.5722	2117.94	20166.29			138.4903		8.36243	154.9888	6.9025	24.996	737.867	46.11	25.77	28.11	13
	3													50.91	23.62	25.47	10
	4	15.0277	261.314	7403.957			116.2691		1.56379	145.9215	5.9911	20.438	401.871				6
	5													39.91	45.28	14.81	
Y	1	41.3809	1718.32	5794.052	684.63		385.538			290.81		10.985	149.509	19	77.08	3.93	2
	2	54.1381	12086.4	9171.079	2176.7		853.6112		36.88588	398.63		14.073	360.45	9.6	80.51	9.89	1
	3													9.93	68.82	21.26	5
	4	75.3137	18581.6	86587.49	1018.47		767.0267		145.5488	782.89	12.614	36.448	930.697	21.81	52.84	25.35	45
	5													12.69	63.53	23.78	25
	6													13.74	70.56	15.7	50
	7	655.226	16574.1	52571.67	2010.63		1330.713	14.42969	245.7596	638.37	74.774	9.0961	1020.42	4.1	94.43	1.46	32
	9																20
	13	3934.43	185004	83880.55	61662.2	32.6295	23925	17.82991	2281.298	15948.95	356.98	30.932	1644.95	6.22	85.36	8.42	
	20													71.3	11.41	17.28	33
	23	1067.36	53043.9	39603.76	10640.51	150.656	3172.669		1803.527	17549.26	428.04	53.523	1347.78	50.65	15.56	33.78	
	27													70.42	11.75	17.82	
Z	1	14.3493	1099.07	3716.362	516.1106	1.32675	264.7174			210.2991	6.5379	12.259	275.243	7.01	82.17	10.82	1
	2	17.9001	1186.54	2832.449	48.79873		248.2527			164.5094	7.062	12.795	163.564	14.68	65.2	20.12	5
	3													12.48	69.89	17.62	25
	4	18.4565	2753.83	20627.46		2.48502	150.2061			179.9238	8.2927	22.304	592.7	25.9	44.81	29.29	13
	5													23.07	52.47	24.46	10
	6													10.65	54.59	34.77	32
	7	10000	77694.1	44356.23	14747.83	65.3212	5702.108	0.6681		1958.48	242.79	25.003	1253.67	28.6	32.87	38.54	50
	9													19.99	72.86	7.15	2
	13	3359.88	53356	42489.39	2588.161	12.9243	2194.781	0.606476		2060.488	59.33	28.516	1772.1	25.41	57.67	16.92	4
	16													19.14	56.87	24	10