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The Role Of Inflammation In Preterm Labour And Perinatal Outcome

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ABSTRACT

There is evidence pointing towards materno-fetal inflammatory pathways with release of pro-inflammatory cytokines as being key to the initiation of preterm labour. However the direct relationship between inflammation and neonatal outcome remains unclear, as histological chorioamnionitis is relatively common in pregnancies that deliver prematurely but significant white matter injury is only seen in a minority of these infants. Expression of the major histocompatibility complex type II (MHC Class II) on the surface of circulating monocytes has been used as a measure of cytokine balance, as it is partly regulated by cytokines and levels of surface expression are decreased by the anti-inflammatory cytokine, interleukin 10 (IL-10). The work presented here examines the hypothesis that monocyte MHC Class II expression is lower in preterm labour, reflecting an anti-inflammatory response, and that the severity of inflammatory challenge may correlate with perinatal morbidity and mortality.

Blood was taken from women in labour at term (n=29), undergoing elective caesarean section (n=23), in preterm labour (PTL) (n=14) or with preterm prelabour rupture of membranes (PPROM) (n=24) at <32 weeks, and a pregnant comparator group of equivalent gestational age (n=57). Blood was also taken from the umbilical cord of infants born following term labour (n=26), term elective caesarean section (n=24), feticides (n=7), PTL (n=16), PPROM (n=9) and caesarean section at <32 weeks for IUGR (n=13). Monocyte MHC Class II expression and plasma cytokine levels of TNFα, IL-1β, IL-6, IL-8, IL-10 and IL-12 were measured using flow cytometry. Plasma levels of $TGF\beta_1$ were assayed using an ELISA. Cytokine production by whole blood in response to *E. Coli* lipopolysaccharide (LPS) was quantified at 24 hours.

In mothers MHC Class II expression was lower in term labour, (94.94%), compared to elective delivery, (98.67%) $p<0.05$. An even greater drop was seen in PTL (84.06%) and PPROM (91.42%), especially in the presence of clinical chorioamnionitis, compared to pregnant controls 99.69%, $p<0.001$. TNFα production following LPS stimulation was decreased in term labour and PTL, $p<0.05$. In cord blood, MHC Class II expression was not affected by term labour, but was lower in neonates born after PTL or PPROM than term infants, $p<0.001$. It was negatively associated with death in

premature neonates, median 44.74% vs 72.0% in survivors, $p<0.05$, and was reduced in term infants with sepsis, $p<0.0001$.

These data suggest that labour represents a period of systemic immune paresis, possibly in response to a preceding immune challenge. Low levels of monocyte MHC Class II expression, perhaps as a result of exposure to an inflammatory stimulus *in-utero*, may partly explain the increased susceptibility to infection observed in premature infants.

ACKNOWLEDGEMENTS

Working for this thesis has been a great opportunity to learn a little about the clinical research, the scientific process and the laboratory work involved. It has been stimulating, challenging and enjoyable experience.

I have been privileged to work with some fantastic people and patients, who have enabled me to carry out this work, particularly my supervisors Donald Peebles and Nigel Klein. I would like to thank Meredith Allen for teaching a complete novice everything in the lab, and all the inspiring conversations and coffee along the way.

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July 2006

DECLARATION

The work presented in this thesis is solely that of the author, who collected all blood samples, performed all laboratory work and statistical analyses.

ABBREVIATIONS

CD14	cluster designation 14
CI	confidence interval
CIITA	MHC Class II transactivator
CLD	chronic lung disease
COX	cyclo-oxygenase enzyme
CRH	corticotrophin releasing hormone
DCDA	dichorionic diamnionitic twins
EDD	expected date of delivery
ELISA	enzyme linked immune sorbant assay
FIRS	Fetal Inflammatory Response syndrome
HPA axis	hypothalamic pituitary adrenal axis
IL	interleukin
IUGR	intrauterine growth restriction
kDa	Kilo Daltons
LAP	latency associated peptide
LMP	last menstrual period
LPS	lipopolysaccharide
M	molar
MCDA	monochorionic diamniotic twins,
mfi	median fluorescence intensity
MHC	major histocompatibility antigen
NF-κβ	nuclear factor kappa B
OR	odds ratio
p	probability

PG	prostaglandin
PGHS	prostaglandin synthetase
PPAR	peroxisome proliferator-activated receptors
PPROM	preterm pre-labour rupture of the membranes
PTL	preterm labour
ROM	rupture of membranes
RPMI	Roswell Park Memorial Institute
SOCS	suppressors of cytokine signalling
SVD	spontaneous vaginal delivery
$TGF\beta_1$	transforming growth factor beta 1
T_h	T helper cells
TLR-4	toll-like receptor-4
$TNF\alpha$	tumour necrosis factor alpha
TNFR	tumour necrosis factor receptor

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CHAPTER 1 - INTRODUCTION

1.1 PRETERM LABOUR

Over the last few decades there have been improvements in the survival rates of extremely preterm infants, as illustrated by Figure 1. However these infants are still at risk of significant morbidity such as neurodevelopmental, sensironeural and respiratory problems. Infection, and the inflammatory processes it precipitates, have been shown to be important in the aetiology of some cases of spontaneous preterm delivery and the associated morbidity (1;2).

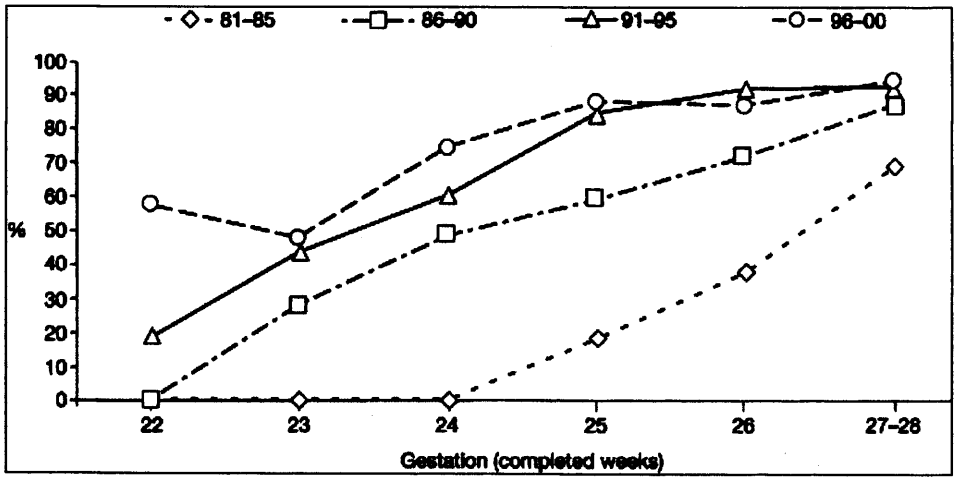

Figure 1: Percentage survival of preterm infants of 22 to 28 weeks gestation admitted to the University of Utah NICU from 1981 to 2000.
Improvements in survival rates were seen over the course of 20 years (3).

One of the major dilemmas facing obstetricians and neonatologists is when is the appropriate time to deliver the fetus of a mother with preterm pre-labour rupture of membranes. Current clinical practice involves monitoring for signs of chorioamnionitis and delivery if these are present, in an attempt to reduce the risk of

inflammation-associated organ damage. However this risk may be small, inflammation may have benefits in terms of survival and early delivery has not been shown to improve outcome. This thesis aims to investigate the role that infection, and the response of the maternal and fetal immune system, may play in preterm labour and prolonged pre-labour rupture of membranes.

1.1.1 Definition & Incidence Of Preterm Labour

Preterm delivery is defined as delivery prior to 37 weeks gestation and complicates about 11% of all pregnancies, see Figure 2 (3-5). Delivery at less than 32 weeks accounts for about 2-3% of all pregnancies, but 60% of the perinatal mortality and morbidity (3;6).

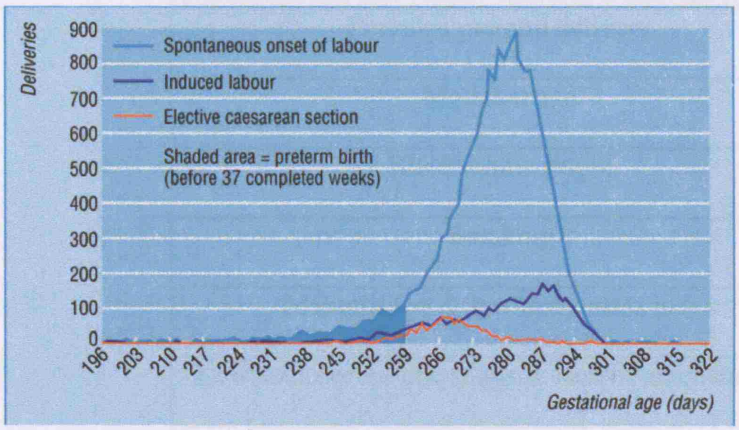

Figure 2: Distribution of deliveries by gestational age (from early ultrasound scanning) in 24675 pregnancies in Nottingham, between 1988 and 1995 (5).

1.1.2 Epidemiology Of Preterm Labour

Preterm delivery can arise from many different aetiologies. Spontaneous preterm labour and prolonged pre-labour rupture of membranes (PPROM) leading to delivery account for about 50% and 25% respectively of all preterm deliveries. Indicated or iatrogenic deliveries, due to problems such as pre-eclampsia and severe intrauterine growth restriction, account for the remaining 25% of all preterm deliveries (7).

1.1.2.1 Established Risk Factors For Preterm Labour

Well-recognised risk factors for preterm labour are listed in Table 1.

Risk Factors For Preterm Labour
Previous preterm delivery
Maternal age
Black ethnic origin
Smoking
Malnutrition & low body mass index
Low socio-economic status & lack of antenatal care
Cervical incompetence
Preterm pre-labour rupture of membranes
Bleeding in pregnancy
Multiple pregnancy

Table 1: Risk Factors for Preterm Labour (6)

Lack of antenatal care has also been shown to be a risk factor although fewer clinic visits may also be a marker of socially excluded groups, or due to preterm delivery before many clinic visits would have occurred. Malnutrition is associated with higher rates of preterm deliveries compared to women with a body mass index within the normal range (8).

Being of black ethnic origin is a risk factor for preterm delivery, after adjustment for socio-economic factors. In USA, the rates of preterm delivery in black women is double that of white women (16% versus 8.4% in 1995) (9). The reasons for this variation in risk with ethnicity are unclear but genetic factors may play a role (10).

Several studies have found a preponderance of male gender amongst infants born prematurely compared to those born at term (11). There are several theories to explain this imbalance, such as an association between fetal sex and the mechanism of labour (12) or due to variation in the timing of fertilisation of the oocyte(13) .

Maternal age is also an important risk factor, with the lowest rates of preterm deliveries seen in women in their twenties (9). First delivery in teenage mothers is not associated with increased risk of spontaneous preterm delivery but second delivery is (14). Older mothers are at increased risk of indicated preterm delivery, probably due to an increase in hypertensive disorders of pregnancy with age (15;16). There is also a significant increase in spontaneous preterm birth below 32 weeks gestation with increasing maternal age (15).

Maternal smoking has been found to be a risk factor for preterm delivery by many studies, with one study showing a relative risk of delivery before 33 weeks of 4.4 (95% CI 2.2-9.1) with maternal cigarette smoking (15;17;18).

Multiple pregnancy is associated with an increased risk of preterm delivery (19). The rates of multiple pregnancy are rising due to increasing numbers of older mothers and assisted conception techniques (20;21).

A history of previous preterm delivery increases the risk of preterm labour in subsequent pregnancies, as does a history of cervical incompetence (5).

1.1.2.2 Epidemiology of Inflammation, Infection & Preterm Labour

Inflammation is a significant component in many cases of preterm labour. Clinical and histological chorioamnionitis increase the risk of preterm delivery (22). Clinical chorioamnionitis has been defined as the presence of two or more of the following; maternal pyrexia (at least 38.2 ^{0}C), fetal tachycardia (>160 bpm), maternal tachycardia (>100bpm), and/or uterine tenderness (23). Histological findings include neutrophil infiltration of the placental tissue and amniotic fluid. About 12% of women in preterm labour have evidence of infection (positive amniotic fluid cultures) (24). The incidence of intrauterine infection seems to be inversely related to gestational age as higher rates of positive amniotic fluid cultures and histological chorioamnionitis have been found with decreasing gestational age at birth (22;25) .

There is also evidence that chronic infection and /or inflammation can be implicated in the pathogenesis of preterm labour. Evidence of infection and inflammation in amniotic fluid samples obtained in the second trimester for genetic analysis has been associated with preterm labour. Several groups have found the presence of organisms such as *Ureaplasma urealyticum, Gardnerella vaginalis, Fusobacterium* sp., and *Clostridium* sp. or inflammatory mediators such as interleukin- 6 (IL-6) in these amniotic fluid samples to be a risk factor for preterm delivery (26-30).

Abnormal colonisation of the lower genitourinary tract is also a risk factor for preterm delivery. The presence of asymptomatic bacteriuria gives an increased risk of preterm delivery, which can be reduced by antibiotic treatment, as shown by two meta-analyses (31;32). Bacterial vaginosis (BV) has also been implicated in preterm delivery and spontaneous abortion (33). A meta-analysis of 18 studies found a two fold increase in preterm deliveries in mothers with BV (34). However antibiotic therapy did not lead to a diminution of this risk (35). Other genital infections during pregnancy, such as *Chlamydia trachomatis* and *Trichomonas vaginalis*, have also been associated with preterm delivery (36-38).

1.2 PROLONGED PRE-LABOUR RUPTURE OF MEMBRANES (PPROM)

PPROM has similar aetiology to preterm labour and is an important cause of perinatal morbidity and mortality. It is associated with a short latency period to delivery, perinatal infection, bleeding, neonatal respiratory distress syndrome, umbilical cord

compression, limb positional abnormalities and pulmonary hypoplasia due to oligohydramnios (39).

1.2.1 Incidence of PPROM

Prolonged pre-labour rupture of membranes (PPROM) occurs in about 3% of pregnancies (39) and is defined as rupture of the membranes before 37 weeks gestation, prior to labour. 50% to 60% of women with PPROM will deliver within one week of rupture of membranes (39). This latency period tends to be shorter the nearer to term that the rupture of membranes occurs.

1.2.2 Epidemiology of PPROM

The epidemiology of PPROM is similar to that of spontaneous preterm labour. Intrauterine infection and inflammation may lead to PPROM and/or PTL (40). Cigarette smoking, low body mass index, history of a previous preterm delivery and being of black ethnic origin are all associated with an increased risk of PPROM, as is a short cervix and detectable cervico-vaginal fetal fibronectin (39;41;42). Cervical surgery such as cone biopsy, vaginal bleeding and uterine distension due to multiple pregnancy or polyhydramnios are also risk factors for PPROM (39).

About one third of women with PPROM have positive amniotic fluid cultures (24). There is epidemiological evidence that intrauterine infection can precede PPROM as well as arising as a result of ascending infection after rupture of the membranes has occurred. There is an association between PPROM and colonisation of the genital

tract with organisms such as Group B *streptococcus*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and the organisms that cause bacterial vaginosis (43;44).

1.3 THE PROBLEMS OF PREMATURITY

1.3.1 Survival of Preterm Infants

The aim of understanding, and subsequently preventing, preterm labour is to avoid the associated perinatal morbidity and mortality. In 2001, in England and Wales, prematurity or extremely low birth weight was the main cause of perinatal mortality, with a neonatal mortality rate of 1.48 per 1000 live births (total neonatal mortality rate 3.6 per 1000 live births) (45). As would be expected, the highest rates of infant mortality are seen in the most preterm infants, see Figure 3.

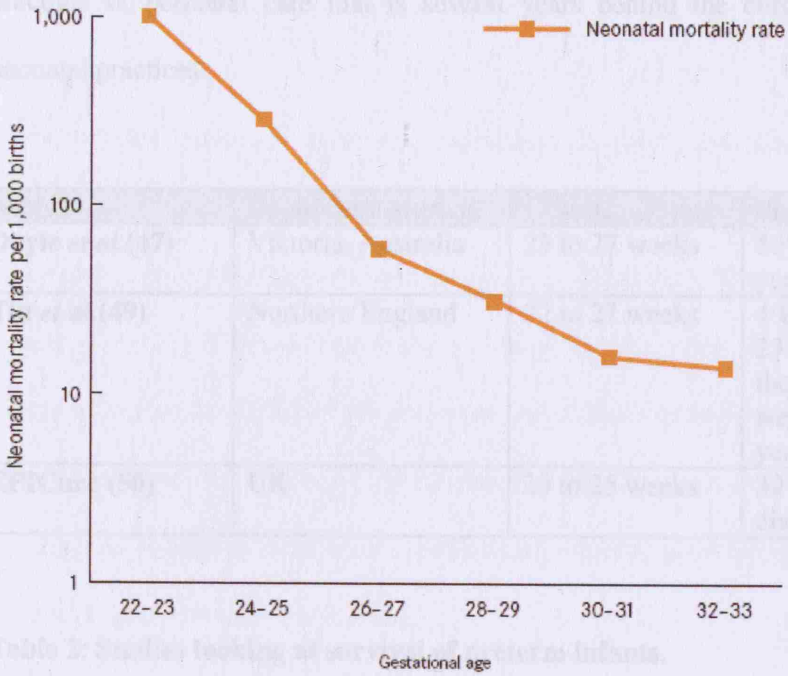

Figure 3: Neonatal mortality rate per 1000 births against gestational age in weeks for Victoria, Australia in 2004 (47).

Survival of infants born at early gestations has increased over the last 15 to 20 years due to improvements in perinatal care such as antenatal steroids, improved ventilatory support and use of artificial surfactant, as shown in Figure 1 (46;47).

Several studies have looked at long-term survival of these preterm infants, see Table 2. These studies provide information for parents and medical staff about outcome of these infants, which aid decisions about management and prognosis. Hack and Fanaroff reviewed the world literature and found that at 23 weeks, survival ranges from 2% to 35% (48). At 24 weeks there is a marked increase in survival with 17% to 62% surviving and a further improvement at 25 weeks to 35% to 72%. Obviously all studies of survival and long-term outcome of preterm infants will be describing the

outcome of perinatal care that is several years behind the current obstetric and neonatal practices.

Author	Population studied	Gestational age	Survival
Doyle *et al.*(47)	Victoria, Australia	23 to 27 weeks	56% alive at 5 years of age
Tin *et al.*(49)	Northern England	22 to 27 weeks	4% of those born 23 weeks & 80% of those born at 27 weeks survived to 1 year of age
EPICure (50)	UK	20 to 25 weeks	39% survived to discharge

Table 2: Studies looking at survival of preterm infants.

Differences between studies in this area in terms of survival rates and rates of disability amongst survivors are due to many factors, including study design. Inclusion or exclusion of stillbirths and infants with lethal anomalies will also affect results. Definitions of viability vary and units differ in their policies of offering intensive care to preterm infants at the limits of viability. Studies that only include infants admitted to the neonatal unit will tend to have better survival rates. It has been suggested that including all fetuses alive at the start of labour will give more informative and statistically consistent results (49).

1.3.2 Morbidity Associated With Prematurity

Despite the improvements in the survival rates of preterm infants, there have not been similar reductions in perinatal morbidity. Morbidity associated with prematurity can be divided into early and late problems. There is epidemiological evidence that inflammation and infection may be linked to preterm labour and PPROM, as discussed in sections 1.2.2. There is also evidence that infection may play a role in the perinatal morbidity associated with preterm labour.

1.3.2.1 Early Morbidity

This includes problems such as respiratory distress syndrome, intraventricular haemorrhage, sepsis and necrotising enterocolitis. Respiratory distress has been reported in over 50% of infants born at less than 1500g (51). Necrotising enterocolitis affects about 11% of infants born extremely prematurely (less than 26 weeks gestation) and about 30% to 50% will have one or more episodes of septicaemia (48). These early morbidities may be life threatening. Survivors can also develop long-term problems as a result of these initial insults, such as cerebral atrophy, secondary to post haemorrhagic ventricular dilatation.

1.3.2.2 Late morbidity

Important long-term morbidity in survivors of preterm delivery includes chronic lung disease and neurodevelopmental disability such as cerebral palsy, cognitive and developmental problems.

1.3.2.3 Chronic Lung Disease

Chronic lung disease (CLD) can be defined as oxygen requirement after 36 weeks corrected gestation. The EPICure study group found 77% of survivors developed CLD, compared to an incidence of approximately 20% in several American studies of very low birthweight infants (<1500g) (51;52). The pathology seen in CLD in the preterm infant includes alveolar hypoplasia, arrest of acinar development and interstitial fluid accumulation. This appears to be due to abnormal growth and repair of immature lung which is exposed to oxygen and chronic inflammation (53).

1.3.2.4 Inflammation, Infection & Chronic Lung Disease

There is some evidence suggesting that antenatal inflammation such as chorioamnionitis may diminish the risk of respiratory distress syndrome as surfactant deficiency is reduced, possibly due to a cortisol surge (54). However other work has shown that chorioamnionitis and postnatal infection increase the risk of long term problems such as CLD (55;56). Increased levels of pro-inflammatory cytokines such as IL-6 and IL-8 have been found in bronchoalveolar lavage fluid and cord blood of infants who develop CLD compared to those who do not (57-59). No increases in cord blood levels of the anti-inflammatory cytokine, IL-10, were seen in infants with CLD. This suggests that an imbalance in homeostasis between pro- and anti-inflammatory arms of the immune response may lead to CLD.

Mechanical ventilation has also been found to be an important risk factor for CLD (55). This may contribute to the inflammatory stimulus that damages the lung, or may be a marker of how unwell the preterm infant is.

1.3.2.5 Perinatal Brain Injury

Perinatal brain injury is an important cause of morbidity in the preterm infant as the white matter of these infants appears to be particularly susceptible to injury due to inflammation (60). Periventricular leucomalacia (PVL) is the term commonly used to describe this white matter injury. Leucomalacia literally means 'softening of the white matter'. PVL refers to necrosis of the white matter adjacent to the angles of the lateral

ventricle (61). Perinatal brain injury can be diagnosed histologically, clinically or by imaging.

1.3.2.5.1 Histology

Histopathological diagnosis of PVL can be made on necropsy. PVL is categorised as cystic or focal, and widespread or diffuse (61). Focal PVL consists of areas of coagulative necrosis in the white matter near to the lateral ventricles. In diffuse PVL, diffuse necrosis is seen in the hemispheric white matter (62). Axonal swelling, activated microglia and astrocytosis can also been seen in the lesions.

Cystic PVL is characterised by coagulation necrosis with axonal swelling, activated microglia and astrocytosis. Subsequently cyst formation may occur within 3 to 4 weeks. Focal PVL is seen more commonly in older infants and diffuse PVL more commonly in extremely preterm infants (63). Diffuse PVL is characterised by the loss of oligodendrocyte (OL) precursors (2).

Histological findings in PVL depend upon the duration between insult and time of death. The neuropathological sequelae of PVL are loss of white matter volume and ventriculomegaly due to the myelin deficiency (2). The distribution of white matter injury is probably more extensive than just 'periventricular' in the preterm infant and can involve the subcortical and callosal regions as well as the internal capsule (64;65).

1.3.2.5.2 Imaging

Ultrasound and magnetic resonance imaging (MRI) have been used to diagnose white matter damage. The quoted incidence of white matter injury in extremely preterm infants ranges from 4 to 13% depends upon the techniques used to diagnose it and the time frame in which it is assessed (2;66;67).

A typical pattern of damage seen in the extremely preterm infant has been decribed. Commonly diffuse white matter damage with later ventriculomegaly and loss of white matter volume is seen (68). Abnormalites of the overlying gray matter abnormalities are also often seen with an increase in the subarachnoid space and immature gyral folding. Fibre development in the white matter is important for gyral development (69).

PVL lesions seen on ultrasound may be hyperechoic, which is thought to be due to vascular congestion or haemorrhage, or alternatively hypoechoic due to removal of necrotic tissue and cyst formation (67).

Prominent hypoechoic periventricular areas on imaging studies predict the later development of motor dysfunction better than any clinical characteristic of the preterm newborn in the neonatal intensive care unit (67). De Vries *et al.* found that serial high resolution ultrasound scans until discharge from the neonatal unit were able to detect abnormalities in 96% of children who developed cerebral palsy (66;70).

There is no consensus on the classification of PVL. De Vries *et al.* developed a grading system for cranial ultrasound examinations in 1992, which has been used by several authors, see Table 3 (71).

Grading	Ultrasound Findings
Grade I	Periventricular echodense area, present for 7 days or more
Grade II	Periventricular echodense areas evolving into localised frontoparietal cysts
Grade III	Periventricular echodense areas evolving into multiple cysts in the parieto-occipital white matter
Grade IV	Echodense areas in the deep white matter, with evolution into multiple subcortical cysts

Table 3: Classification of periventricular and subcortical leucomalacia based on cranial ultrasound findings, from De Vries *et al.* (71)

Using MRI techniques, white matter injury has been seen as diffuse, excessive, hyperintense areas on T2 weighted images. This diffuse high intensity signal is also associated with the development of cerebral atrophy (72). Cysts, ventriculomegaly and myelination defects can also be assessed using MRI. The gray matter can also be examined to look for signal abnormalities, gyral pattern and subarachnoid space (68). MRI studies have been performed at varying time points, such as in the first few days of life, when the infant reaches term gestational age, and at 1 year of life and beyond. Using MRI, abnormalities in the white matter have been seen in 40% to 68% of preterm infants (68;72).

At term gestational age, MRI has been found to be more sensitive in detecting subtle white matter injury (68;72). Therefore MRI may be the gold standard in terms of delininating white matter injury but information from cranial ultrasound imaging may be easier for many units to obtain sequentially.

1.3.2.5.3 Clinical Diagnosis

About 10% of extremely preterm infants will develop severe neurodevelopmental problems such as cerebral palsy, and about 40% will have other cognitive and learning difficulties (2;73).

A recent consensus definition states that cerebral palsy is an "umbrella term covering a group of non-progressive, but often changing, motor impairment syndromes secondary to lesions or anomalies of the brain arising in the early stages of its development" (74). Cerebral palsy is classified according to the extremities involved (monoplegia, hemiplegia, diplegia, and quadriplegia) and the characteristics of the neurological dysfunction (spastic, hypotonic, dystonic, athetotic, or a combination. In preterm infants, spastic diplegia is most common form of cerebral palsy diagnosed (75). The incidence of this form of cerebral palsy has increased in recent years in line with the increased survival rates of preterm infants (76;77)

There is reasonable correlation between white matter abnormalities detected on imaging and subsequent development of neurodevelpmental problems such as cerebral palsy (66;78).

1.3.2.6 Inflammation, Infection & Perinatal Brain Injury

Clinical evidence of maternal sepsis, such as pyrexia and clinical chorioamnionitis, is associated with a increase in the risk of neonatal encephalopathy, white matter injury seen on MRI, cognitive difficulties and cerebral palsy (68;79-87). A recent meta-analysis demonstrated an association between clinical and histological chorioamnionitis and both white matter damage and cerebral palsy in preterm infants (88).

Fetal inflammation is a stronger risk factor, with funisitis (the presence of polymorph leucocytes in the blood vessels walls of the umbilical cord) having a relative risk of 3.0 of white matter damage or cerebral palsy (86;89;90).

Elevated levels of pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6) in post-mortem brain tissue (63;91), amniotic fluid (92), umbilical cord blood (92-94) and neonatal blood (95;96) have been associated with white matter damage diagnosed on histological, ultrasound and magnetic resonance imaging criteria. Raised levels of such cytokines in umbilical cord blood and amniotic fluid have also been associated with an increased risk of cerebral palsy (97).

However some studies have failed to show this association between inflammation and adverse neurodevelopmental outcome (73). Baud *et al.* found levels of IL-1β, TNFα and IL6 in the amniotic fluid of women who delivered prematurely, correlated with

neonatal sepsis, but not with the development of PVL as diagnosed on MRI or cranial ultrasound (98).

Rupture of the membranes (ROM) is also associated with increased risk of cerebral palsy (99;100). This is thought to be due to intrauterine infection as much higher rates of infection are seen in preterm deliveries with ROM than in those without (100).

It is sometimes difficult to elucidate whether infection or inflammation is the causative factor of perinatal brain injury in human studies. Animal models have demonstrated that administration of LPS or microbes leads to white matter injury (101-103). White matter damage may be induced whether the bacteria or endotoxin is given directly into the fetal brain (104), systemically to the fetus (105-107) or to the pregnant mother via several routes including intraperitoneal and cervical administration (102;108). Some of the earliest work was done by Gilles *et al.* who used *E.coli* LPS in neonatal cats and found white matter injury, the severity of which correlated to the dose of LPS given (101). Young *et al.* also demonstrated LPS induced white matter injury in neonatal dogs (106). Peebles *et al.* found that E.coli LPS given to fetal sheep produced white matter lesions (107).

There are several possible mechanisms by which infection may lead to white matter damage, such as neurotoxicity and cardiovascular effects of LPS and inflammatory pathways.

Animal models have also demonstrated the presence of cytokines in the fetal brain, following maternal administration of microbes or LPS. Cai *et al.* found increased levels of TNFα and IL-1β mRNA in fetal rat brains in a dose dependant manner,

following maternal intraperitoneal LPS administration (102). Bell *et al.* gave LPS intracervically to pregnant rats and found increases in fetal brain homogenate levels of TNFα and IL-1β and a decrease in IL-10 (109). These pro-inflammatory cytokines are toxic to neurones and OL precursors, they can induce apoptosis and mitochondrial dysfunction, as well as inhibiting maturation (110-112).

There is some evidence from human studies that infection and hypoxia-ischaemia may act synergistically. Nelson and Grether found that exposure to combined infection and hypoxia-ischaemia increased the risk of developing cerebral palsy compared to either insult alone (odds ratio 78, 95% CI 48 – 406) (113).

The syngergistic relationship between LPS and hypoxia-ischaemia has also been demonstrated in animal models, with administration of LPS prior to hypoxia ischaemia results in increased white matter injury (114;115).

Mallard *et al.* have demonstrated that both LPS and asphyxia cause activation of microglia, loss of OL precursors and damaged astrocytes in the white matter of fetal sheep (116). Both these insults can induce cytokines in the fetal brain (63;91;117), suggesting that these inflammatory mediators may be a common pathway to white matter injury following these insults.

Interestingly, neuroprotection has been described using repeated hypoxic-ischaemic insults if the initial ischaemia occurs 24 to 4 hours prior to subsequent insults (118;119). This may be due to protein synthesis, such as growth factors. It has also been postulated that tolerance to the excitotoxic effects of glutamate may be induced

or there may be release of inhibitory neuromodulators such as adenosine. In contrast to this work, recent animal models have shown a synergistical effect of repeated insults, whether they be LPS administration or hypoxia-ischaemia (120).

There is evidence from human studies and animal models that anti-inflammatory cytokines can be neuroprotective. $TGF\beta_1$ is minimally expressed in healthy brain tissue but has been found to be raised in human stroke (121). IL-10 has been shown to be neuroprotective during cerebral ischaemia (122).

Rodts-Palenik *et al* found that maternal treatment with IL-10 reduced the white matter injury seen in the rat fetus (108). The mechanism for this neuroprotection is not clear but same group have shown IL-10 reduces IL-1 and IL-6 levels in the fetal brain. The cellular changes such as hypomyelination, apoptosis, OL precursor loss and astrocyte and microglial activation seen after *E.coli* administration were reduced with administration of IL-10 (123). IL-10 prevented secondary energy failure in neonatal piglets following LPS and prior to a hypoxic-ischaemic insult (124). Lyng *et al.* were unable to demonstrate any beneficial effects in brain metabolism or microcirculation with administration of IL-10 following exposure to LPS and prior to a hypoxia-ischamic insult and so suggested that IL-10 may act through different mechanisms (125).

These anti-inflammatory cytokines may explain the preconditioning seen in repeated insults, leading to protection from subsequent insults (119;126). Boche *et al.* showed in a mouse model that LPS injected into the hippocampus led to an increase in $TGF\beta_1$ at 24 hours, which reduced the extent of injury due to a subsequent excitotoxic insult

(127). They also found that antibodies to $TGF\beta_1$, given 24 hours after LPS injection, resulted in larger lesions than LPS alone. $TGF\beta_1$ can be neuroprotective via several mechanisms. It reduces the production of TNFα and IL-1β. *In-vitro*, $TGF\beta_1$ can stabilise neuronal calcium homeostasis in ischaemic conditions, and encourage neuronal survival through activation of the gene Bcl2 in the face of glutamate toxicity (128;128).

There is a paucity of data regarding $TGF\beta_1$ and IL-10 levels in neonates with white matter injury. Duggan *et al.* found increased levels of umbilical cord IL-10 in infants who had white matter lesions seen on MRI (93). Ellison *et al.* found an increase in the CSF levels of IL-10 in infants with white matter lesions (129). In animal models neuronal expression of $TGF\beta_1$ has been found following an excitotoxic insult (130). Bell *et al.* found that IL-10 levels fell in fetal brain following LPS administration (109)

The neuroprotection effect of an anti-inflammatory response may explain the findings of a retrospective cohort study, where antenatal betamethasone led to lower rates of PVL in infants delivered between 24 and 31 weeks gestation, 4% versus 8.4% in those who did not receive antenatal corticosteroids (131). Steroids induce a Th2 response, leading to production of IL-10, which could provide a degree of neuroprotection (132).

1.3.2.7 Sensorineural Deafness and Retinopathy of Prematurity

Sensorineural deafness and blindness, due to retinopathy of prematurity, are also major problems in survivors of preterm delivery. High frequency hearing loss occurs in about 4% of extremely preterm infants. The aetiology is unclear but probably multifactorial, including problems such as ventilation, hypoxia and ototoxic drugs (133). Retinopathy of prematurity occurs in about 25% to 50% of infants born at 23 weeks gestation (48). The development of retinopathy in preterm infants following use of supplemental oxygen was described in the 1950s (134). The first phase of retinopathy of prematurity is failure of normal vascularisation of the retina (135). Phase two is involves a reactive neovascularisation in response to local hypoxia. Oxygen therapy is believed to interfere with the normal process of angiogenesis and development of the retina (136).

1.4 CYTOKINES

As discussed previously, there is some epidemiological evidence linking infection and inflammation with preterm labour and perinatal morbidity. Cytokines may play a role in the link between inflammation and the resulting damage in preterm infants. In this thesis, I have analysed the following cytokines, TNFα, IL-1β, IL-6, IL-8, IL-10, IL-12 and $TGF\beta_1$. An overview of these cytokines follows.

Cytokines are proteins of low molecular weight that can be produced by virtually every nucleated cell in the human body (137). They regulate processes such as cell growth, cell activation, inflammation, immunity and tissue repair. Cytokines are

effective at low concentrations as they bind to high-affinity receptors on cell surfaces, which transmit signals to the nucleus.

1.4.1 Tumour Necrosis factor α (TNFα)

TNFα is a pro-inflammatory, cytotoxic cytokine.

1.4.1.1 Synthesis

TNFα can be produced by many cells including monocytes, T & B cells, neutrophils, glia, neurones and smooth muscle cells, but is mainly secreted by activated macrophages (138). Production of TNFα can be stimulated by a variety of chemical, biological and physical factors. Viruses and bacterial products such as endotoxin can induce TNFα production, as can cytokines such as IL-1, IFNγ, GM-CSF and TNFα itself. X-ray radiation, trauma and ischaemia can also induce TNFα production.

The synthesis of TNFα is tightly controlled. In the normal healthy human, it is produced in very small quantities so that there is virtually no TNFα detectable. However it is one of the first cytokines to be produced in response to stimulation such as sepsis or trauma (139).

TNFα exists in two forms; a 26 kDa transmembrane proTNF and a 17 kDa secreted TNF. The trans-membrane TNF is the newly secreted form of TNF and is proteolytically cleaved to release mature TNF (140). It can function as active TNFα through direct cell-to-cell contact. Most biological activity of TNFα is attributed to

the soluble form but the membrane bound form TNFα does have some activity and is more able to activate TNF receptor 2 (141).

1.4.1.2 Regulation

TNFα production is regulated during transcription, translation and at a post-translation stage. The TNFα gene has a promoter TATA sequence. Alongside this are regulatory sequences for NF-κB sites. Nuclear factor -κβ (NF-κβ) is a regulatory protein that promotes the transcription of pro-inflammatory cytokines such as TNFα, IL-1 and IL-6. IL-1, IL-6 and TNFα can activate NF-κβ (142).Corticosteroids and IL-10 can lead to inactivation of NF-κB, leading to inhibition of TNFα transcription. At a post-translational stage, TNFα is secreted as a prohormone and is proteolytically cleaved by TNFα coverting enzyme (TCE). This is a metalloprotease enzyme, which is upregulated by cytokines including TNFα itself and substances such as LPS, plasminogen and plasmin. TCE is downregulated by IL-4 and IL-10.

1.4.1.3 Receptors & Signalling

There are two TNF receptors, TNFR 1 (55-kDa) and TNFR 2 (75-kDa). Both are type I transmembrane glycoproteins and are members of the TNF receptor superfamily. They are high affinity receptors, found on most cell types apart from red blood cells. TNFR 1 is thought to be responsible for most TNFα biological activity. TNFR 2 is thought to potentiate the effects of TNFR1 and also has been reported to promote the proliferation of T cells as well as playing an important role in oligodendrocyte regeneration (138;143;144).

TNFR 1 has heterogeneous intracellular domains, leading to a variety of actions such as inflammation and apoptosis. TNFR 1 signals through protein kinases such as I kappa B (IκB) kinase. IκB is an inhibitory protein that controls nuclear factor kappa B (NF-κB) activity. Binding of TNFα leads to phosphorylation of IκB. This in turn leads to nuclear translocation of freed NF-κB, which is futher regulated by protein kinases such as mitogen activated protein kinases (MAPKs) (145). NF-κB acts to increase pro-inflammatory gene expression. Little is known about the signalling pathways of TNFR 2.

Soluble receptors for TNFα (sTNFR) are produced by proteolysis of extracellular domains of the membrane bound TNFR 1 & 2. TNFα and LPS all increase the cleavage rate of TNFRs, leading to an increase in sTNFR in the circulation. When sTNFR levels are high, they act to inhibit TNFα by competing for ligand with the membrane bound TNFR(146).

1.4.1.4 Actions

- **Systemic**

TNFα produces fever and anorexia due to its effects on the hypothalamus. It also leads to release of acute phase proteins from the liver. TNFα is a procoagulant and increases permeability and MHC class I expression of vascular endothelium. These actions are important for local host defence as increased endothelial adhesiveness to white cells and platelets helps to limit spread of bacteria. However on a systemic level this can lead to disseminated intravascular coagulation and shock.

- **Monocytes and macrophages**

The actions of TNFα vary depending upon the target cell. In monocytes and macrophages, TNFα acts synergistically with IFNγ and inducing production of IL-1, IL-6, IL-8, GM-CSF, IFNγ and TGFβ. It also induces further release of TNFα. TNFα causes degranulation, superoxide release and increased adhesion in granulocytes.

- **T and B cells**

In B cells, TNFα induces superoxide production. In T cells it induces cytotoxic invasiveness.

1.4.2 Interleukin -1β (IL-1β)

IL-1 is an important pro-inflammatory cytokine, produced in two forms, IL-1α and IL-1β. These two cytokines are structurally different but bind to the same receptors and have the same actions. IL-1β is secreted whereas IL-1α is cell associated.

1.4.2.1 Synthesis & Regulation

IL-1β is a tetrahedral globular protein, with a β pleated sheet tertiary structure. It is produced as a precursor that is cleaved by IL-1β converting enzyme to form mature IL-1β. IL-1β is produced by many cells such as epithelial cells, mononuclear phagocytes, dendritic cells, smooth muscle, fibroblasts, T and B cells. LPS, cytokines such as TNFα, T cell/antigen presenting cell interactions and immune complexes induce the production of IL-1β.

1.4.2.2 Receptors & Signalling

There are two receptors for IL-1β, IL-1RI and IL-1RII. IL-1RI leads to signal transduction whereas IL-1RII binds IL-1β but does not result in a signal. IL-1RI is a 80Kda transmembrane glycoprotein, that is a member of the Ig superfamily of receptors. It is found on many cells, including smooth muscle, endothelium, epithelial cells, fibroblasts and T lymphocytes. It is relatively sparce in number on cell surfaces. Signal transduction occurs through a series of protein kinases, leading to NKκB activation, which in turn increases pro-inflammatory gene transcription.

1.4.2.3 Actions

IL-1β induces an acute phase response. It causes pyrexia and hypotension. IL-1β activates the vascular endothelium by inducing adhesion molecules and procoagulants and increases permeability. IL-1β increases prostaglandin production in macrophages. It induces T and B lymphocyte proliferation and differentiation. It is chemotactic for neutrophils and increases production of IL-6 and TNFα.

1.4.3 Interleukin-6 (IL-6)

1.4.3.1 Synthesis & Regulation

Many cells including T and B cells, macrophages, fibroblasts and endothelial cells, produce IL-6. IL-6 is usually produced in combination with TNFα and IL-1, and these cytokines induce the production of acute phase proteins from the liver. IL-6, a 30-kDa glycoprotein with a 4 anti-parallel helical structure, is present at low levels in the

circulation of healthy adults (147). It is induced by TNFα and insults such as endotoxaemia, lung injury, trauma and acute infections (139;148;149). IL-6 is also present in low levels in diseases such as diabetes, obesity and cardiovascular disease (150;151).

1.4.3.2 IL-6 Receptors & Signalling

IL-6 binds to a plasma membrane receptor, IL-6 receptor. The IL-6 receptor is part of the class I cytokine receptor superfamily. It consists of 2 subunits, the α subunit is called IL-6 receptor (gp80) and is of low affinity and does not signal after binding IL-6. The β subunit is a homodimer of gp130 receptor, which does not directly bind IL-6, but associates with the IL-6/ α-chain complex. Activation of gp130 leads to signal transduction via JAK (Janus kinase) tyrosine kinase pathway which leads to activation of the STAT (signal transducers and activation of transcription) pathways (152;153). The IL-6 receptor is expressed on activated B cells, plasma cells, T cells, monocytes, epithelial cells, fibroblasts, hepatocytes and neural cells.

Soluble forms of IL-6Rα and gp130 are found in human serum (153). These may be generated by shedding of membrane bound receptors or through translation of alternatively spliced mRNA (153). Soluble gp130 receptor has been shown to have an inhibitory action on circulating IL-6. Soluble IL-6R has also been shown to potentiate this antagonism of IL-6 actions (153). This may play a role in modulating the response to IL-6.

1.4.3.3 Actions

- **Systemic**

IL-6 is found in the circulation of septic patients but it is unclear whether it is merely an acute phase reaction-inducing cytokine or whether it has any other pro or anti-inflammatory actions (139). Together with TNFα and IL-1, it induces fever and production of acute phase proteins (148).

- **Monocytes / Macrophages**

IL-6 is secreted by monocytes following sepsis and trauma. It has a negative feedback effect inhibiting further pro-inflammatory cytokine secretion, discussed below (149).

- **T and B cells**

IL-6 acts synergistically with IL-1 and TNFα to induce T cell proliferation and differientiation. It also stimulates B cells to differientiate into plasma cells. This pro-inflammatory role may be important in chronic infections in order to lead to the development of specific humoral and cellular immune responses (149).

- **Anti-inflammatory properties of IL-6**

IL-6 has been shown to inhibit the synthesis of TNFα and IL-1 but has little effect on anti-inflammatory cytokines IL-10 or TGFβ production. In a mouse IL-6 knock-out model, local and systemic pro-inflammatory reactions were more marked in the absence of IL-6 (149).

1.4.4 Interleukin-8 (IL-8)

1.4.4.1 Synthesis & Regulation

IL-8, a 77 amino acid glycoprotein dimer, is synthesised as a 99 amino acid precursor by activated monocytes, lymphocytes, fibroblasts, epithelial cells and hepatocytes. It can be induced by IL-1β, TNFα, LPS and viral RNA. LPS is particularly effective in inducing production of IL-8 by monocytes. IFNγ inhibits the production of IL-8.

1.4.4.2 Receptors & Signalling

IL-8 acts through two high affinity G-protein coupled receptors, IL-8 receptor type A and IL-8 receptor type-B. IL-8 receptor type A, otherwise known as CXCR1, is a 44 to 59Kda receptor, present on many cells including neutrophils, T cells, monocytes, platelets and fibroblasts. IL-8 receptor type B or CXCR2 is a 67 to 70Kda receptor, present on neutrophils, monocytes, platelets and astrocytes. Binding of IL-8 to its receptor leads to an increase in intracellular calcium and superanion production.

1.4.4.3 Actions

IL-8 is important for cell-mediated response to infection. It is a chemotactic cytokine that activates neutrophils, leading to degranulation and release of collagenase and elastase containing granules.

1.4.5 Transforming Growth Factorβ_1 (TGFβ_1)

The transforming growth factor β family of cytokines includes TGFβ isoforms 1, 2 & 3, and macrophage inhibitory cytokine-1 (MIC-1). These cytokines are important in immunoregulation and embryonic growth.

1.4.5.1 Synthesis & Regulation

TGFβ_1, a homodimer of two 12.5kDa peptides, is secreted as an inactive precusor and is converted to the active form extracellularly. It circulates both in the active form and as its inactive precusor. The inactive precusor is a 75-kDa complex of active TGFβ_1, which is non-covalently associated with a precursor molecule latency-associated peptide (LAP). TGFβ_1 must be cleaved from LAP to become biologically active. The precise mechanism of cleavage in vivo is not understood but may be mediated by macrophages (154). TNFα and LPS can induce TGFβ_1 production (127).

1.4.5.2 Receptors

TGFβ_1's effects are mediated by type I and II receptors, present in most cells in the body. The type II receptor binds TGFβ_1 molecule, which enables the type I receptor to bind TGFβ_1, resulting in induction of intracellular signalling (155).

1.4.5.3 Actions

$TGF\beta_1$ stimulates cell growth in hepatocytes and haematopoietic cells, but inhibits growth in osteoblasts and granulocytes. $TGF\beta_1$ is immunoregulatory, inhibiting T and B cell proliferation, natural killer cell and T cell cytotoxicity (156). It also inhibits T helper type 1 and 2 ($T_{H1\&2}$) cell cytokine production and decreases IFNγ induced MHC Class II expression (157;158).

$TGF\beta_1$ is minimally expressed in normal healthy brain but has been found to be increased after stroke in humans (121).

1.4.6 Interleukin-10 (IL-10)

IL-10 is an important deactivating, immunosuppressive and anti-inflammatory cytokine. It was first described as a cytokine synthesis inhibitory factor that has an inhibitory effect on Th_1 (T $helper_1$) cells but a stimulatory effect on cytotoxic T cells and B cells. IL-10 is present in the circulating blood of patients with severe infections and high levels predict morbidity in febrile patients (159).

T helper (T_h) cells are CD4+ T cells. CD is the *cluster designation* and refers to groups (clusters) of monoclonal antibodies that bind to a specific cell marker. These cells have T cell receptors (TCRs) on their cell surface that, in co-operation with CD4+, recognise peptides bound by MHC Class II molecules on the surface of antigen presenting cells and B lymphocytes. CD4+ T cells are subdivided based on the cytokines they produce, into T_h1 and T_h2 cells.

T_h1 cells recognise antigens presented by macrophages and act by increasing cell-mediated immunity, through the production of IL-2 and IFNγ. These cytokines enable CD8+ T cells to differientiate into cytotoxic T cells, capable of destroying infected host cells, activating macrophages, activating complement and increasing monocyte production in the bone marrow.

T_h2 cells recognise antigens presented by B lymphocytes. They produce cytokines such as IL-2, 4,5,10 and 13 that promote antibody production. These cytokines enable B cell proliferation and activation. IL-10 is known to selectively suppress T_h1 responses, by inhibiting the production of IL-1, IFNγ, TNFα and IL-12. T_h1 cytokines inhibit T_h2 cell proliferation, whereas T_h2 cytokines block the activation of T_h1 cells.

1.4.6.1 Synthesis & Regulation

IL-10, a 18.5kDa homodimer of two polypeptide chains is produced by macrophages, T and B cells and various other cells such as mast cells and keratinocytes (160). LPS, catecholamines and glucocorticoids are known to increase IL-10 secretion (161).

1.4.6.2 Receptors & Signalling

The IL-10 receptor has 2 subunits which are members of the cytokine receptor family type 2 (162). The ligand-binding unit IL-10R1 (or IL-10Rα) binds IL-10 with high affinity. IL-10R1 is expressed on haemopoietic cells and is down regulated by T cell activation. Constitutive expression of IL-10R1 has been demonstrated in cytotrophoblast cells. IL-10R2 is the subunit of the IL-10R involved in the signalling

pathways and does not directly bind IL-10. IL-10R2 is constitutively expressed in most cells and signals via a JAK kinase pathway. IL-10 also acts to inhibit NFκB, a transcription factor involved in the production and signalling of pro-inflammatory cytokines (163).

1.4.6.3 Actions

- **Monocytes and macrophages**

IL-10 inhibits the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNFα by activated monocytes and macrophages (164). It also has a negative feedback loop, reducing its own production. Following LPS stimulation, monocytes produce TNFα, which in turn stimulates the production of IL-1β and IL-6. Within 8 hours of LPS exposure, IL-10 is produced, down regulating the production of pro-inflammatory cytokines (through NF-κβ) and inhibiting the activation of NK cells and PBMCs (160). IL-10 also enhances the expression of antagonists to these inflammatory cytokines, such as IL-1RA and soluble TNFRs.

IL-10 inhibits the production of prostaglandin E_2 (PGE_2) through downregulation of cyclooxygenase-2 (COX-2) expression. This in turn reduces the expression of the matrix metalloproteases, which are regulated by a PGE_2-cAMP pathway. Matrix metalloproteases (MMPs) are enzymes involved in cervical changes during in labour and membrane rupture. IL-10 also downregulates expression of toll-like receptor-4 (TLR-4) and enhances expression of CD14 and CD64. TLR-4 is the signal transducing receptor for LPS. CD14 is known as LPS binding protein, and promotes

the ability of TLR-4 to bind LPS. CD64 is also known as FcγRI, a high affinity IgG receptor expressed on monocytes that binds immune complexes.

IL-10 has been shown to down regulate MHC class II molecules at monocyte cell surface through post translational effects(165). IL-10 inhibits the transport of MHC class II molecules to the cell surface.

- **Neutrophils**

IL-10 inhibits the LPS induced production of IL-1β, IL-8 and TNFα by neutrophils. IL-10 blocks the gene transcription of these pro-inflammatory cytokines by inhibiting NFκB activation. It also suppresses killing of phagocytosed bacteria and enhances production of IL-RA (163).

- **T and B cells**

IL-10 enhances expression of MHC class II molecules on B cells. It also increases antibody production and B cell survival. IL-10 strongly inhibits CD4+ cytokine production, whilst stimulating CD8+ cells into cytotoxic activity and proliferation.

1.4.7 Interleukin-12 (IL-12)

IL-12 enhances T_h1 immunity and is important for defence against intracellular pathogens.

1.4.7.1 Structure

IL-12 is a heterodimeric protein with a heavy chain (p40) and a covalently bound light chain (p35).

1.4.7.2 Synthesis and Regulation

IL-12 is produced by B cells and dendritic cells, in response to stimulation by T-cells and antigens or bacteria. It is also produced in response to LPS stimulated macrophages, microglia, neutrophils and astrocytes.

1.4.7.3 Receptors & Signalling

The IL-12 receptor is a high affinity receptor with 2 chains, β_1 and β_2.It is a member of the type I cytokine receptor superfamily. The receptor is expressed on activated T cells and NK cells. T_{h1} cells have both β_1 and β_2 chains and lead to signalling via protein kinases, STAT-4 and JAK2. T_{h2} cells only have β_1 chains and do not signal. Il-10 inhibits production of IL-12 by monocytes

1.4.7.4 Actions

IL-12 acts in synergy with IL-2 to induce IFN production by T cells and NK cells. It enhances NK cell activity and costimulates blood lymphocyte proliferation. It also stimulates the proliferation and activation of T_h1 cells.

1.5 PREGNANCY AND THE IMMUNE SYSTEM

Changes in the immune response occur during pregnancy, altering the cytokine response. These variations are described below.

In pregnancy, there is a shift in T_h1 and T_h2 cell ratios towards a T_h2 dominance. Placental products such as progesterone, prostaglandin E2, IL-4 and IL-10 suppress the T_h1 immune response (166), and there is a relative lymphopaenia (167). Progesterone also reduces the oxidative burst and pro-inflammatory cytokine production of monocytes, and alters T cell cytokine production leading to increased IL-10 production (168).

The suppression of T_h1 response does leave the pregnant woman more susceptible to certain types of infection, especially intracellular pathogens such as *Listeria*. This may be partially compensated for by activation of the innate immune system, such as an increase in neutrophils and monocytes from the first trimester onwards (169;170). As well as being increased in number, these cells are activated, as demonstrated by an increase in intracellular reactive oxygen species and increased expression on the cell surface of CD14, CD11b and CD64 (171-173). CD11b is involved in leucocyte adhesion and binds to ICAM-1. CD64 is also known as FcγRI, a high affinity IgG receptor expressed on monocytes, which binds immune complexes.

Failure to initiate this shift towards a T_h 2 cytokine profile has been demonstrated in pregnancies that miscarry (174). IL-10 production by peripheral blood mononuclear cells was lower in women that miscarried, and production of the T_h1 cytokines, IL-2 and IFNγ, was higher than in women with normal ongoing pregnancies.

In-vitro stimulation of peripheral blood mononuclear cells from pregnant women leads to increased production of IL-4 and IL-10 in the third trimester and a decrease of IL-2 and IFNγ, compared to non-pregnant controls (174). IL-12 production by peripheral blood mononuclear cells has been shown to decrease during pregnancy (175). Progesterone favours the development of T_h2 lymphocytes and inhibits the development of T_h1 lymphocytes, and thus may explain why the highest levels of IL-4 and IL-10 are seen in the third trimester, when progesterone levels are highest (166;176). This alteration in the balance between T_h2 and T_h1 immunity could explain the pregnancy induced remission observed in cell-mediated autoimmune diseases such as rheumatoid arthritis (177) and systemic lupus erythematosus (178).

1.6 CYTOKINES DURING PREGNANCY

Many cytokines are produced by the placenta during pregnancy (179). Cytokines are involved in placental growth and development such as trophoblast invasion. IL-1β, IL-6 and TNFα stimulate production of placental hormones such as oestradiol, human placental lactogen (HPL) and human chorionic gonadotrophin (HCG). Trophoblast apoptosis can be induced by TNFα. Interferons also play an important role in protecting the fetus from viral infections.

1.6.1 Pro-Inflammatory Cytokines During Normal Pregnancy

1.6.1.1 Maternal Serum & Plasma

Some studies have found higher levels of pro-inflammatory cytokines such as IL-6, TNFα and IL-1β in maternal serum than in the non-pregnant population (180), and an increase in these cytokines over the course of pregnancy (181;182). Other groups have, in contrast, found no difference between the pregnant and non-pregnant population (183;184).

1.6.1.2 Amniotic Fluid

TNFα, IL-1β, IL-6 and IL-8 have all been demonstrated in low levels in amniotic fluid from the first trimester onwards, and in some studies have been found to increase with gestation (28;180;181;184-186).

1.6.1.3 Placental Tissue

Dudley *et al.* detected IL-8 mRNA from the deciduas, chorions and amnions, and IL-1β in the deciduas of women at term and not in labour. They detected very little TNFα or IL-6 from any of the placental tissue (187)

1.6.1.4 Cervicovaginal Secretions

Cox *et al.* found low levels of IL-6 and IL-1β in the cervicovaginal secretions of women at term but not in labour (188).

1.6.2 Anti-inflammatory Cytokines In Pregnancy

1.6.2.1 TGFβ$_1$

TGFβ$_1$ has been found in higher levels in maternal plasma compared to non-pregnant controls, and to increase with gestation (189;190). It has also been found in amniotic fluid in mid gestation (186;189). TGFβ$_1$ plays an important role in trophoblast growth and invasion, as well as control and development of the immune system (191). It has an inhibitory effect on human placental lactogen (HPL) and human chorionic gonadotrophin (HCG) production (179). TGFβ$_1$ levels have been found to be lower in fetuses with intrauterine growth restriction. It is hypothesised that lower levels of TGFβ$_1$ lead to impaired placentation and thus problems such as growth restriction and pre-eclampsia (191). However some studies have shown no changes in levels of TGFβ$_1$ in women with pre-eclampsia compared to controls (190).

1.6.2.2 IL-10

Low levels of IL-10 have been described in maternal serum and amniotic fluid by several authors (186;189;192). One group found an increase in amniotic fluid levels of IL-10 with increasing gestational age (193).

1.7 INFLAMMATION AND LABOUR

Labour is the physiological process by which the fetus is expelled from the uterus. It is defined as regular uterine contractions, leading to the effacement and dilatation of the cervix. Labour has been postulated to be an inflammatory event, with escape from factors such as progesterone that maintain uterine quiescence during pregnancy.

There are three main processes occurring during labour; cervical remodelling, rupture of the membranes and uterine contractions (168). The factors initiating labour both at term and prematurely are not completely understood. The neuroendocrine systems of both mother and fetus are thought to play a role. Corticotrophin releasing hormone (CRH), a hypothalamic neuropeptide, increases in maternal plasma a few weeks prior to delivery (194). A switch from a progesterone dominant milieu to an oestrogen dominant state occurs. This leads to a sequence of events that promote labour, such as the synthesis of prostaglandins and expression of oxytocin receptors.

The human placenta cannot synthesise oestrogens from progesterone as it lacks the necessary enzyme. Therefore the fetal adrenal gland produces dehydroepiandrosterone sulphate (DHEA-S), which can be used by the placenta to synthesise oestrogens. CRH stimulates DHEA-S secretion, increases prostaglandin release from the placenta and augments the action of oestrogen on the uterus and cervix (194).

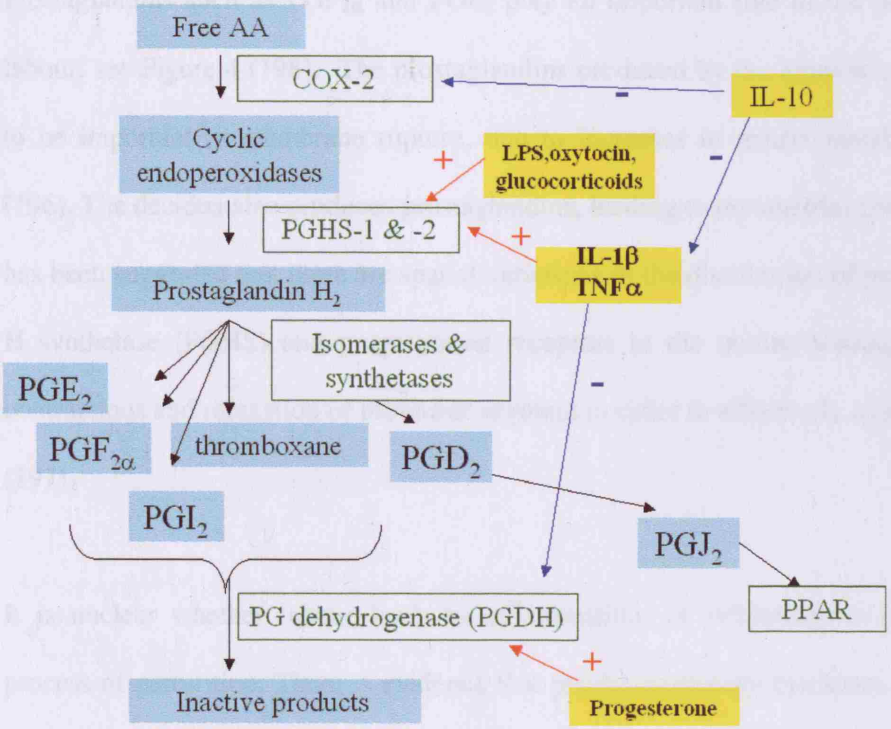

Figure 4: Prostaglandin metabolism.

Progesterone maintains uterine relaxation, by upregulating prostaglandin dehydrogenase (PGDH) in the chorion, via glucocorticoid receptors. Levels of PGDH fall at term in the chorion, especially in the area overlying the internal cervical os, allowing an increase in prostaglandin (PG) concentrations. In rodents, this rise in PG may be due to a progesterone withdrawal preceding labour. In humans there is no corresponding fall in progesterone levels, but a functional progesterone withdrawal appears to occur. This may be due to a reduction in the numbers of progesterone receptors in the decidua and increases in cortisol, which displaces progesterone from its receptors (195-197). Cortisol levels at term are raised due to increases in CRH and conversion of cortisone to cortisol by trophoblasts.

Prostaglandins such as $PGF_{2\alpha}$ and PGE_2 play an important role in the processes of labour, see Figure 4 (198). The prostaglandins produced by the amnion are believed to be important in membrane rupture, due to increases in matrix metalloproteases (196). The decidua also produces prostaglandins, leading to myometrial contraction. It has been suggested that there are spatial variations in the distribution of prostaglandin H synthetase (PGHS) and progesterone receptors in the uterus, leading to fundal contractions and relaxation of the lower segment in order to effectively expel the fetus (197).

It is unclear whether labour leads to inflammation, or inflammation initiates the process of parturition. There is evidence that pro-inflammatory cytokines play a role in the propagation of labour and so their role will be discussed in more detail

1.7.1 Pro-Inflammatory Cytokines And Labour

1.7.1.1 Maternal Blood

Some studies have shown a pro-inflammatory response to term labour, with increases in maternal serum levels of cytokines such as TNFα, IL-1β, IL-6 and IL-8 (181;183;199;200). Other groups have not seen any change in serum levels of pro-inflammatory cytokines in women in established labour, compared to those not in labour at term (181;184).

1.7.1.2 Amniotic Fluid

Amniotic fluids levels of TNFα, IL-1β, IL-8 and IL-6 have been found to be higher in women in labour at term than those not in labour by many groups (181;184;200-204). Even higher levels of pro-inflammatory cytokines have been found if evidence of infection (bacterial endotoxin) was present (205).

1.7.1.3 Cervicovaginal Secretions

Elevated levels of IL-1α, IL-6, IL-8 and TNFα were found in the cervicovaginal secretions during labour compared to women not in labour at term (188;205-207).

1.7.1.4 Placental Tissues

Increased amounts of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IL-8, have been found in amnion and chorion of placentas following term labour (205;206;208;209). Evidence from cDNA array studies shows modest up regulation in IL-1β, IL-6, IL-8 and TNFα expression in amniochorion tissue during labour at term (210). Increased levels of mRNA for IL-1β and IL-6 in amnion, chorion and decidua of placentas from women in term labour have also been described (187). Messenger RNA for TNFα was only found in 20 to 50% of samples from women in labour.

Macrophages infiltrating the decidua and placental tissues during labour have been shown to stain positively for pro-inflammatory cytokines. Therefore these cells may

be the source of the increase in cytokine levels (205). IL-8 can be produced by amnion, chorion and amniotic fluid cells (211). The anti-inflammatory prostaglandin PGD_2 suppresses IL-8 production by the placenta (210). Stimulation of placental tissue with substances such as LPS or IL-1β leads to the production of cytokines such as IL-10, Il-6, IL-8 and IL-1β (210).

1.7.1.5 Cord Blood

Following labour at term, Seghaye *et al.* found higher levels of TNFα in cord blood compared to maternal blood but lower levels of IL-6 and IL-10 (212). Rizos *et al.* found IL-6 and IL-1β to be increased in infants who had a spontaneous delivery compared to those delivered by elective caesarean section (199).

1.7.1.6 Correlation Between Compartments

Studies that have looked at cytokine levels in more than one compartment have failed to show any correlation between levels (181). However blood and amniotic fluid samples were not always taken at the same time point and so duration of labour may influence cytokine levels, which have short half-lives and may be rapidly changing.

1.7.2 Action Of The Pro-Inflammatory Cytokines In Labour

1.7.2.1 Myometrial Contractility

In-vitro studies have also demonstrated that amnion, chorion and decidual cells, when stimulated with pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β, will produce prostaglandins $F_{2\alpha}$ and E_2 (187;213-215). TNFα, IL-6 and IL-1β act by increasing expression of COX-2 and PGHS-2, and decreasing the activity and expression of the enzyme PGDH, see Figure 4 (216-218). As discussed above, $PGF_{2\alpha}$ and PGE_2 are important in stimulation of myometrial activity. TNFα, IL-6 and IL-1β also increase production of endothelin from the amnion, leading to myometrial activation and increased prostaglandin production (219).

1.7.2.2 Membrane Rupture

Membrane rupture is one of the processes occurring in normal labour and has been shown to be due to a localised weakness in the membrane overlying the cervix. Matrix metalloproteases (MMPs) and neutrophil-derived elastases and collagenases digest the extracellular matrix of the membrane, leading to rupture. There is also evidence that apoptosis in the amniochorion plays a part in the weakening and subsequent rupture of the membranes (179). Exposure to vaginal flora at this point, inducing an inflammatory response, is believed to be one of the factors involved, leading to increases in TNFα, IL-6, IL-8 and IL-1β. Meisser *et al.* found that TNFα, IL-6 and IL-1β increased levels of matrix metalloproteases (220). TNFα has been shown to induce apoptosis in placental tissues and so may also lead to membrane

rupture via this mechanism (221). Stretching of the amnion increases IL-1β and IL-8 (210). PGE_2, TNFα and IL-1β can also stimulate release of IL-8 from the membranes (222-224). IL-8 is a chemokine and neutrophil activator, leading to the production of neutrophil-derived elastases and collagenases (225).

1.7.2.3 Cervical Ripening

Cervical ripening occurs through similar processes to those causing rupture of the membranes. Levels of IL-8 are increased in the pregnant cervix compared to the non-pregnant cervix (179). TNFα, IL-6 and IL-1β increase with cervical dilatation, stimulating IL-8 production. The chemokine effects of IL-8 leads to the large neutrophil influx to the cervix during ripening and labour (206). The neutrophils degranulate, releasing collagenases and metalloproteases (225). These substances alter the structure of the cervix. There is an increase in water content and glycosaminoglycans and the previously regular bundles of collagen become disorganised, leading to softening of the cervix and eventually effacement.

1.7.3 Role Of Anti-Inflammatory Cytokines In Labour

1.7.3.1 Interleukin-10

There are conflicting reports in the literature regarding IL-10 levels and normal pregnancy and labour. Dudley *et al.* and Greig *et al.* found no difference in amniotic fluid IL-10 levels between women in labour and those not in labour at term (193;226). Simpson *et al.* showed that exposure to labour reduced IL-10 production by choriodecidual tissue but increased IL-10 responsiveness to stimuli such as LPS and

TNFα (227). However other groups have shown no change in IL-10 expression during labour (228;229). In vitro production of IL-10 by choriodecidual tissue is increased by IL-1β and LPS (230;231).

Endogenous IL-10 appears to ameliorate TNFα expression and PGE_2 production and decreasing expression of COX-2 enzymes following LPS exposure (232). It has been hypothesised that the withdrawal of anti-inflammatory cytokine effects may be important in the mechanism of labour, similar to the proposed withdrawal of progesterone, allowing pro-inflammatory cytokines to increase production of substances such as prostaglandins (210). Cytokines are also regulated by a family of proteins called suppressors of cytokine signalling (SOCS). SOCS act as intracellular inhibitors of cytokine signalling (210). A decrease of SOCS proteins has been found in placental tissue after labour, suggesting that reduced expression of SOCS may be a mechanism via which pro-inflammatory cytokines form a positive feedback loop in labour (210;233).

However recent work by Mitchell *et al.* reveals IL-10 acting as a pro-inflammatory stimulus in fetal membranes (234). They found that IL-10 behaved in anti-inflammatory manner in choriodecidual tissue, inhibiting production of IL-8 and TNFα. However in amnion, IL-10 increased production of TNFα, IL-8 and PGE_2. They postulate that this pro-inflammatory behaviour of IL-10 may be of benefit in situations of overwhelming intrauterine infection, enabling the fetus to escape from a hostile environment, or in the course of normal established labour, to give positive feedback to the pro-inflammatory factors, leading to delivery. There is little data

regarding cord levels of IL-10; one study found low levels of IL-10 in term infants (192).

1.7.3.2 Transforming Growth Factor β_1

Changes in expression of TGFβ_1 receptors in pregnancy and labour have been shown but the role of TGFβ_1 is not clearly understood (235). Increased levels of TGFβ_1 have been found in labouring myometrial tissue, compared to non-pregnant and non-labouring myometrial samples (236). In contrast, TGFβ_1 receptor types I and II were found at higher levels in non-labouring myometrial tissue compared to labouring tissue (236). An in-vitro model showed that oestrogen and progesterone increased the release of TGFβ_1 from myometrial cells but also to decreased the levels of TGFβ_1 receptor expression (237). Thus it has been hypothesised that TGFβ_1 may operate as an autocrine signal in the myometrium. As TGFβ_1 has been shown to be under hormonal control, it may play a role in myometrial cell growth and differientiation in preparation for labour.

TGFβ_1 has been shown to reduce production of MMP-1 and MMP-3 in myometrial cell culture (220;238). Other work has shown that TGFβ_1 inhibits production of TNFα, which increases MMP-1 and MMP-3 (235). It has been suggested that lower TGFβ_1 expression in labour may lead to increased MMP production, leading to the extracellular matrix degration seen in membrane rupture.

On the other hand, there is some evidence that TGFβ_1 may play a pro-inflammatory role in labour as it has been shown to upregulate the contraction associated protein

(CAP) gene, RyR2, which is an intracellular Ca^{2+} release channel protein (239). It also increases COX-2 enzyme levels, increasing production of PGs (237). In fetal membranes $TGF\beta_1$, in conjunction with IL1β, increases levels of COX-2 enzyme, leading to increased PGE_2 production (240).

1.7.4 What Stimulates The Production Of Cytokines In Labour?

It is still unclear what is the initiating signal for human labour, and what triggers the cytokine responses discussed above. It has been postulated that normal labour may involve a low-grade inflammatory response to vaginal flora. The extent of leucocyte infiltration of placental tissue was related to the levels of pro-inflammatory cytokines in amniotic fluid following term labour (241).

1.8 PRETERM LABOUR AND INFLAMMATION

As discussed in section 1.1.2, there is epidemiological evidence that infection such as chorioamnionitis and funisitis play a role in the aetiology of preterm labour, especially below 32 weeks gestation. The presence of microbes in amniotic fluid at the time of genetic amniocentesis predicts those who will go on to deliver prematurely (29). There is also evidence from animal models to support this with administration of microbes or endotoxin to pregnant animals causing preterm delivery (242;243)

Microorganisms may reach the amniotic cavity and fetus via several routes. Possible pathways include haematogenous transplacental spread, during invasive procedures such as amniocentesis, and via ascending pathways, from the vagina and cervix.

The most common route of infection is the ascending pathway. Evidence to support this includes the fact that histological chorioamnionitis is more common at the site of membrane rupture. Bacteria identified in congenital infections of the neonate are often similar to those in the lower genital tract (40). In twin pregnancies, histological choriamnionitis is more common in the first-born twin, whose membranes are opposed to the cervix (244).

The infective agent can then infiltrate the decidua and chorion, producing an inflammatory reaction and leucocyte infiltration. This can proceed to infection of the amniotic cavity and fetus. Cytokines released by inflammatory cells amplify the inflammatory process and can lead to prostaglandin release, causing myometrial contractions and production of MMPs, causing membrane rupture. A variety of organisms have been associated with PTL, such as *Ureaplasma urealyticum, Gardnerella vaginalis, Fusobacterium* sp., and *Clostridium* sp. etc (28). Therefore it may be that inflammatory mediators, such as cytokines, that are the link between infection and preterm labour, rather than the organism itself.

1.8.1 Pro-inflammatory Cytokine Levels In PTL & PPROM

1.8.1.1 Maternal Blood

Some groups have found increased levels of IL-6 and IL-8 in the serum of women in preterm labour compared to those in labour at term or gestation matched controls (183;245-247). Greig *et al.* found higher levels of IL-6 in those women in PTL who delivered prematurely compared to those who did not (248). However others have not found a correlation between maternal serum cytokine levels and PTL (249;250).

Differences may occur due to sensitivities of assays used and the time point at which the samples were taken, for example Bahar *et al* .who failed to find an association between IL-6, IL1β or TNFα levels and PTL, took samples from women in early labour whereas Greig *et al.* took samples from women in preterm labour with regular contrations and cervical dilatation of >1cm (248;249).

Maternal serum IL-6 levels have been correlated to neonatal sepsis in some work but not in others (249;251). Similarily Stallmach *et al.*(252) and Maeda *et al.*(253) found that maternal serum IL-6 was postivitely associated with evidence of histological chorioamnionitis whereas Lencki *et al.* and Salafia *et al.* found no correlation (253-255) .

1.8.1.2 Amniotic Fluid

IL-1β, IL-6, TNFα and IL-8 have been found at higher levels in the amniotic membranes of women in preterm labour compared to term labour and when compared to gestation matched controls not in labour (184;202;208;256;257). IL-6 has been found to be particularly predictive with higher levels in the amniotic fluid of women in threatened preterm labour who went on to deliver early compared to those who did not (203;256;257).

Pro-inflammatory cytokine levels have also been found to be higher in pregnancies that deliver prematurely with signs of infection, than those without. IL-6, IL-8, IL-1β, and TNFα have been shown to be elevated in the amniotic fluid of pregnancies that deliver prematurely with chorioamnionitis compared to spontaneous preterm

deliveries without chorioamnionitis (202;204;205;256;257). Levels of IL-6, IL-1β, and TNFα were also higher in several studies in cases of PTL with positive amniotic fluid cultures compared to those with negative cultures (28;256).

Increased levels of IL-6 have also been found at the time of genetic amniocentesis in women who later go on to deliver prematurely (30;258;259). This supports the theory that a chronic inflammatory process can be associated with preterm labour.

1.8.1.3 Placental Tissue

Keelan *et al.* found increased levels of IL-1β, IL-6 and IL-8 in amnion and choriodecidual tissue from women in PTL compared to term labour. There was no change in cytokine levels from villous placental tissue, suggesting that villous placental tissue may not be the key source of cytokines in PTL (208). Dudley *et al.* found increases in mRNA levels of IL-1β, IL-6, IL-8 and TNFα from amnion, chorion and decidua of women in PTL and term labour, compared to women undergoing elective caesarean section (187). cDNA arrays show upregulation of IL-1β, IL-1α, IL-6, IL-8 and TNFα in amnion and choriodecidual tissue in preterm labour (210). Activated leucocytes have been found on the amnions of women who deliver prematurely (260). Furthermore cytokine levels in amniotic fluid, choriodecidua and amnion tissue correlate with leucocyte count (241;261). Therefore these activated leucocytes may be the source of cytokines detected in PTL or may stimulate their production from other cells in these tissues.

1.8.1.4 Cervicovaginal Secretions

Inglis *et al.* investigated levels of TNFα, IL-6 and fetal fibronectin in cervicovaginal secretions as a predictor of preterm labour (262). In women with threatened preterm labour they found an increased risk of 6.19 ($p < 0.005$) of preterm delivery with raised TNFα, and increased risk of 4.81 ($p < 0.05$) of preterm delivery with positive fetal fibronectin test. The Preterm Prediction study also found increased IL-6 levels (>90th centile) in cervicovaginal fluid to be associated with increased risk of PTL (263). Increased levels of IL-1β have been found in the vaginal secretions of women with bacterial vagnosis (207).

In contrast, Simhan *et al.* found lower concentrations of IL-1β, IL-6 and IL-8 in cervical swabs taken early in pregnancy were associated with an increased risk of chorioamnionitis later in gestation (264). They postulate that this 'hyporesponsiveness' may allow infections to become chronic. These chronic infections may then go on to cause chorioamnionitis and preterm labour.

1.8.1.5 Cord Blood

Several groups have found increased levels of pro-inflammatory cytokines in umbilical cord blood in fetuses who are born prematurely. Huang *et al.* found increased levels of IL-8 in cord blood of preterm infants compared to those born at term, but mode of delivery was not recorded (265). Dammann *et al.* recorded higher levels of IL-1, IL-6, IL-8 and TNFα than term infants (266).

Chorioamnionitis has been associated with a pro-inflammatory cytokine response in the fetus, particularly if funsitis is present (267-270). Viscardi *et al.* looked at 276 infants born at less than 33 weeks and measured cord or venous cytokine levels taken within 12 hours of life (271). They found that IL-6, IL-1β and TNFα were raised in those pregnancies with chorioamnionitis. Others have also reported increases in IL-6, IL-8, IL-1β and TNFα levels in cord blood of those infants born with chorioamnionitis (252;254;272-274). There is better correlation between fetal cord cytokine levels and chorioamnionitis than with maternal serum levels (255;275).

This pro-inflammatory response in the fetus has been likened to the adult systemic inflammatory response syndrome seen in patients on intensive care units and associated with serious morbidity and mortality. The term *fetal inflammatory response syndrome* or FIRS has been coined for those fetuses who have an elevated serum IL-6 level (276). Almost 50% of preterm infants with PPROM were found to have FIRS. It has been proposed by the same group that FIRS may initiate PTL in the context of intrauterine infection (277). The elevated levels of pro-inflammatory cytokines are likely to be produced by cells in cord blood as LPS stimulated cord blood, and in particular mononuclear cells, are capable of producing IL-1β, IL-6, IL-8 and TNFα (278;279). Romero *et al.* studied a cohort of patients with PPROM. They found that fetuses with evidence of FIRS were more likely to deliver than those without (275). They suggest that the infected fetus may initiate labour in order to escape from a hostile environment by generating pro-inflammatory cytokines that trigger parturition (275;280).

1.8.2 Actions Of Pro-Inflammatory Cytokines In PTL & PPROM

LPS and bacteria stimulate placental tissue to secrete cytokines such as IL-1β, IL-1α, IL-6, IL-8, IL-10 and TNFα, with *E.coli* being particularly potent (281;282). IL-1β is also capable of inducing further production of pro-inflammatory cytokines such as IL-8 from amnion cells (223). These cytokines will have similar effects as described in term labour (see section 1.7.2), leading to uterine contractions, membrane rupture and cervical remodelling. Reduced expression of PGDH in the chorion of women in PTL has been shown (283), which could be due to increases in pro-inflammatory cytokines such as IL-1β and TNFα, see figure 4.

1.8.3 Anti-Inflammatory Cytokines

The evidence regarding anti-inflammatory cytokines and preterm labour is unclear. Greig *et al.* found amniotic fluid IL-10 levels were increased in women in PTL with evidence of infection compared to those in PTL with no infection (193). However Dudley *et al.* found no difference in amniotic fluid IL-10 levels between women in PTL who delivered within 7 days and those who did not, or between those with or without chorioamnionitis (226).

Trautman *et al.* found that the decidual tissue, but not amnion or chorion, produced IL-10 in response to stimulation with IL-1β (230). Blanco-Quiro *et al.* found increased levels of cord IL-10 in preterm infants compared to term infants, but this was a mixed group including vaginal and operative deliveries (192).

IL-10 has been shown to reduce LPS induced preterm delivery in a rat model (284). Fortunato *et al.* found that IL-10, but not TGFβ_1, was capable of reducing LPS induced production of IL-8 in amniochorionic tissue (282). These findings suggested that IL-10 might play a role in dampening down the pro-inflammatory process in intrauterine infection.

Very little is known about the role of TGFβ_1 in PTL. Chegini *et al.* demonstrated increased levels of mRNA from myometrial tissue from women in PTL compared to an elective caesarean section group, but decreased expression of TGFβ_1 type II receptor (235).

1.9 GENETICS & INFLAMMATION

As a clinician, it is particularly intriguing to observe the different responses of mothers and fetuses to preterm labour. Some infants are delivered in frankly septic situation but seem to do very well in the long term, whereas other infants are born in better clinical condition, but go on to develop problems such as white matter injury and overwhelming sepsis. One obvious explanation is that the insult received is variable, however similar organisms and histological findings are reported in these women (29;30), so it is reasonable to presume that they may face a similar insult but respond differently.

In other clinical settings, this biological variation to a similar stimulation has been investigated, such as trauma or surgical insults received by patients on intensive care units (142). Individual variations in aspects of the immune system such as CD14, toll-

like receptors and cytokines have been investigated. Polymorphisms in cytokine genes have been demonstrated to influence outcome. Polymorphisms are allelic variations that exist stably in a population at a frequency of 1% or more. The most common type of polymorphism in DNA is caused by a single base-pair substitution (SNP). If the SNP is within a protein-coding region of the gene, it can lead to amino acid substitution that may alter the function of the protein. More commonly SNPs occur in non-translated regions but may affect promoter regions, altering binding affinity of transcription factors such as NF κB.

IL-6 production is tightly controlled by a number of transcription factors that bind to the IL-6 promoter site. A SNP polymorphism has been identified in the –174 position on the IL-6 promoter, deriving from a C to a G substitution (285). IL-6-174 C allele has been associated with lower plasma levels of IL-6. Hartel *et al.* found an increased frequency of IL-6 –174 G alleles in mothers who delivered prematurely (286). An increased frequency of IL-6 –174 G allele has also been found in association with PPROM (287). However there was no difference in IL-6 polymorphisms in those who had evidence of chorioamnionitis and those who did not.

TNFα is regulated both transcriptionally and post-transcriptionally. A G to A susbstitution at position –308 in the promoter region can occur and the A allele enhances transcriptional activation, leading to increased TNFα levels (288). Roberts *et al.* found an association between TNFα-308 A allele in mothers and an increased risk of PPROM but no increased risk of PTL (10). Macones *et al.* found TNFα-308 A allele was associated with PTL (289). They also found evidence of gene-environment interaction, in that symptomatic bacterial vaginosis and the presence of TNFα-308 A

allele greatly increased the risk of PTL, compared to either factor alone. Annells et al failed to show an association between the TNFα-308 A allele and PTL (287).An increased risk of clinical chorioamnionitis in term pregnancies with maternal TNFα-308 A allele has also been found (290). Kalish *et al.* found an association between TNFα-308 A allele in the first twin in multiple pregnancies and PPROM (291). This association was not seen for the second twin.

Several polymorphisms in the promoter region of the IL-10 gene have been described, at positions –1082, -819 and –592. Three major haplotypes have been reported, -GCC, ACC and ATA. The term haplotype denotes ordered combinations of alleles on a single chromosome. Highest levels of IL-10 have been reported with the GCC haplotype (292;293). Kalish *et al.* found no relationship between maternal IL-10 polymorphisms and PTL (294). However Annells *et al.* found the GCC haplotype to be associated with PPROM and the ATA haplotype to be associated with PTL (287;295).

Variations in results from different studies will depend upon the ethnicity of the populations studied (296), the power of the studies and other variables such as the gestational ages of the patients studied.

1.10 CYTOKINE BALANCE

As discussed in the previous section, there is evidence from animal and human work that pro-inflammatory cytokines may play a role in preterm labour and may also be detrimental to the developing brain, but there is some conflict within the data. There

does not appear to be one identifiable marker that will predict poor outcome. Clinical markers of inflammation such as C-reactive protein perform poorly as a predictor of preterm delivery and neonatal outcome (297). Therefore in this thesis, I have looked at the concept of cytokine balance as a possible tool for aiding clinical decision-making regarding the management of women in preterm labour or with PPROM.

The concept of cytokine balance has developed alongside interest in the influence of individual variability (295). Initially the immune response to insults such as sepsis, trauma or surgery was believed to be one of uncontrolled inflammation but Bone revised this concept and raised the possibility that an excessive anti-inflammatory response may also be detrimental to the patient (295). Research in the intensive care setting has moved away from identifying and blocking a single mediator of inflammation and towards understanding the state of cytokine balance that exists and trying to restore homeostasis (298;299).

1.10.1 The Balance Between TNFα And IL-10

The balance between TNFα and IL-10 has been investigated. In healthy individuals circulating levels of TNFα are negligible but rapidly rise within 15 to 30 minutes of stimulation such as LPS exposure (139). TNFα acts through a positive feedback mechanism to increase its own production. In contrast IL-10 is constitutively produced at low levels by monocytes and macrophages and inhibits its own production via a negative feedback loop. LPS induced IL-10 production by monocytes takes several hours to occur (160). IL-10 inhibits TNFα production. It inhibits proteolytic cleavage of membrane bound TNFα molecule and NF-κB activation of

TNFα transcription. On the other hand, TNFα increases IL-10 production by monocytes and macrophages.

1.10.2 Cytokine Balance And Sepsis

The concept of cytokine balance has been most extensively studied in the field of sepsis. Studies have shown that high or low levels of TNFα or IL-10 to be associated with poor clinical outcome (159). However IL-10 levels have been reported to be highest in critically ill patients with sepsis and in non survivors (300). This may be a marker of the severity of the disease or may represent the opposite end of the spectrum and excess anti-inflammation. Patients with high levels of IL-10 are more likely to die of meningococcemia and other infections (159). Recent work from ICH has demonstrated that in children undergoing cardiopulmonary bypass, excessive anti-inflammation is associated with prolonged stay on ITU and a greater risk of subsequent sepsis (301). Therefore it appears that too much or too little of either pro-inflammation or anti-inflammation in the immune response can be associated with a poor outcome.

1.10.3 Cytokine Balance, Preterm Labour & Perinatal Brain Injury

Animal models have demonstrated that IL-10 may be neuroprotective, as discussed in section 1.3.2.6. However the neonate is vulnerable to sepsis due to immaturity of their immune system (302;303). There are several aspects of the immune system that appear to be immature, such as delayed B and T cell maturation and deficiencies in neutrophil chemotaxis (302;304). Therefore it may be that an excessive anti-

inflammatory response may have some benefits to the preterm infant in terms of neuroprotection but may leave them more vulnerable to sepsis in the neonatal period.

1.10.4 Quantitating Cytokine Balance

The role of cytokine balance in the setting of preterm delivery and perinatal brain injury therefore needs to be addressed. The interactions of cytokines are highly complex and poorly understood. Thus it is unlikely that assays of individual circulating cytokines will describe the inflammatory response. Serum levels of particular cytokines may not be representative of levels in other compartments such as the amniotic fluid and fetal brain tissue. As cytokines have short half-lives, the ratio between two individual cytokines at a set time point may not be representative of the overall balance of the immune response. Two alternative methods of quantifying cytokine balance have emerged:

- Reduced surface expression of Major Histocompatibility Complex (MHC) Class II on monocytes.
- Monocyte hyporesponsiveness to LPS stimulation.

Both of these measurements were investigated in this thesis, as well as assays of individual plasma cytokine levels, in order to assess cytokine balance (or imbalance) in preterm labour.

1.10.5 Monocytes And MHC Class II Expression

Individuals vary in their expression of monocyte MHC Class II expression (305). Reduced monocyte MHC Class II expression has been found in both adult and paediatric patients on intensive care units following major trauma, surgery or sepsis and has been shown to predict outcome (298;301). Monocyte MHC Class II expression has been shown to fall following an insult such as surgery but then to quickly recover in those who made an unevental post-operative recovery (306). However in those who developed a secondary infection, monocyte MHC Class II expression took longer to recover, and in fact monocyte MHC Class II expression never recovered in those patients who developed sepsis and died (305).

1.10.5.1 Monocytes

Monocytes constitute 5-8% of total circulating lymphocytes in whole blood (2-8 $x10^5$ monocytes/ml of blood). The monocyte measures 16-20μm in diameter with a deeply indented or U-shaped nucleus (figure 1.2). Monocytes circulate in blood for about a day ($t_{1/2}$ = 17 hours), and are extremely sticky, attaching to plasticware if left at room temperature in concentrated numbers.

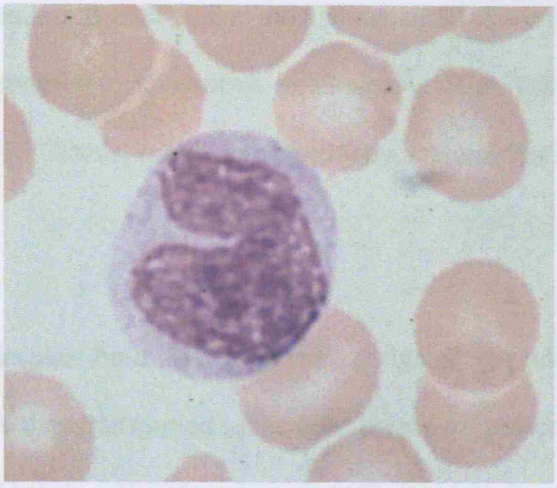

Figure 5: Normal peripheral blood monocyte
(Courtesy of Haematology Department, Great Ormond Street Hospital).

1.10.5.2 MHC Class II Expression

The major histocompatability complex (MHC) is a locus on the genome involved in rejection of non-self tissue. In the human, MHC molecules are called the human leucocyte antigens (HLA). These are proteins that bind to fragments from pathogens (antigens) and display them on the cell surface for recognition by T cells. HLA molecules are divided into Class II and I. MHC Class I molecules are encoded by HLA-A, -B and –C genes. Most cells in the body express MHC Class I molecules and these present endogenously synthesised antigens to CD8+ cytolytic T cells. These cytolytic T cells destroy the cell presenting the antigen and thus this is a mechanism for killing intracellular microbes.

MHC Class II molecules are expressed on antigen presenting cells, such as macrophages, dendritic cells and B-lymphocytes. The HLA-D region encodes genes

for MHC Class II molecules. Three loci, DP, DQ and DR, encode the major expressed products of the human lass II region. The most abundant of these is HLA-DR. Whilst many clinical studies refer to alterations in monocyte HLA-DR expression, some studies use a pan-MHC Class II monoclonal antibody, staining for HLA-DR, -DP and –DQ (301;307;308).

MHC Class II molecules bind to exogenous antigenic peptides in intracellular compartments and are then transported to the cell surface for presentation to the T cell receptor (TCR) on T helper cells. Thus they provide a system for displaying peptides from extracellular microbes.

Together with other costimulatory receptors, this interaction between the MHC Class II molecule, antigen and TCR stimulates CD4+ cells to secrete cytokines. This leads to macrophage activation and activation of B cells leading to antibody production.

1.10.5.3 MHC Structure

MHC Class II molecules are cell surface glycoproteins with peptide binding groove. They consist of 2 chains, α and β glycoproteins. MHC polymorphisms lead to changes in the amino acid sequence of the binding cleft.

1.10.5.4 MHC Class II Production And Transport

MHC Class II molecules are synthesised and assembled on the rough endoplasmic reticulum. 3 newly synthesised MHC Class II αβ complexes associate with 1 invariant chain (CD74) trimer, which stabilises their spatial structure, targets them to Golgi apparatus with the help of 2 sorting signals, and prevents premature peptide loading (309). The invariant chain is cleaved by endopeptidases in an endosomal or lysosomal compartment, leaving MHC Class II invariant chain derived peptides referred to as CLIP (Class II-associated invariant chain peptides), see Figure 6 (310). CLIP occupies the peptide binding groove of the Class II αβ dimer.

MHC Class II loading with antigenic peptides occurs in the lysosomal like MHC Class II loading compartment with the help of the non classical MHC Class II product HLA-DM. HLA-DM promotes the dissociation of CLIP, stabilisation of the empty MHC Class II complex and loading with high affinity antigenic peptides (311). The MHC Class II molecule and bound antigen are then transported to the cell surface for expression, with a lag time of 1 to 3 hours.

1.10.5.5 Recycling

Once expressed on the cell surface, Class II molecules are internalised and exchange antigenic peptides. They may then be recycled to the cell surface again.

1.10.5.6 CIITA

The Class II transactivator (CIITA) induces transcription of MHC Class II genes, see Figure 7. It is the master switch for Class II, HLA-DM and the invariant chain. CIITA production is induced by IFNγ, IL-4 and LPS and injury(312). It is downregulated by IL-10 and TGFβ. Polymorphisms in CIITA or MHC may influence cytokine profiles or Th1 / Th2 balance (312).

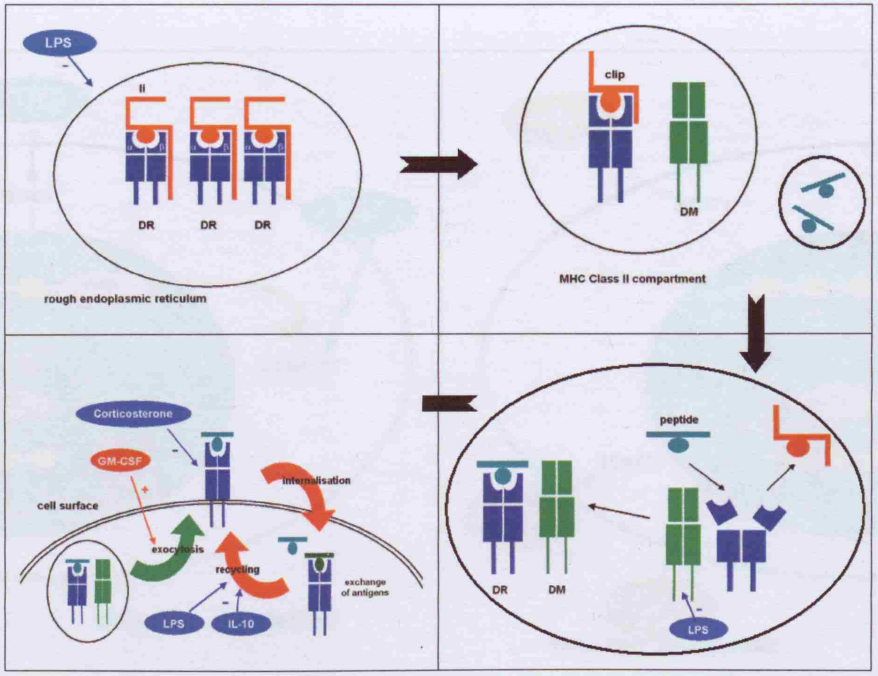

Figure 6: Synthesis and transition of MHC Class II from endoplasmic reticulum through protein loading, to antigen presentation on the surface of the monocyte. Positive and negative regulatory factors are illustrated.

The first box shows 3 newly synthesised HLA-DR molecules in association with the invariant chain (Ii). The second box shows cleavage of the invariant chain to leave CLIP (Class II-associated invariant chain peptides). The MHC Class II loading compartment fuses with endosomes containing antigen. The third box shows removal of CLIP and peptide loading, facilitated by HLA-DM. The fourth box then shows transport of MHC Class II molecule to the cell surface. The Class II molecules are then internalised and exchange antigen peptides. They may then be recycled to the cell surface again.

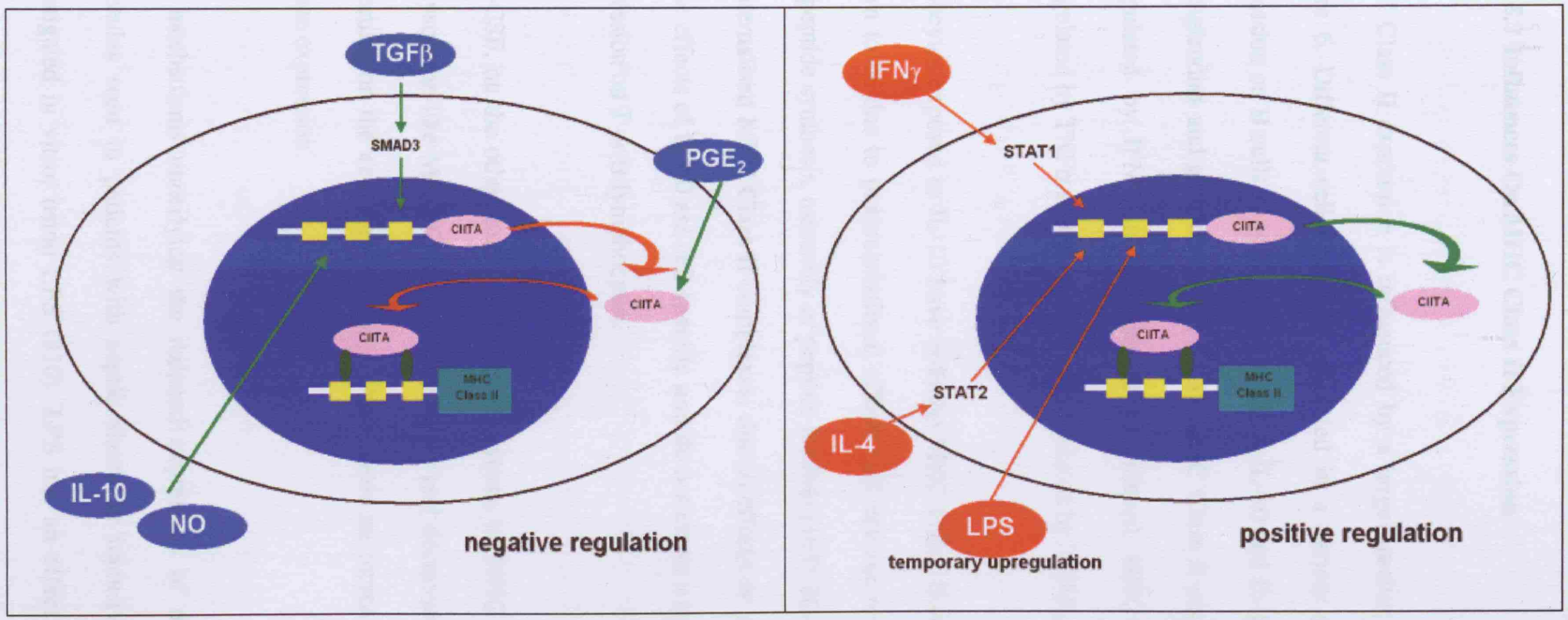

Figure 7:Positive and negative regulation of CIITA.

Positive regulators include IFNγ, IL-4 and LPS temporarily. Negative regulators include TGFβ, PGE, IL-10 and Nitric Oxide (NO). (Adapted from Ting JP and Trowsdale (312)

1.10.5.7 Influences On MHC Class II Expression

MHC Class II expression is influenced by a large number of different stimuli, see Figure 6. Different cell types are regulated in a variety of ways. MHC Class II expression on B cells is upregulated by IL-4, IL-10 and IL-13 and downregulated by prostaglandins and glucocorticoids (313). MHC Class II expression on monocytes is upregulated by IFNγ. In turn this IFNγ induced MHC Class II expression is upregulated by TNFα and IL-4 and downregulated by $TGF\beta_1$ (158;307).

Monocytes exposed to IL-10 have reduced MHC Class II expression. This has been shown to be due to posttranslational effects and not due to effects on transcription, polypeptide synthesis, assembly or peptide loading (165). IL-10 leads to accumulation of internalised MHC Class II complexes, due to effects on recycling and exocytosis. These effects of IL-10 are cell-specific and do not cause a reduction in MHC Class II expression on T or B lymphocytes.

GM-CSF, on the other hand, increases exocytosis of MHC Class II molecules from the lysosome-like MHC Class II compartment and decreased internalisation of those molecules on the cell surface (165). This causes an increase in MHC Class II cell surface expression.

The mechanisms underlying the reduced expression of monocyte MHC Class II molecules seen in patients with septic shock in intensive care units have been investigated in vitro, using LPS (310). LPS has an effect at various stages in the

production of MHC Class II molecules. Synthesis of MHC Class II molecules is reduced and the expression of CD74 is altered. HLA-DM levels are reduced, leading to decreased removal of CLIP from MHC Class II molecules.

LPS increases intracellular accumulation of MHC Class II molecules. This accumulation may occur due to reduced degradation of invariant chain and / or because of alterations in the vesicular traffic of mature MHC Class II – peptide complexes.

The neuroendocrine system may also play a role in control of monocyte MHC Class II expression as glucocorticoids have been found to reduce levels in a mouse model (314).

1.10.6 Monocyte MHC Class II Expression In Pregnancy

There is a paucity of data regarding monocyte MHC Class II expression during pregnancy and labour. Two groups have found no difference in expression between non-pregnant and pregnant women, or over the course of pregnancy itself (173;315). Naccasha *et al.* found lower HLA-DR expression in pregnant women compared to non-pregnant controls (172).

It has been long recognised that the maternal immune system undergoes changes during a normal pregnancy. The fetus is a semi-allograft, expressing human leucocyte antigens (HLA) of maternal and paternal origin, which could lead to rejection of the fetus by the maternal immune system. However in normal pregnancies this does not

occur, partly due to mechanical barriers that reduce interactions between fetal tissues and maternal lymphocytes, and also due to immunological mechanisms. The fetal villous cytotrophoblast and synctiotrophoblasts, which are in direct contact with the maternal circulation, do not express MHC Class I or Class II molecules. The non-classical MHC Class I antigens HLA-G and E are expressed on extravillous trophoblast but do not seem to stimulate a cytotoxic T cell response and can in fact block natural killer cells' cytotoxicity (316).

It has been hypothesised that there is a suppression of the adaptive immune system during pregnancy, in order to prevent rejection of the fetus. This idea is supported by the fact that total T cell numbers and cytotoxic lymphocyte activity are decreased during pregnancy (317) whereas moncyte numbers and phagocytosis are increased (169;318).

During pregnancy, there appears to be some upregulation of the innate immune system. This is hypothesed to be a compensatory reaction to the suppression of the adaptive immune system (316). However, not all aspects of the innate immune system are upregulated in pregnancy, peripheral natural killer (NK) cell proliferation and cytotoxic activity is reduced (319). This reduction in activity may be due to the fact that NK cells are regulated by T_h 1 cytokines, IL-2 and IFNγ (320).

1.10.7 Monocyte MHC Class II Expression In The Fetus & Neonate

In the fetus, MHC Class II expression first occurs on lymphocytes in the second trimester (321). Several studies have found reduced monocyte MHC Class II

expression in neonates as compared to a healthy adult population (322-326). Jones *et al.* found an inverse relationship between monocyte MHC Class II expression and gestational age at delivery, but factors such as mode of delivery, sepsis, chorioamnionitis etc, were not taken into account (229;322). Birle *et al.* also found a positive correlation between monocyte MHC Class II expression and gestational age (322).

Respiratory distress and evidence of sepsis (raised CRP and clinical signs) were also found to be associated with lower monocyte HLA-DR expression, but mode of delivery or maternal factors such as pre-eclampsia were not related to HLA-DR expression.

1.10.8 The Concept of Immune Paresis

We also chose to assess cytokine balance by measurement of monocyte responsiveness to stimulation. This may be a useful method to provide more information about the inflammatory processes occurring in mother and fetus during preterm labour.

In adult patients with sepsis in intensive care, monocytes have been shown to have not only reduced levels of MHC Class II expression but also a diminished capacity to produce TNFα, IL-1α, IL-1β, IL-6, IL-10 and IL-12 (327;328). This alteration in monocyte function has been termed *immune paresis* or *monocyte deactivation. Immune paresis* has been defined as a reduction in HLA Class II expression and reduced ability to produce LPS-induced TNFα *in vitro* and has been associated with

poor outcome in adult patients (298). The mechanism underlying *immune paresis* may be explained by the concept of *endotoxin tolerance*. This was initially described in animals, which survived a lethal dose of LPS if they had previously been treated with a sublethal dose (329). Prior *in-vivo* inflammatory challenge may cause alterations in the patient's immune balance that can be unmasked in *in-vitro* whole blood stimulation.

The ability of monocytes to respond to stimulation *in-vitro* can be assessed using whole blood or isolated monocytes. LPS stimulation of whole blood has been used by many groups in order to take into account the influence of plasma constituents and leucocyte interactions (330;331). Although it does not provide information about cytokine production from individual cells, it reflects the *in-vivo* situation in the patient and the possible regulatory effects of plasma constituents.

Beckmann *et al.* found similar amounts of TNFα production with whole blood LPS stimulation, in healthy pregnant and non-pregnant women (332). Amory *et al.* showed that blood from women with a history of previous preterm delivery produced less TNFα than blood from women who had delivered at term (333). Stimulation of peripheral blood mononuclear cells (PBMCs) with mitogens has shown some variance between women in preterm labour and healthy controls (334). Those in PTL produced more Th_1 cytokines such as IL-2 and IFNγ, whereas the healthy pregnant controls produced more IL-10, a Th_2 cytokine. This study suggests there may be a difference in response to stimulation in women in PTL.

The neonate's cellular immunity is immature compared to adults and they are more susceptible to infection (303). There are varying reports of the ability of cord blood to produce cytokines in response to LPS stimulation. Several groups have found similar or slightly lower levels of pro-inflammatory cytokine production by term infants compared to adults (302;335-337). Decreased production of these cytokines has been described in preterm infants compared to adults and term infant controls(279;336;338-340). This low level of pro-inflammatory cytokine production may explain the lack of a febrile response often seen in neonates. Decreased production of IL-8 may explain the immaturity of the neutrophil response seen in preterm infants, and their susceptibility to infection, as IL-8 is important for chemotaxis of neutrophils.

Neonatal monocytes also seem to also have a reduced capacity for production of anti-inflammatory cytokines. Schultz *et al.* found fewer monocytes staining positively for IL-10 and TGFβ_1 in neonatal blood than adult blood, following LPS stimulation (341). Lower levels of IL-10 production in neonatal cord blood compared to adults were also reported by Kotiranta-Ainamo *et al.* (342).

The low MHC Class II expression and reduced capacity for cytokine production in response to stimulation seen in preterm infants may mean that they are unable to mount a response to further challenges such as nosocomial infection on the neonatal unit. Exposure to inflammation as a result of intrauterine infection may reduce their ability to mount an appropriate response to further insults such as the hypoxic challenge of labour (343). Manimtim *et al.* provide some evidence that the cytokine balance may be altered by exposure to infection perinatally. They found that *in-vitro* exposure to *Ureaplasma urealyticum* led to reduced production of IL-10 and IL-6

from LPS stimulated monocytes from preterm infants (344). There was however enhanced production of TNFα. This alteration in the balance of pro- and anti-inflammatory cytokines may influence the outcome of the neonatal in terms of sepsis or white matter injury.

1.11 HYPOTHESES

1.11.1 Maternal Inflammatory Response

Monocyte MHC Class II expression will be lower in mothers in preterm labour, especially in the presence of an inflammatory condition such as chorioamnionitis. This will be reflected by reduced *in-vitro* response to LPS stimulation. The degree of inflammation, and therefore time to delivery, will be reflected by a greater fall in MHC Class II expression.

1.11.2 Fetal Inflammatory Response

Preterm infants, owing to the inflammatory nature of preterm delivery, will have lower levels of monocyte MHC Class II expression. Monocyte MHC Class II expression will correlate with neonatal outcome.

1.12 AIMS OF THIS THESIS

- To investigate the effects of labour on monocyte MHC Class II expression and the cytokine response to whole blood LPS stimulation in a) maternal blood and b) fetal blood.
- To determine if these responses differ between term and preterm labour.
- To investigate the relationship of monocyte MHC Class II expression and evidence of intrauterine infection (clinical or histological chorioamnionitis).
- To investigate whether MHC Class II expression and response to LPS stimulation can identify a subgroup of neonates at increased risk of adverse outcome.

CHAPTER 2 - MATERIALS AND METHODS

2.1 INTRODUCTION

The following chapter describes established scientific methods that have been used during the course of this MD. Many of these methods required optimisation for use with very small samples of blood, to ensure reproducibility of results (345).

2.2 MATERIALS AND REAGENTS

2.2.1 Chemicals

Chemical	Supplier
Bovine serum albumin (fraction V)	Sigma, Poole, UK
CellFix™	BD Bioscience, Oxford, UK
Cell lysing solution	BD Bioscience, Oxford, UK
di-Sodium hydrogen phosphate (Na_2HPO_4)	Sigma, Poole, UK
CBA human inflammatory cytokine beads kit	BD Bioscience, Oxford, UK
E.coli 0111:B4 lipopolysaccharide	Sigma, Poole, UK
Heparin 5000IU/ml (preservative free)	CD Pharmaceuticals, Wrexham, UK
Hydrochloric acid	Sigma, Poole, UK
Phosphate buffered saline tablets	Oxoid, Basingstoke, UK
Potassium di-hydrogen phosphate (KH_2PO_4)	Sigma, Poole, UK
Potassium chloride (KCl)	Sigma, Poole, UK
RPMI 1640 with L-glutamine	Invitrogen /Life Technologies, Paisley, UK
Sodium Chloride	Sigma, Poole, UK
Sodium hydroxide	Sigma, Poole, UK
Streptavidin HRP	Biosource International Inc, California, USA
Sulphuric acid	Sigma, Poole, UK
Tetramethyl benzidine (TMB)	Biosource International Inc
Transforming growth factor β (human)	BD Bioscience, Oxford, UK
Tween 20	Sigma, Poole, UK

2.2.2 Buffers And Solutions

All solutions were made up to 1000mls with double deionised water unless otherwise indicated.

Buffer or Solutions	Composition	Concentration	pH
Coating Buffer for ELISA	NaCl $Na_2HPO_4.2H_2O$ KH_2PO_4 KCl	140mM (8g/litre) 8.0mM (1.42g/litre) 1.5mM (0.2g/litre) 2.7mM (0.2g/litre)	7.4
Blocking solution for ELISA	Coating buffer as above Bovine serum albumin (fraction V)	 0.5% (5g/litre)	7.4
Standard Diluent for ELISA	Blocking solution as above Tween 20	 0.01%	7.4
Wash Buffer for ELISA (distilled water)	NaCl Tween 20	150mM (9.0g/litre) 0.01%	7.4
Stop Solution for ELISA	H_2SO_4	1.8N	
Phosphate buffered saline	PBS tablet	1 per 100mls distilled water	7.4
FACS washing buffer	PBS Sodium azide Bovine serum albumin	 0.02% 0.5% (5g/litre)	7.4
Sodium Hydroxide	NaOH	1N (36.5g)	
Hydrochloric acid	HCl	1N (40g)	

2.2.3 Antibodies For Flow Cytometry

Antibody	Fluorochrome	Isotype	Clone	Supplier	Code
CD14	RPE	Mouse IgG1	TUK4	Dako	R0864
HLA-DR	FITC	Mouse IgG2a	AB3	Dako	F7266
HLA-DP,DQ,DR	FITC	Mouse IgG1	CR3/43	Dako	F0817
IgG1 isotype control	FITC	mouse	DAK-G01	Dako	X0927
IgG2a isotype control	FITC	mouse	DAK-G05	Dako	X0933

2.2.4 BD Cytometric Bead Array Human Inflammation Kit

Supplied by BD Bioscience, Oxford, UK, catalogue number 551811

2.2.5 Antibodies For Elisa

Antibody pairs and standards used for ELISAs are listed below

Antibody	Isotype	Clone	Supplier	Code
Multispecies TGFβ_1 cytoset			**Biosource International, USA**	CHC1683
Capture TGFβ_1	human	RS02B11/A	Biosource International, USA	58.168.09
Detection TGFβ_1	human	RS02B11/B	Biosource International, USA	58.168.03

2.3 GENERAL EQUIPMENT AND CONSUMABLES

Consumables	Supplier
BD cytometric bead array software	BD Bioscience, Oxford, UK
Bijou (5mls)	SLS, Wilford, Nottingham, UK
FACScalibur Flow Cytometer	BD Bioscience, Oxford, UK
FACScan Research and Lysis II Software	BD Bioscience, Oxford, UK
FACS tubes	BD Bioscience, Oxford, UK
Falcon tubes (15ml)	BD Bioscience, Oxford, UK
Maxisorb 96 microwell plates	Nalgene Nunc, Rochester, NY, USA
MRX ELISA microplate reader	Dynatech Laboratories, Chantilly, VA, USA
Multichannel pipette	SLS, Wilford, Nottingham, UK
Screw cap microtubes (1.8mls)	SLS, Wilford, Nottingham, UK
Universal tubes (20mls)	SLS, Wilford, Nottingham, UK

2.4 PATIENT DATA COLLECTION

Patients were prospectively recruited from October 2003 until July 2005 from the Elizabeth Garrett Anderson Obstetric Hospital (EGA), University College London Hospital (UCLH) NHS Trust and the Whittington NHS Trust. Ethical approval for this work was obtained from UCLH / UCL Joint Ethics Committee and the Whittington Hospital's Ethics Committee. All women recruited were given an information leaflet to read and their written consent was obtained.

This was a prospective observational study carried out from October 2003 to July 2005. This work was a pilot study and so it was not possible prospectively to perform a power calculation. Pregnancies with a major fetal abnormality or aneuploidy or with

maternal complications, such as diabetes mellitus were excluded. The study groups recruited in the following chapters are listed in Table 4 and Table 5. *Term* was defined as gestational age of 37 completed weeks or more.

	Maternal Groups	Description
1	**PTL**	Women in preterm labour (PTL) at less than 32 weeks
2	**PPROM**	Women with preterm pre-labour rupture of membranes (PPROM) at less than 32 weeks
3	**Antenatal Controls**	Women over the three trimesters of normal pregnancy
4	**Term Labour**	Women in spontaneous labour at term
5	**Elective Caesarean Section**	Women having prelabour elective caesarean section at term

Table 4: Maternal Study Groups Recruited

	Neonatal Groups	Description
1	**PTL**	Infants born following PTL at less than 32 weeks
2	**PPROM**	Infants born following PPROM at less than 32 weeks
3	**Feticide**	Fetuses undergoing feticide for structural or chromosomal abnormalities
4	**Term Labour**	Infants born by spontaneous vaginal delivery at term
5	**Term Caesarean Section**	Infants born by prelabour elective caesarean section at term
6	**IUGR**	Infants born by elective caesarean section for intrauterine growth restriction at less than 32 weeks

Table 5: Neonatal Study Groups Recruited

A data collection sheet was completed for each woman and neonate who took part in the study (appendix 1). Data was collected from existing NHS systems. An Access Database (Microsoft Office'98/XP) was developed for use in this work. Patient data was password protected and managed according to the data protection policy of University College London.

2.5 BLOOD COLLECTION AND HANDLING

Whole blood was collected and placed immediately into sterile bijous containing preservative free heparin (final concentration 10 U/ml). Processing of blood samples was undertaken within 30 minutes to avoid cellular activation. Aliquots of this whole blood were used for the LPS stimulation studies and monocyte MHC Class II expression studies. The remaining sample was spun at 1200g for 10 minutes and the plasma fraction aliquoted and stored at -70^0C in sterile tubes for cytokine assays.

Whole blood for genetic analysis was collected and anticoagulated with 4.2 mM ethylenediaminetetraacetic acid. These samples were stored at –70°C until DNA extraction.

2.6 FLOW CYTOMETRY

Flow cytometry was used to select and analyse MHC Class II expression on monocytes and to assay several cytokines in plasma samples using a multiplex bead technique.

A BD FACS flow cytometer with a 488nm laser was used and its performance was monitored through out the period of this thesis with weekly calibration checks. These were undertaken with CaliBrite3 Beads, (Becton Dickinson, UK) and analysed with the automated calibration programme, FACSComp (BD, UK).

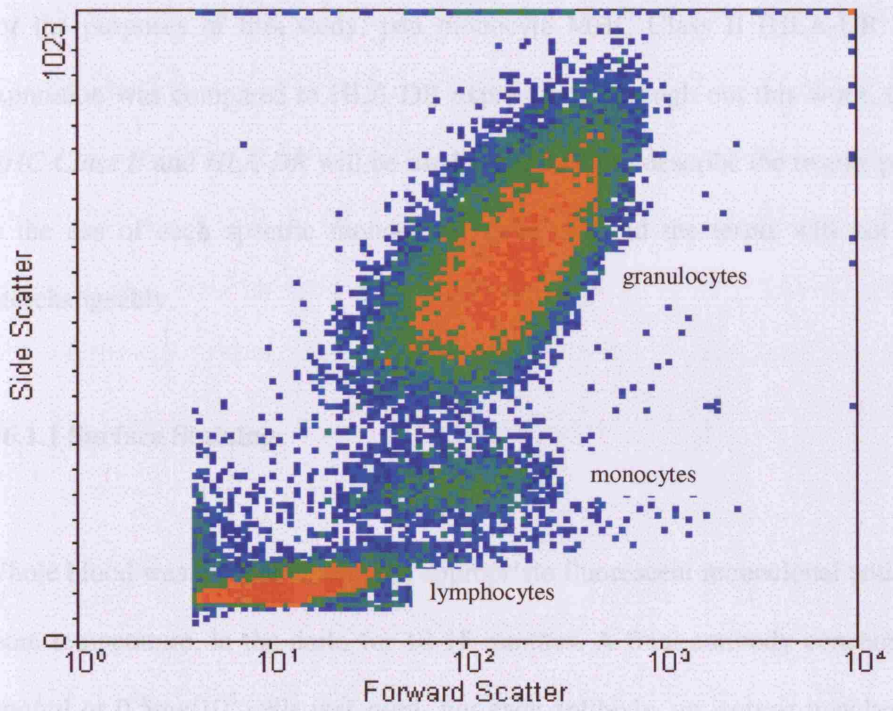

Figure 8: Flow cytometric profile of whole blood after lysis of red blood cells

Anticoagulated whole blood is incubated with the appropriate monoclonal antibodies before the red blood cells are lysed and the remaining cells fixed. The sample is then passed through the flow cytometer where each particle is assessed and plotted according to its size (forward scatter) and granularity (side scatter). Monocytes are readily recognisable as the smaller cloud of cells located between granulocytes and lymphocytes, see Figure 8.

2.6.1 Measurement Of Monocyte MHC Class II Expression

For the purposes of this study, pan monocyte MHC Class II (HLA-DR DP DQ) expression was compared to HLA-DR expression. Through out this work, the terms *MHC Class II* and *HLA-DR* will be used specifically to describe the results pertaining to the use of each specific monoclonal antibody, and the terms will not be used interchangeably.

2.6.1.1 Surface Staining

Whole blood was incubated with the appropriate fluorescent monoclonal antibodies at room temperature, in the dark, for 10-15 minutes. A final antibody concentration of 5mg/ml or $0.5mg/10^6$ cells was used. For each antibody, an isotype matched control was used to control for non-specific binding. 1ml of cell lysis buffer was added to lyse red blood cells, before the remaining cells were pelleted by centrifuging at 350g for 5 minutes. The supernatant was discarded and the pellet resuspended in 250ml of cell fix solution. To conserve fluorescence, samples were protected from the light and stored at 4^0C until analysed.

2.6.1.2 Measurement of Monocyte MHC Class II and HLA-DR Expression

Monocytes were identified by a double gating technique, using physical characteristics (size and granularity) and positive CD14 staining using a R-phycoerythrin (RPE) conjugated monoclonal mouse anti-human CD14 monoclonal antibody, see Figure 8. Monocyte cell surface MHC Class II expression was identified using a fluorescein isothiocynate (FITC) monoclonal antibody to HLA-DR DP DQ. Non-specific staining was determined with isotype-matched control, mouse IgG1 monoclonal antibody raised against keyhole limpet haemocyanin. HLA-DR expression was identified using a fluorescein isothiocynate (FITC) monoclonal antibody to HLA-DR. Non-specific staining was determined with isotype matched control, mouse IgG2a monoclonal antibody, raised against Aspergillus niger glucose oxidase. Monocyte MHC Class II expression or HLA-DR expression was determined on 2500 events, and expressed as the percentage of cells positive for fluorescence (%) or as the median fluorescence intensity (mfi). Whenever a new antibody was used, there was a period of co-staining to confirm reliability.

2.6.2 Cytometric Bead Array

In order to maximise the information obtained from the small volumes of blood available from very preterm neonates in this study, a cytometric bead array system, the BD CBA human inflammation kit, was used to assay the majority of cytokines investigated in this work. The advantages of using a cytometric bead array system are that very small samples can be analysed more reliably than with ELISA techniques.

An existing human inflammatory cytokine kit was chosen as it provided assays for the pro- and anti-inflammatory cytokines of interest during pregnancy and neonatal life. In the array used for this work, there were six beads, each with distinct fluorescence intensity, see Figure 9. Each bead was coated with a capture antibody specific for one of six cytokines, TNFα, IL-1β, IL-6, IL-8 IL-10 and IL-12p70. The beads were mixed together to form a suspension that allowed for the detection of multiple cytokines in small volume samples of about 50μls. Figure 10 shows an overview of the assay procedure.

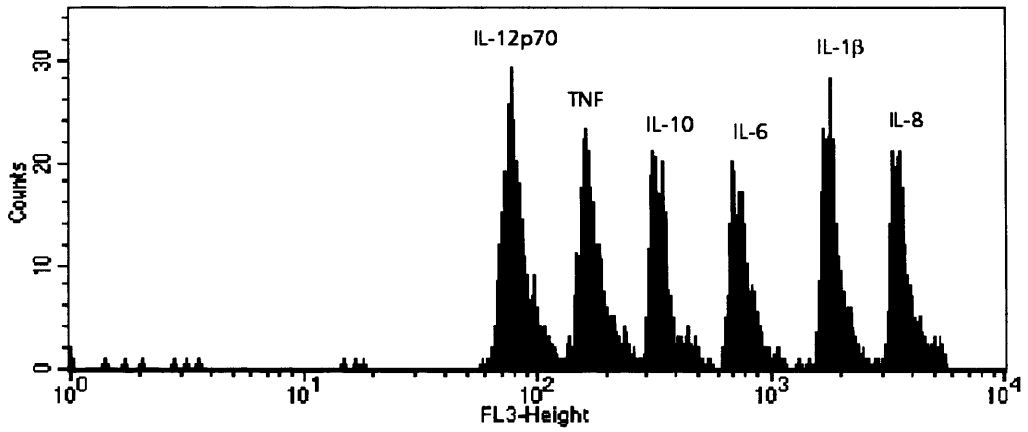

Figure 9: The six bead populations with distinct fluorescent intensities, coated with capture antibodies for six cytokines in the BD CBA human inflammation kit. (From BD CBA Human Inflammation Kit instruction manual)

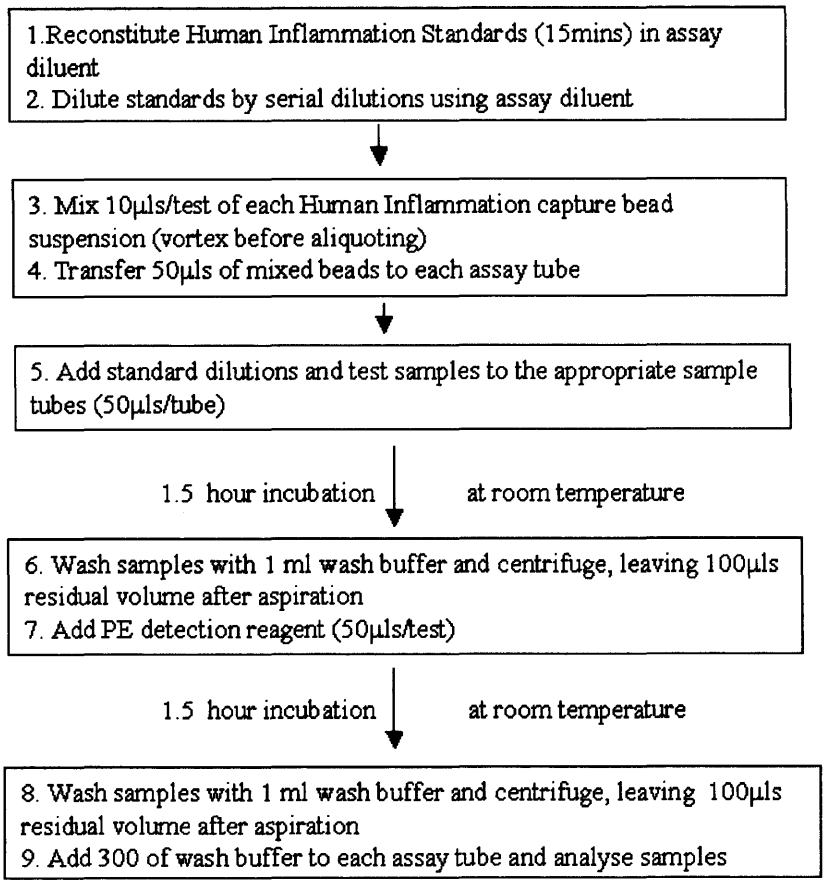

Figure 10: Plasma Assay Procedure for CBA Human Inflammation Kit

2.6.2.1 Sample Preparation

Plasma samples were taken and stored as described in section 2.5. Samples were defrosted and vortexed well prior to analysis. All samples from a single patient, including the maternal, umbilical cord and neonatal samples, were analysed on the same run of the CBA assay. This eliminated intra-assay variability as a potential confounder of the results obtained.

2.6.2.2 Data Acquisition and Analysis

Flow cytometry was used for quantification of fluorescence, using a FACScalibur. For each experiment, the compensation settings for the flow cytometer were optimised using cytometer 'set up' beads. 1800 events were recorded to ensure each sample file contained approximately 300 events per capture bead, see Figure 11. Standard curves were generated from known concentrations, using a data programme from BD CBA software, and then samples were quantified using these curves, see Figure 12.

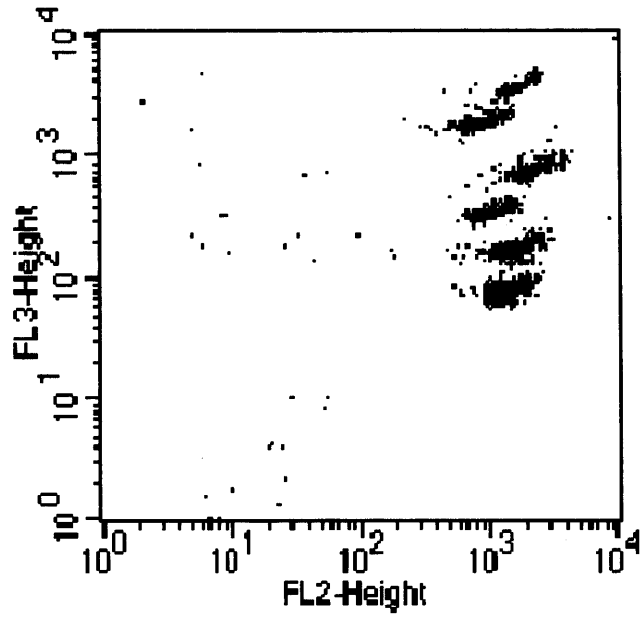

Figure 11: Example of sample acquisition plot

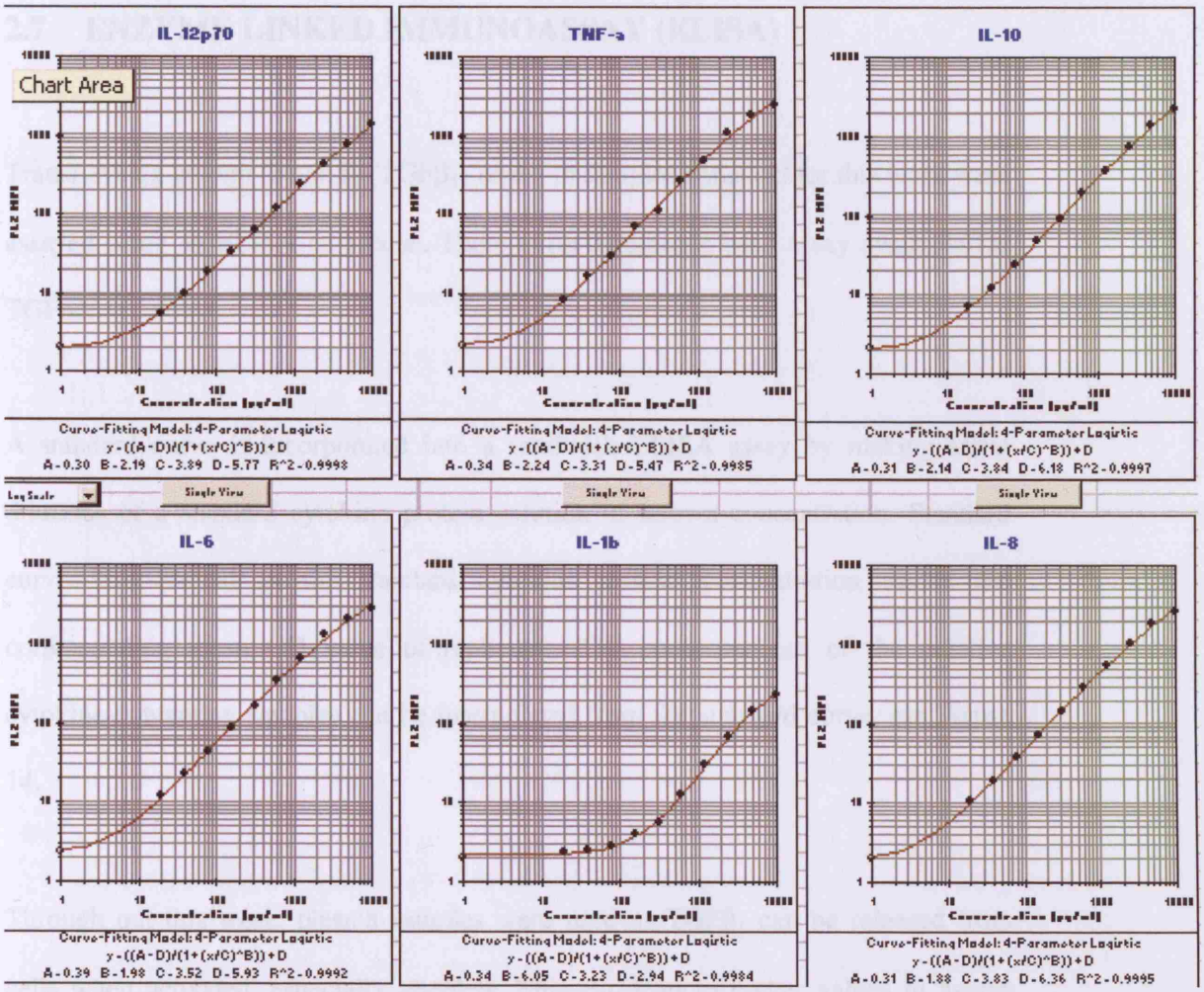

Figure 12: Examples of the standard curves generated

The sensitivity of the assay was 3pg/ml for TNFα, IL-8, IL-10, IL-6 and IL-12p70 and 100pg/ml for IL-1β. The manufacturers analysed samples containing a single recombinant protein and found no cross-reactivity.

2.7 ENZYME LINKED IMMUNOASSAY (ELISA)

Transforming growth factor β_1 ($TGF\beta_1$) levels in samples collected for this work were assayed using an ELISA technique. There is no cytometric bead assay available for TGFb1.

A standard curve is incorporated into a sandwich ELISA assay by making serial dilutions of a standard cytokine protein solution of known concentration. Standard curves are plotted as the standard cytokine protein concentration versus the corresponding mean OD value of replicates. The concentrations of the putative cytokine-containing samples can be interpolated from the standard curve, see Figure 14.

Through out this work, plasma samples were used as $TGF\beta_1$ can be released from cells when activated, especially platelets. This can lead to higher values in assays using serum rather than plasma (346). In the circulation, $TGF\beta_1$ exists both in the active form and as its inactive precursor, non-covalently bound to latency-associate peptide (LAP), as discussed in chapter 1. The anti-TGF antibodies available in commercial ELISA kits only bind the active form of $TGF\beta_1$. Therefore an acid activation step was used in order to release active $TGF\beta_1$.

2.7.2 Sample preparation

Blood samples were collected as described in 2.5 and the plasma stored at -70^0C until use.

2.7.3 ELISA procedure

	Step	Description
1	**Dilution of sample**	Sample diluted 1 in 5 with PBS
2	**Acid activation**	1N HCL added in a concentration of 1:25
3	**Incubation**	Sample incubated at 4^0C for 60 mins
4	**Neutralisation**	1N NaOH added in a concentration of 1:25
5	**CAPTURE ANTIBODY**	capture antibody coated onto Maxisorp plates for 12 to 18 hours at 4^0C
6	**Wash X 3**	saline with Tween 20
7	**BLOCK**	0.5% BSA in PBS at room temperature for 2 hours
8	**Wash X 3**	saline with Tween 20
9	**SAMPLES added to plates**	Standards and acid activated samples for 2 hours at room temperature
10	**Wash X 3**	saline with Tween 20
11	**DETECTION ANTIBODY**	biotinylated antibody added to plates for 2 hours at room temperature
12	**Wash X 6**	saline with Tween 20
13	**STREPTAVIDIN**	streptavidin-HRP in PBS for 30 minutes at room temperature
14	**Wash X 3**	saline with Tween 20
15	**SUBSTRATE**	TMB with plate covered for 30 minutes at room temperature
16	**STOP**	2N H_2SO_4 added
17	**PLATE READING**	Plate read at an absorbance 450nm

Figure 13: General Scheme for TGFβ_1 ELISA

The ELISA protocol followed is summarised in Figure 13. Biosource multispecies $TGF\beta_1$ CytosetsTM were used. 96 well polystyrene microplates were coated with 100mls per well of capture antibody diluted in coating buffer to 2mls/ml for 18 hours at 2-8^{0}C. After 3 washes and aspiration, 300mls of blocking solution was added to each well and the plates were covered and incubated at room temperature for 2 hours. After 3 washes and aspiration, 100mls of diluted standards diluted in standard diluent and samples were added to the plates. A substrate blank and a positive control were used on all plates, and all standards, samples and controls were run in duplicate. The plates were covered and incubated for 2 hours at room temperature, with continual shaking at 700rpm.

After 3 washes and aspiration, 50mls of biotinylated detection antibody diluted in standard diluent to 0.8ml /ml was added to each well. The plates were covered and incubated for 2 hours at room temperature, with continual shaking at 700rpm. After 6 washes and aspiration, 100mls of Streptavidin-HRP diluted 1:500 with standard diluent per well were added to the plates. The plates were covered and incubated for 30 minutes at room temperature, with continual shaking at 700rpm. After 3 washes and aspiration, 100mls of Substrate solution was added to each well and the plates incubated in the dark at room temperature for 30 minutes, with continual shaking at 700rpm. 50mls of acid solution was added to each well to stop the reaction and then the plates read at 450nm within 30mins. ELISA solutions used are listed in 2.2.2. The standard curve is shown in Figure 14, range 3.9pg/ml to 8000pg/ml.

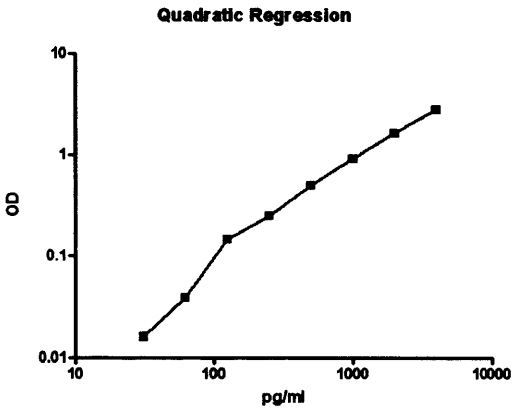

Figure 14: Standard curve for TGFβ_1 ELISA.

The standard curves were generated using serial dilutions of a known concentration of recombinant TGFβ_1. The optical densities were measured using a Dynatech MRX plate reader and analysed by quadratic regression. All plasma TGFβ_1 concentrations for a single patient (mother and fetus) were calculated from the curve.

2.8 WHOLE BLOOD STIMULATION WITH ENDOTOXIN

Whole blood stimulation *in-vitro* with lipopolysaccharide (LPS) is an established technique to assess the reactivity of leucocytes in blood (333).The amount and types of cytokine produced after incubation with LPS are measured. Commercially available *Escheria coli* lipopolysaccharide serotype 0111:B4 was used to stimulate whole blood as this organism has been identified in amniotic fluid and placentas of patients in preterm labour and with chorioamnionitis (202;347).

2.8.2 Methods

E.coli 0111:B4 lipopolysaccharide was suspended in sterile saline and stored in aliquots at -70^0C. The aliquots were thawed and vortexed well prior to use. Over the course of this work, one batch of *E.coli* 0111:B4 LPS was used to avoid possible variation in activity between batches and from freeze-thaw cycles.

Whole blood was collected in a heparinised bijou as described in section 2.5. 0.5mls of whole blood was added to 0.5mls of RPMI 1640 with L-glutamine. *E.coli* 0111:B4 LPS was added in a concentration of 100ng LPS /ml of blood. The sample was incubated at 37^0C in 5% CO2 for 24 hours. The sample was then spun at 1200g for 6 minutes at room temperature and the supernatant aspirated. The supernatant was stored at -80^0C in sterile polypropylene tubes. Cytokine assays were performed as described sections 2.7 and 2.8, using flow cytometric analysis and ELISA techniques. For each sample, a control sample was also incubated for 24 hours, without LPS, to control for background in-vitro production of cytokines.

CHAPTER 3 – MATERNAL CYTOKINE BALANCE IN NORMAL PREGNANCY

3.1 INTRODUCTION

It has been recognised for many years that pregnancy alters the maternal immune system. More recently, labour has been described as a pro-inflammatory event (348). However the concept of cytokine balance and the role of the anti-inflammatory arm of the immune system have not been so extensively described during pregnancy and labour.

As discussed in chapter 1, monocyte MHC Class II expression may be used as a measure of cytokine balance. Therefore this chapter aims to investigate this aspect of the maternal immune response during pregnancy and labour. Cytokines play an important role in regulation of monocyte MHC Class II surface expression and so plasma cytokine levels were also assayed. As well as a reduction in the expression of MHC Class II, altered responsiveness of monocytes to LPS stimulation has been reported in situations of immune dysregulation (328). Therefore maternal cytokine response to whole blood stimulation with LPS was also investigated.

There have been a small number of studies looking at monocyte MHC Class II expression over the course of normal pregnancy and labour, with one demonstrating a decrease in monocyte HLA-DR expression in normal pregnancy compared to non-pregnant controls (172). Others have found no difference in monocyte HLA-DR expression between non-pregnant controls and early pregnancy, or over the three trimesters of pregnancy (173;315). There are no descriptions of monocyte MHC Class

II expression during labour at term. Some variation in stimulated cytokine production by monocytes or whole blood has been described in normal and pathological pregnancies (174;332).

Over the course of normal pregnancy, some authors have reported an increase in pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8, with a further increase during labour (180-182;199;200). Low levels of the anti-inflammatory cytokines IL-10 and $TGF\beta_1$ have been found in maternal blood during pregnancy, with some increase in $TGF\beta_1$ with increasing gestation (186;189).

This chapter sets out to describe the effect of pregnancy and labour on monocyte MHC Class II expression, plasma cytokine levels and whole blood cytokine response to LPS stimulation.

3.2 METHODS

3.2.1 Optimisation Of Sample Collection & Assays

The techniques of measurement of monocyte MHC Class II expression, LPS stimulation and cytokine assays have been described in chapter 2. Prior to collecting patient samples, a series of experiments were undertaken to assess the stability of the techniques used. Deliveries, and therefore blood sampling, took place at various times of the day and night. The author of this work approached and recruited all patients for this work, and also collected all samples. Due to the workload this entailed and the unpredictability of sample acquisition, these experiments were performed in order to

establish the most practical methods of sample collection, and to assess whether samples could be stored prior to staining and analysis. Being able to store samples would enable staff on the delivery suite to collect samples, and then staining and analysis could be performed at a later time point. The aim of these series of experiments was to find a technique, which was practical for clinical collection of samples and that also gave reliable, reproducible results.

3.2.2 Optimisation Of Measurement Of Monocyte MHC Class II Expression

Blood samples were collected from 3 healthy non-pregnant female volunteers for optimisation experiments. The following experiments performed to assess the feasibility of the clinical study proposed.

3.2.2.1 Titration Of Heparin Concentration

Blood collected from the 3 adult controls was anticoagulated with a range of concentrations of heparin, from 1 IU/ml to 10000IU/ml. The samples were then stained for monocyte MHC Class II expression as described in section 2.6.1. Each control sample at each concentration of heparin was run in duplicate to assess reproducibility. Samples were then analysed on the flow cytometer and a dose response curve plotted.

3.2.2.2 Optimisation Of Staining Interval For MHC Class II Expression

One aliquot of blood from each of the 3 healthy adult controls was stained with monoclonal antibodies immediately. Further aliquots were stored at 4^0C and stained at hourly intervals for four hours. Samples were then immediately analysed on the flow cytometer.

3.2.2.3 Optimisation Of Interval From Staining To Cell Lysis For Measurement Of MHC Class II Expression

The effect of delaying cell lysis by 24 hours was investigated. One aliquot of blood from each control was stained and lysed after 10 minutes incubation. A second aliquot was stained and then left for 24 hours at 4^0C in the dark, prior to cell lysis. Samples were then analysed on the flow cytometer.

3.2.2.4 Investigation Of The Effect Of Increasing Interval From Sample Preparation To Analysis On MHC Class II Expression

Blood from each adult control was collected and multiple aliquots stained for MHC Class II expression, lysed and fixed as described in section 2.6.1. Samples were then stored at 4^0C in the dark and analysed on the flow cytometer at increasing time intervals, from time = 0 to 72 hours.

3.2.2.5 Optimisation Of Antibody Preparation For MHC Class II Expression Measurement

An experiment was performed to investigate whether assay tubes containing monoclonal antibodies could be prepared in advance and then left in the labour ward fridge. This would enable midwives to add blood to the antibodies when a baby was delivered, minimising any delay in time to stain. Blood from 3 healthy adult volunteers was collected into heparinised syringes similar to that used for umbilical cord gas sampling. To assess the feasibility of the method for use on the labour ward where it is not possible to pipette samples accurately, one drop or three drops of blood were added to the assay tubes, as it may be difficult to add a single drop of blood to the tubes. One drop was estimated to be equivalent to 50µls of blood and three drops of blood to be approximately 150µls.

3.2.2.6 Investigation Of The Reliability And Reproducibility Of The Technique For Measurement Of MHC Class II Expression

The first 10 patient samples were prepared and analysed in duplicate to assess reliability and reproducibility.

3.2.3 Optimisation Of Enzyme Linked Immunoassay

The ELISA technique used to assay transforming growth factor β_1 was optimised for this work. Different concentrations of capture and detection antibodies were investigated, see Figure 15

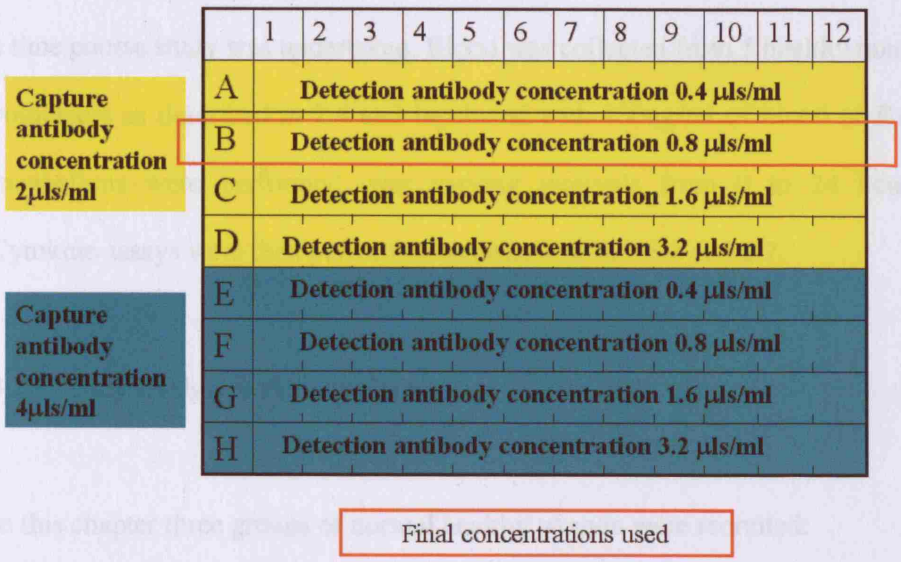

Figure 15: ELISA optimisation plate template.

A plate was run using different concentrations of capture and detection antibodies. A standard curve of serial dilutions of a known concentration of recombinant TGFβ_1 was run on each row of the plate.

The length of time of incubation with standards and samples was also investigated. Serial dilutions of a known concentration of recombinant TGFβ_1 were either incubated at room temperature for 2 hours or overnight for 12 to 18 hours at 4^0C.

To determine assay precision, the coefficient of variation (standard deviation of replicate responses x 100/ mean of the response) was determined using replicas on one plate (intra-plate precision) as well as multiple plates (inter-plate precision).

3.2.4 Optimisation Of Incubation Period For LPS Whole Blood Stimulation

In order to establish the optimal length of stimulation of whole blood with endotoxin, a time course study was undertaken. Blood was collected from 5 healthy non pregnant volunteers as described in 2.4 and incubated with 100ng/ml of blood of *E.coli* LPS. Incubations were performed over varying intervals from 0 to 24 hours (349). Cytokine assays were then performed as described in 2.6.2 and 2.7.

3.2.5 Study Design & Patient Group

In this chapter three groups of normal healthy women were recruited:

- Women in the three trimesters of pregnancy, not in labour
- Women in spontaneous labour at term
- Women having pre-labour elective caesarean section at term

Women with a normal pregnancy, attending the Elizabeth Garrett Anderson Obstetric Hospital, were invited to participate in the study. Pregnancies with a major fetal abnormality or aneuploidy or with maternal complications, such as diabetes mellitus or pre-eclampsia, were excluded.

For the term labour group, maternal blood was taken once a diagnosis of established labour was made (regular uterine contractions with cervical dilatation of 4cm or more).

For those women having elective caesarean section, blood was taken at the time of cannulation, prior to induction of spinal anaesthesia. For the purposes of this study, the following outcome measures were recorded: spontaneous onset of labour, mode of delivery.

3.2.6 Clinical Care

The Elizabeth Garrett Anderson (EGA) Obstetric hospital is a NHS hospital in central London, with 3000 deliveries per annum. Antenatal care is shared between the hospital and primary care.

Low risk women with normal pregnancies attend the EGA for an initial booking visit with the antenatal clinic midwives. Subsequent visits are with the community midwives or general practitioners, with a visit to the obstetricians at 41 weeks if still undelivered. All women are offered a dating ultrasound scan at 10 to 13 weeks gestation and integrated screening test for Downs syndrome, along with screening for HIV, hepatitis B, syphilis and haemoglobinopathies. They are then offered an anomaly ultrasound scan at 20 weeks gestation.

Entonox, diamorphine and epidural anaesthesia are available to women for analgesia during labour. The National Institute for Clinical Excellence (NICE) guidelines for

caesarean section are adhered to, with women receiving metoclopramide and ranitidine pre-operatively, prophylactic co-amoxiclav intraoperatively and thromboprophylaxis with dalteparin post operatively (350). Combined spinal and epidural anaesthesia is the unit's preferred technique used for caesarean section.

Clinical care was unaffected by participation in the study, as blood samples were only collected at the same time as clinical samples, and results of investigation were not available to the clinical team.

3.2.7 Sample Collection

Blood collection and handling has been described in 2.5.

3.2.8 Monocyte MHC Class II Expression & Cytokine Assays

The following assays were performed on all blood samples collected:

- Monocyte MHC class II and HLA-DR expression, as described in 2.6.1.
- Cytokine assays, as described in 2.6.2 and 2.7
- Whole blood LPS stimulation studies, as described in 2.8

3.2.9 Statistics

As MHC Class II and HLA-DR were expressed as percentages, and not normally distributed, comparisons between groups were performed with Mann Whitney test.

Analysis was performed with the Statistical Package for the Social Sciences (version 12.0 SPSS, Chicago, IL.)

3.3 RESULTS

3.3.1 Optimisation Of Measurement Of Monocyte MHC Class II Expression

The results of the optimisation experiments are presented below.

3.3.1.1 Titration Of Heparin Concentration For Blood Collection

A concentration of 10 IU heparin/ml was used in all subsequent work as Figure 16 illustrates this concentration gave the most reproducible results. At higher levels heparin appeared to reduce monocyte MHC Class II surface expression.

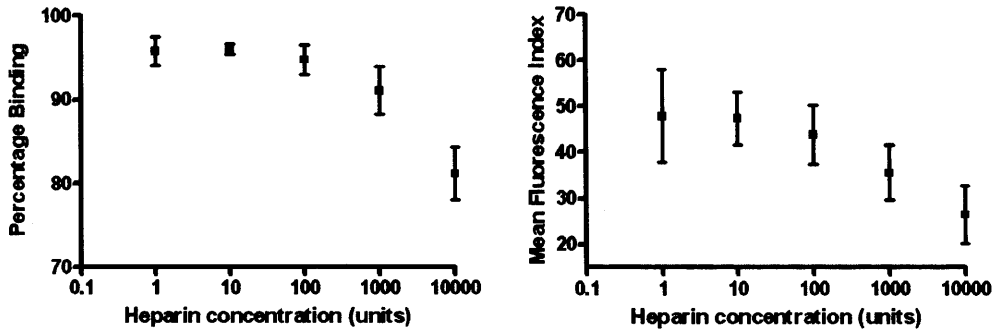

Figure 16: Mean monocyte MHC Class II expression (% and mfi), with standard deviations, using blood collected into different concentrations of heparin.

Data from a single control are shown. The levels of monocyte MHC Class II expression, as measured by percentage and median fluorescence intensity, begin to decrease with heparin concentrations of 100U/ml and above. The most consistent results (smallest standard deviation) was seen with 10U/ml of heparin. Therefore all subsequent work was performed using 10U/ml of heparin.

3.3.1.2 The Effects Of The Interval Between Blood Sampling And Staining For MHC Class II

The MHC Class II expression measurements remained stable up until an interval of 2 hours between blood sampling and staining, see Figure 17. A downward trend in intensity of MHC Class II expression (mfi) was seen if there was a delay of 2 hours or more from blood sampling to antibody staining. There was also some variation in the percentage of cells expressing MHC Class II after 2 hours delay. Therefore all study samples were stained within 1 hour of blood sampling.

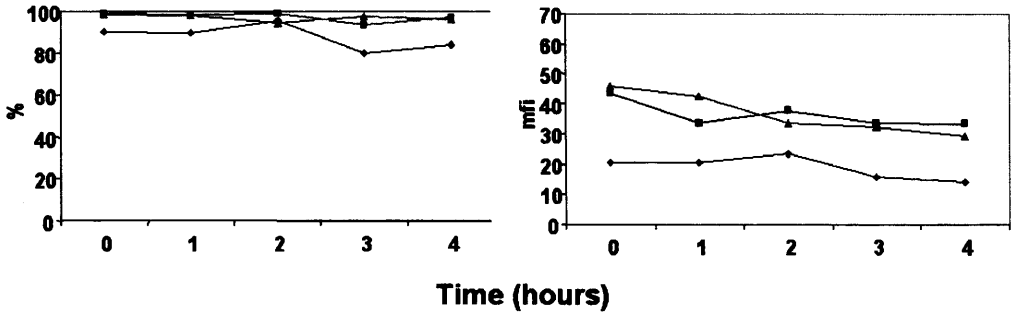

Figure 17: The effect of delay in antibody staining of whole blood.

Blood from 3 healthy volunteers was stored at 4^0C until staining for MHC Class II expression, which was performed at increasing time intervals. Results for each individual are shown in the graphs. There was variability in the percentage and median fluorescence intensity of monocytes expressing MHC Class II after 2 hours when both percentage of class II expression and median fluorescence intensity decreased.

3.3.1.3 The Effect Of Delaying Cell Lysis

Delayed lysis had a marked effect on the results obtained. There was a greater than 30% fall in the percentage of cells expressing MHC Class II and more than 70% decrease in the intensity of expression if samples were stained but left for 24 hours prior to cell lysis, compared to those prepared immediately. Therefore all patient samples were treated with cell lysis 10 minutes after staining.

3.3.1.4 The Effects Of Increasing Interval Between Staining And Analysis On MHC Class II Expression

Analysis of control samples showed a marked reduction in surface MHC Class II expression (both % and mfi) with an interval of greater than 24 hours between

staining and analysis. Samples analysed within 24 hours showed minimal variation, see Figure 18. Therefore all study samples were analysed on the flow cytometer within 24 hours of antibody staining.

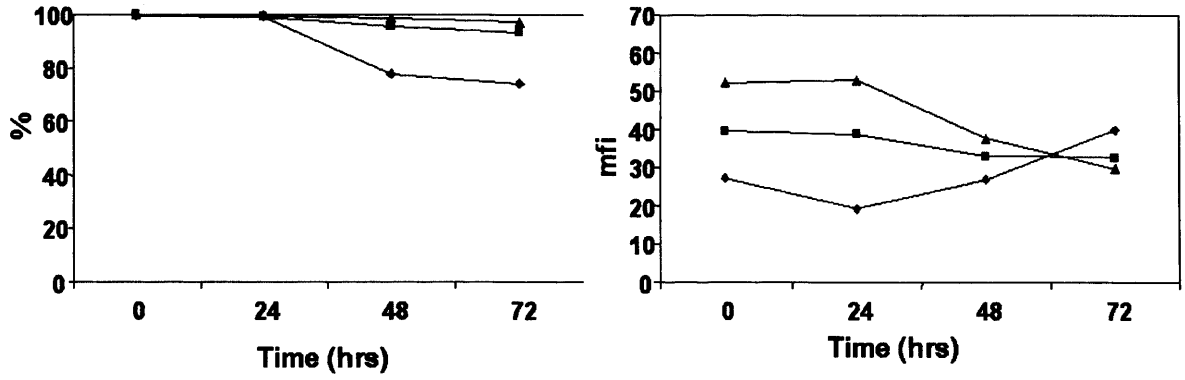

Figure 18: The effect of delay in analysis of MHC Class II Expression.

Blood from 3 volunteers was stained immediately for MHC Class II expression. Samples were stored at 4^0C and analysed on the FACS flow cytometer at increasing intervals. Both MFI and percentage class II expression remained stable for the first 24 hours.

3.3.1.5 The Effect Of Antibody Preparation On MHC Class II Expression

A reduction in the intensity of expression of MHC Class II on the surface of monocytes (mfi) was seen using antibodies that had been prepared 1 day or more in advance, see Figure 19. This experiment demonstrates that it was not possible to

prepare tubes containing the antibodies in advance and gain reliable results. Therefore prepared tubes of antibodies were not used in this work.

In addition, a lower level of MHC Class II expression was seen using three drops of blood compared to one drop, this may be due to binding sites in excess of the antibody concentration, see figure 3.7. Therefore 50µls aliquots of blood were pipetted for subsequent samples in this work.

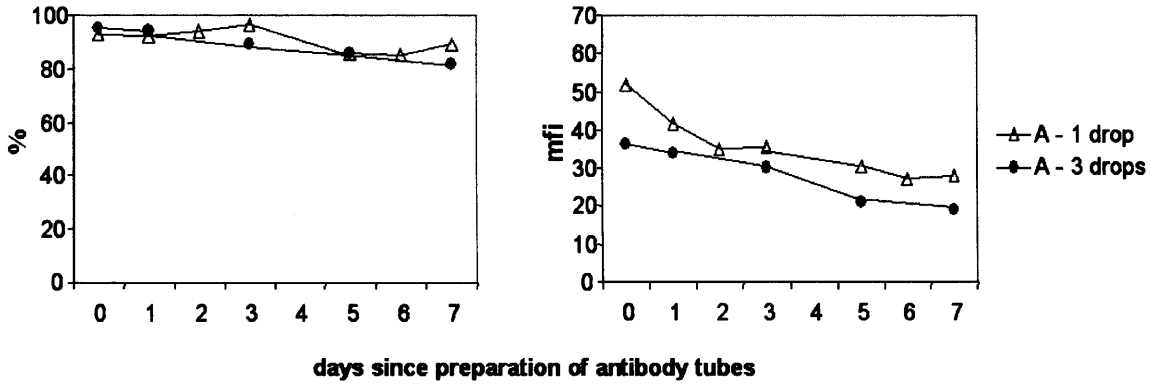

Figure 19: Effect on MHC Class II expression (% and mfi) using 1 drop or 3 drops of blood per assay tube.

Results are shown from one of the healthy volunteers. Blood was added to the antibody containing tubes that were either just prepared, or prepared 1 to 7 days in advance. Analysis of the samples was carried out immediately after addition of blood. There was a fall in MHC Class II expression, particularly in mfi, over time. Use of three drops of blood rather than one gave lower levels of MHC Class II expression.

3.3.1.6 Reproducibility of MHC Class II Staining

Analysis of duplicates from the first 10 patients showed a variability of less than 2% on replicated samples, see Figure 20. There was less than 0.5% variability for re-analysis of the same sample on the same day. This was independent of the level of MHC Class II or HLA DR expression

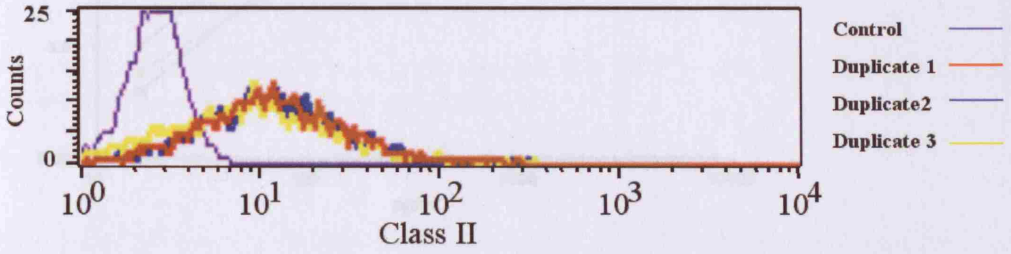

Figure 20:Histogram showing MHC Class II expression for one patient.
Duplicate staining of initial patient samples shows high reproducibility for the technique used.

3.3.2 TGFβ1 ELISA Optimisation

Figure 21 illustrates that there was little difference between the various combinations of different concentrations for capture and detection antibodies. Therefore the

manufacturers' recommendation of 2µls/ml of capture antibody and 0.8 µls / ml of detection antibody were used for all samples.

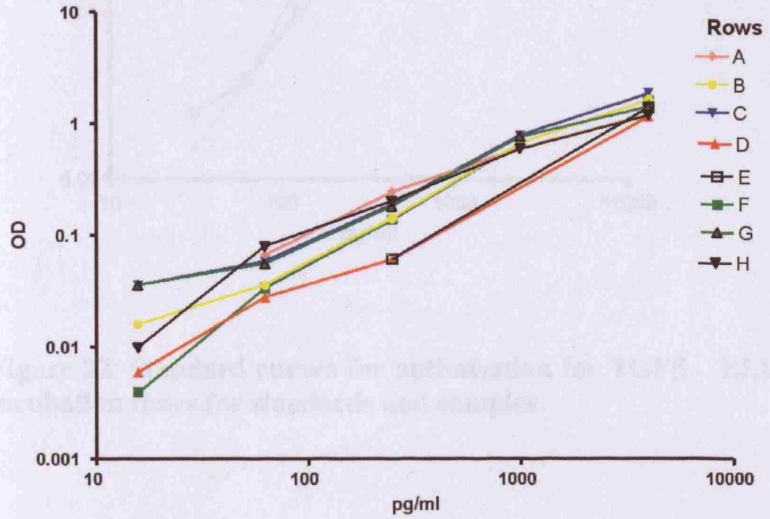

Figure 21: Standard curves for optimisation for TGFβ_1 ELISA, using different concentrations of capture and detection antibody.

See figure 15 for key

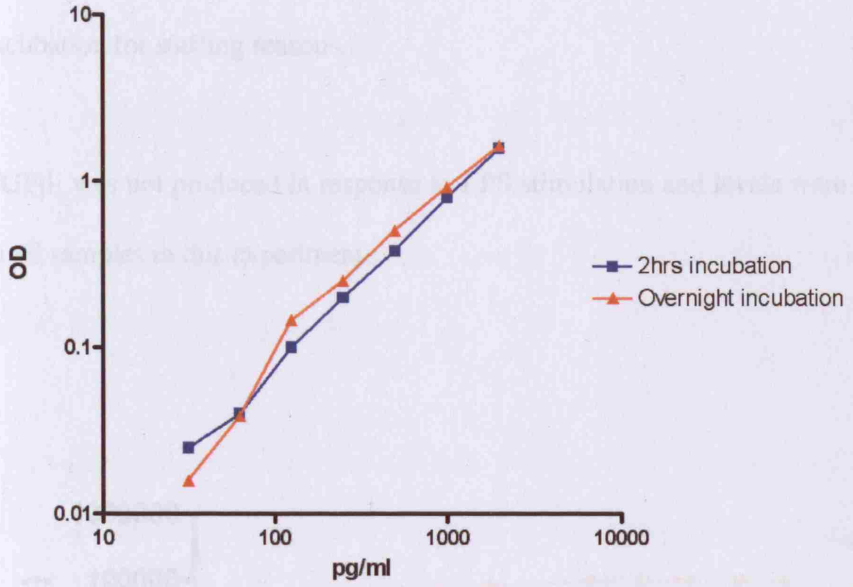

Figure 22: Standard curves for optimisation for TGFβ_1 ELISA, using different incubation times for standards and samples.

As Figure 22 shows there was little difference in results obtained between the two incubation times so a 2-hour incubation period was used for study samples.

The highest coefficient of variation was 13 for intra-plate precision (n=6) and 17 for interplate precision (n= 6). The sensitivity of the assay was 3 pg/ml. Samples and standards were run in duplicate.

3.3.3 Optimisation Of Whole Blood Stimulation With LPS

Figure 23 shows the production of cytokines at different time points from one individual. Peak levels of TNFα, IL1β, IL6, IL-8, IL-10 and IL-12 were produced by

12 hours and maintained until 24 hours. 24 hours was chosen as the optimal length of incubation for staffing reasons.

$TGF\beta_1$ was not produced in response to LPS stimulation and levels were undetectable in all samples in this experiment.

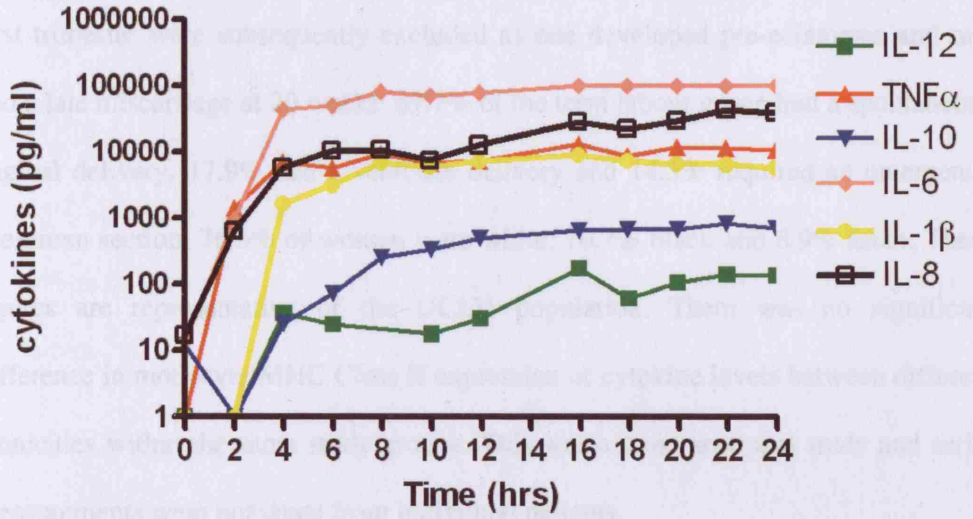

Figure 23: Production of cytokines at varying time intervals following incubation of whole blood with 100ng/ml of blood of LPS.

TNFα, IL1β, IL8 and IL6 all peaked by 6 hours of incubation and remained elevated by 24 hours. IL-12 was not greatly elevated but peaked by 4 hours and remained at a constant level until 24 hours. IL-10 reached its peak by 20 hours and was still elevated at 24 hours.

3.3.4 Patient Characteristics and Outcomes

Once optimal conditions for monocyte MHC Class II staining and analysis, $TGF\beta_1$ ELISA and LPS stimulation had been determined, patient samples were collected. 126 patients were studied over 21 months. An additional 6 patients were approached but declined to participate in the study.

The patient characteristics are summarised in Table 6. Two patients recruited in the first trimester were subsequently excluded as one developed pre-eclampsia and one had a late miscarriage at 20 weeks. 69.7% of the term labour group had a spontaneous vaginal delivery, 17.9% had a ventouse delivery and 14.3% required an emergency caesarean section. 76.5% of women were white, 10.7% black and 8.9% asian. These figures are representative of the UCLH population. There was no significant difference in monocyte MHC Class II expression or cytokine levels between different ethnicities within the same study groups. This was a cross sectional study and serial measurements were not taken from individual patients.

Patient groups	n	Maternal Age in years median (range)	Parity median (range)	Gestational age in days median (range)
Non pregnant	17	31 (26-42)	0	-
1st trimester	26	34 (24–41)	0 (0 – 1)	79 (71- 83)
2nd trimester	18	33 (23–41)	0 (0-2)	112 (86 – 193)
3rd trimester	13	30 (28 38)	0 (0 –2)	236 (198 – 279)
Term labour	29	32 (22-41)	0 (0-7)	282.5 (259-293)
Elective caesarean	23	36 (25 –40)	1 (0-3)	273 (259 –290)

Table 6: Patient Characteristics

3.3.5 Monocyte MHC Class II Expression Over Course Of Normal Pregnancy Compared To Non-Pregnant Controls

There was no variation in monocyte MHC Class II or HLA-DR expression over the course of normal pregnancy, see

Table 7. There was also no difference between monocyte MHC Class II expression in the non-pregnant group and the pregnant groups. There was no correlation with age or ethnicity.

However there was a significant increase in the intensity of surface expression of HLA-DR of the monocytes of women in the first trimester of pregnancy compared to the non-pregnant controls, median 25.0 compared to 11.7 (Mann Whitney $p<0.001$) see Figure 24.

		Non-pregnant Controls	1st trimester	2nd trimester	3rd trimester	Elective Caesarean Section	Spontaneous Labour
MHC Class II	**%**	99.81 (97.48-99.89)	99.01 (94.74-99.54)	99.45 (91.26-99.94)	98.67 (90.62-99.34)	99.17 (97.45-99.71)	94.94* (82.32-98.19)
	mfi	34.60 (26.55-39.24)	30.65 (24.04- 30.65)	28.64 (19.63-45.32)	29.16 (15.13-37.12)	32.78 (25.54– 46.58)	16.78* (11.51-26.54)
HLA DR	**%**	77.96 (63.28-90.83)	90.3 (72.09 – 90.30)	82.16 (77.69-88.06)	85.79 (59.81-90.10)	84.12 (72.81-93.69)	75.5 (60.10-87.65)
	mfi	11.65 (7.92-14.76)	25.03*** (17.00 – 28.01)	14.79 (11.29-16.51)	13.82 (6.86-17.03)	18.82 (10.84-22.73)	7.91* (5.85-15.2)

Table 7: Median MHC Class II and HLA –DR expression as measured by mfi and % (interquartile ranges) for non-pregnant controls and women in the 1st, 2nd and 3rd trimesters of pregnancy and term elective caesarean section and term spontaneous vaginal elivery.

Key: * = compared to 3rd trimester, Mann Whitney $p<0.05$

*** = compared to non-pregnant controls, Mann Whitney $p<0.001$

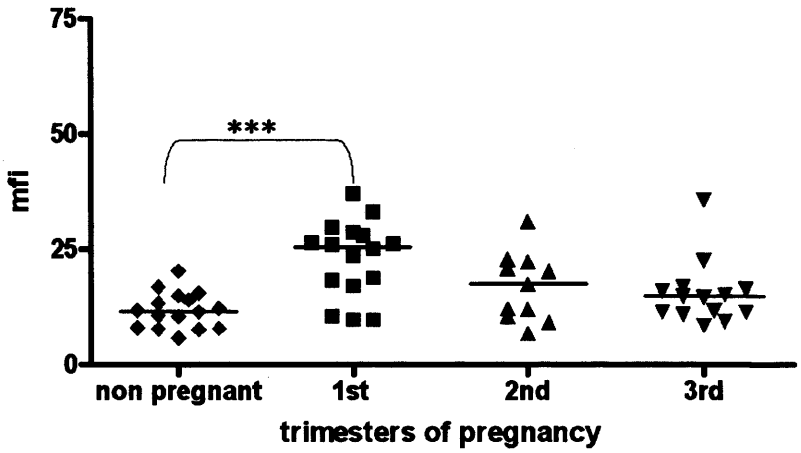

Figure 24: Monocyte HLA-DR expression (mfi) through out the 3 trimesters of pregnancy compared to non-pregnant women.

The mfi of monocytes expressing HLA-DR was significantly higher in the 1st trimester of pregnancy than in the non-pregnant controls (Mann Whitney $p<0.001$).

3.3.6 Cytokine Levels In Maternal Plasma During Pregnancy

There was no change in maternal plasma cytokine levels with gestational age and all cytokines were at very low levels or undetectable, see Table 8.

Cytokine (pg/ml)	1st	2nd	3rd
IL-1β	Undetectable	Undetectable	Undetectable
IL-6	1.0 (1.0- 6.7)	1.0 (1.0-5.4)	Undetectable
IL-8	7.5 (4.8-9.6)	7.8 (6.4-9.9)	6.1 (5.0-7.1)
IL-12	2.0 (1.0-8.4)	1.0 (1.0- 5.8)	1.0 (1.0-3.3)
TNFα	1.0 (1.0-3.9)	1.0 (1.0-4.1)	Undetectable
IL-10	1.0 (1.0-3.7)	3.3 (1-3.9)	1.0 (1.0-3.1)
$TGF\beta_1$	11.9 (2.2-23.7)	8.0 (1.0-24.4)	7.2 (1.0-21.5)

Table 8: Median maternal serum cytokines (pg/ml) during the three trimesters of pregnancy (interquartile range).

3.3.7 Whole Blood Cytokine Production In Response To LPS Stimulation In Normal Pregnancy

There was no significant change over the three trimesters in the cytokine production following 24 hour LPS stimulation of whole blood, see Table 9. Very little IL-12 was produced in all cases.

Cytokine (pg/ml)	1st trimester	2nd trimester	3rd trimester
IL-1β	2801.0 (1447.0-7574.0)	1603.0 (831.0-4157.0)	1917.5 (1116.8-4003.0)
IL-6	59871.0 (48536.0-84351.0)	53126.0 (44269.0-78778.0)	51847.5 (40029.5-75215.5)
IL-8	71782.0 (35262.0-100000.0)	58949.0 (20927.0-100000.0)	61334.0 (37617.8-95086.3)
IL-12	47.0 (34.0-92.0)	51.0 (1-101.0)	36.5 (24.8-80.5)
TNFα	4216.0 (2570.0-8569.0)	1538.0 (1311.0-5215.0)	3292.5 (1633.0-5355.3)
IL-10	701.0 (623.0-1286.0)	651.0 (565.0-1297.0)	688.0 (571.3-1093.8)

Table 9: Median maternal plasma cytokine levels (pg/ml) following whole blood stimulation with LPS, in 1st, 2nd and 3rd trimesters of pregnancy (interquartile range).

3.3.8 Monocyte MHC Class II And HLA-DR Expression Versus Mode Of Delivery

There was no significant difference in the expression of monocyte MHC Class II or HLA-DR between the 3rd trimester group and the elective caesarean group, see Table

7. The elective caesarean section group had a more advanced gestational age than the third trimester group (median gestational age 39 weeks compared to 32 weeks).

There was a significant fall in monocyte MHC Class II expression (% and mfi) and in the intensity of HLA-DR expression (mfi) in the term labour group compared to both the 3rd trimester and the elective caesarean section groups (Mann Whitney $p<0.05$), see Table 7and Figure 25. There was a trend towards a significant reduction in the percentage of monocytes expressing HLA-DR in the spontaneous labour group compared to the 3rd trimester group, median 75.5% and 82.2%, Mann Whitney $p=0.0996$.

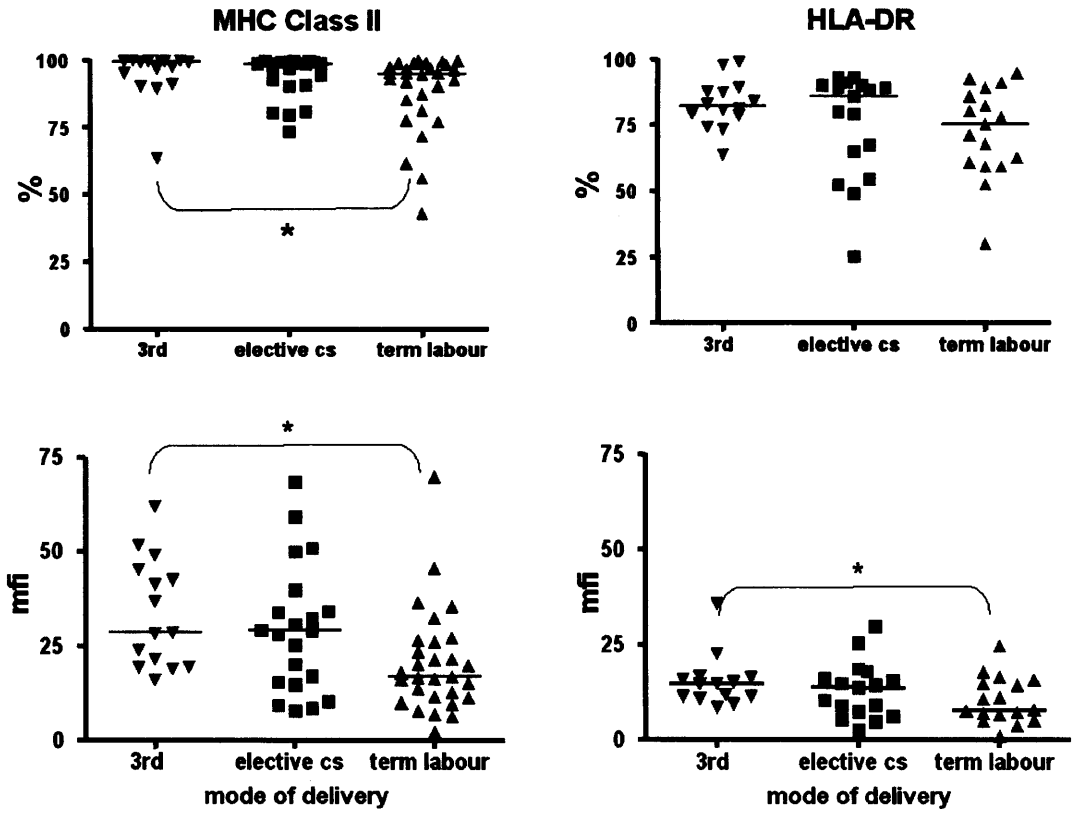

Figure 25: Monocyte MHC Class II and HLA-DR expression in women in 3rd trimester of pregnancy, in spontaneous labour and undergoing pre-labour elective caesarean section at term.

MHC Class II and HLA DR expression was significantly lower in those in labour at term compared to the 3rd trimester group, both in terms of percentage and mfi.

Key: *'elective cs'* – elective caesarean section at term, *'term labour'* – spontaneous labour at term. * Mann Whitney $p<0.05$

Within the spontaneous labour group, there was no difference in MHC Class II or HLA-DR expression and the different modes of delivery.

3.3.9 Maternal Plasma Cytokine Levels During Labour

An increase in IL-8 levels was seen between the 3rd trimester and elective caesarean section group, with a further increase in spontaneous labour (Mann Whitney $p<0.05$), see Table 10. There were significant elevations in maternal plasma IL-6 and IL-8 in the spontaneous labour group compared to both the 3rd trimester and elective caesarean section group, (Mann Whitney $p<0.001$), see Figure 26. IL-1β was undetectable in all three groups and no increase in TNFα during labour was seen.

IL-10 was significantly elevated in the spontaneous labour group compared to the 3rd trimester group and the elective caesarean section group, (Mann Whitney $p<0.001$), see Figure 26. A rise in $TGF\beta_1$ levels was seen between the 3rd trimester and elective caesarean section group (Mann Whitney $p<0.05$), but not in spontaneous labour.

Within the spontaneous labour group, there was no difference in any of cytokine levels between different modes of delivery.

Plasma IL-10 levels did not correlate with monocyte MHC Class II or HLA-DR expression, but IL-6 and IL-8 did show some weak positive correlations with monocyte MHC Class II and HLA-DR expression.

Cytokine	3rd Trimester	Elective Caesarean	Spontaneous Labour
IL-1β	Undetectable	Undetectable	Undetectable
IL-6	Undetectable	1.0 (1.0-4.0)	37.2 (11.8-70.4)*** †
IL-8	6.1 (5.0-7.1)	9.4 (7.8-11.9) †	17.5 (10.6-24.2)*** †
TNFα	Undetectable	1.0 (1.0-3.3)	1.0 (1.0-4.3)
IL-10	1.0 (1.0-3.1)	1.0 (1.0-4.0)	5.5 (3.7-7.7)*** †
TGFβ$_1$	7.2 (1.0-21.5)	60.0 (17.9-116.3)*	28.6 (1-95.0)
IL-12	1.0 (1.0-3.3)	Undetectable	1.0 (1.0-3.5)

Table 10: Median maternal plasma cytokine levels (interquartile range) in 3rd trimester, elective caesarean section and spontaneous labour at term.

Key: * = compared to 3rd trimester, Mann Whitney *p<0.05*.
*** = compared to elective caesarean section, Mann Whitney *p<0.001*
† = compared to 3rd trimester, Mann Whitney *p<0.001*.

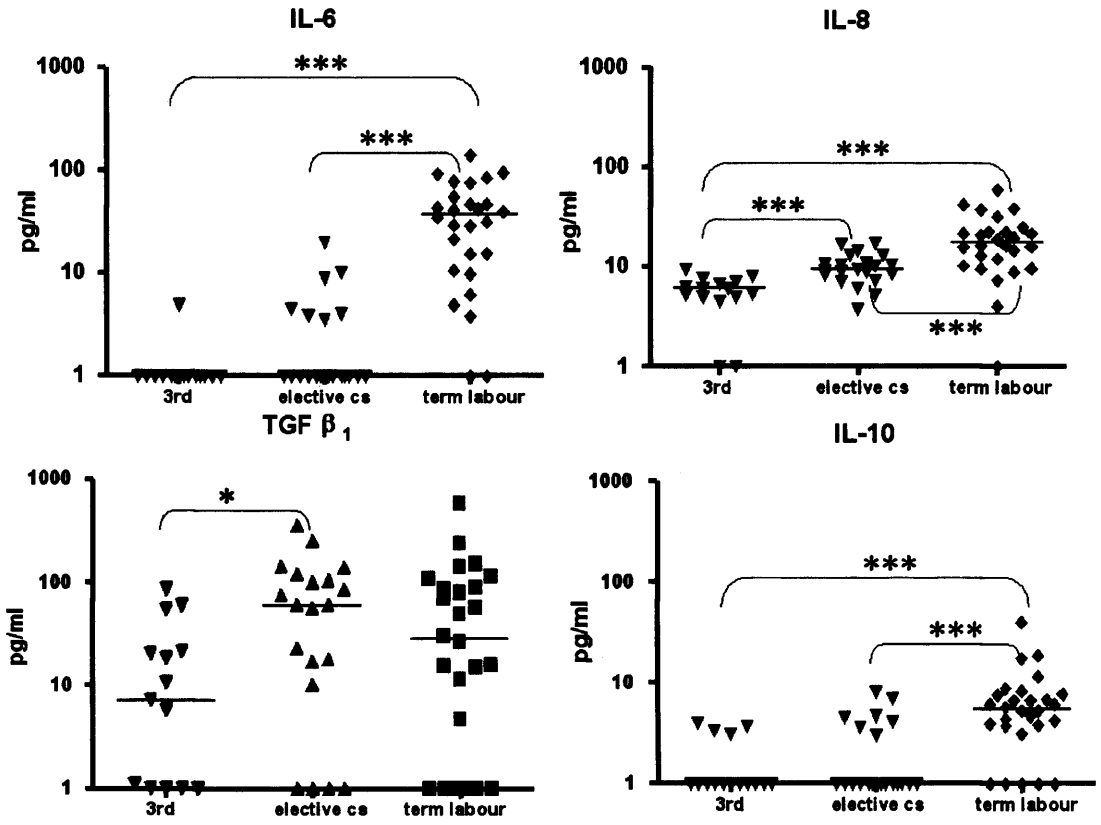

Figure 26: Maternal plasma cytokine levels during 3rd trimester of pregnancy, at elective caesarean section, and during spontaneous labour at term.

Pro-inflammatory cytokines IL-6 and IL-8 levels were significantly higher in the term labour group compared to both elective caesarean section and the third trimester groups. The anti-inflammatory cytokine IL-10 also showed a rise in term labour.

Key: * = Mann Whitney $p<0.05$,
*** = Mann Whitney $p<0.001$.

3.3.10 Whole Blood Stimulation During Pregnancy And Labour

There was a significant reduction in the amount of TNFα produced with LPS stimulation in the spontaneous labour group, compared to the elective caesarean

section group, Mann Whitney *p=0.0465* and a trend towards lower levels of TNFα compared to the 3rd trimester group, Mann Whitney *p= 0.0565,* see Figure 27.

There were no significant differences between the groups in the levels of production of the other cytokines assayed, see Table 11.

Cytokine (pg/ml)	3rd Trimester	Elective Caesarean	Spontaneous Labour
IL-1β	1917.5 (1116.8-4003.0)	1364.7 (700.3-2060.3)	647.5 (311.8-1232.8)
IL-6	51847.5 (40029.5-75215.5)	30673.0 (14057.3-53113.3)	30125.5 (10000.0-50735.5)
IL-8	61334.0 (37617.8-95086.3)	34060.0 (14352.0-98890.0)	42307.0 (10000.0-94574.5)
IL-12	36.5 (24.8-80.5)	22.5 (1-40.75)	5.3 (1-29.8)
TNFα	3292.5 (1633.0-5355.3)	2244.5 (1206.0-5987.3)	714.2* (187.3-1666.3)
IL-10	688.0 (571.3-1093.8)	584.0 (439.3-1213.0)	416.5 (277.3-657.4)

Table 11: Median maternal plasma cytokine levels (pg/ml) following whole blood stimulation with LPS, in 3rd trimester, elective caesarean section and spontaneous labour at term (interquartile range).

Reduced production of TNFα, IL-12 and IL-1β was seen in term labour but only the fall in TNFα levels reached statistical significance. Levels of IL-6 and IL-8 production did seem to fall with increasing gestational age but this did not reach statistical significance.

Key: * = Mann Whitney *p<0.05*.

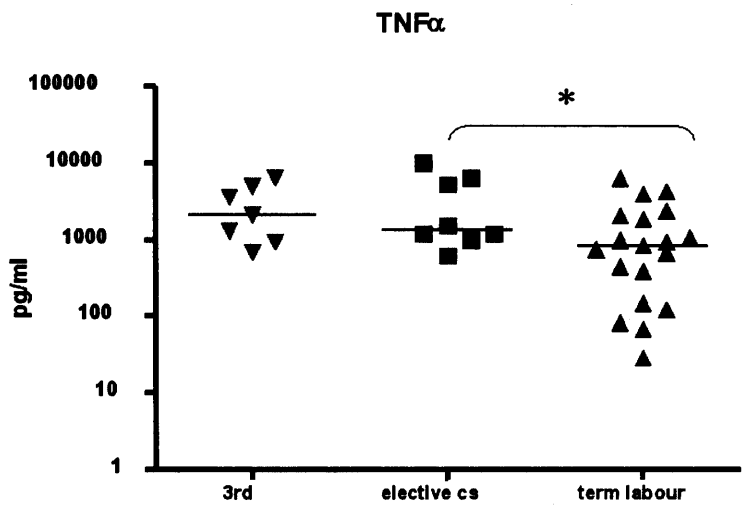

Figure 27: TNFα production (pg/ml) following 24hr whole blood stimulation with LPS in women during the 3rd trimester of pregnancy, elective caesarean section and term labour.

During term labour, maternal blood production of TNFα was reduced compared to those having elective caesarean section.

Key: * = Mann Whitney $p<0.05$.

There were weak correlations between the amount of TNFα produced following whole blood LPS stimulation and maternal monocyte MHC Class II and HLA DR expression, see Table 12. There were no individuals with low levels of maternal monocyte MHC Class II and HLA DR expression that produce high levels of TNFα.

		Spearman's Rank Correlation	p value
MHC Class II expression	**%**	0.315	0.023
	mfi	0.485	<0.001
HLA DR expression	**%**	0.417	0.022
	mfi	0.543	0.002

Table 12: Correlations between maternal monocyte MHC Class II and HLA DR expression and levels of TNFα produced following whole blood LPS stimulation.

One possible explanation for the reduction in cytokine response to whole blood LPS stimulation seen in the term labour group could be due to a fall in the number of circulating monocytes. However there were no significant differences in monocyte count between the elective caesarean section group and the term labour group, meaning these results are not due to dilutional effects (data not shown).

3.4 DISCUSSION

The initial optimisation experiments demonstrate that this study and the studies described in subsequent chapters were feasible. They show that clinical samples could be collected and analysed, giving robust results. The difficulties of the unpredictable timing of sample acquisition were addressed, and a protocol developed that could give reliable, reproducible results by adhering to criteria pertaining to the timing and conditions of sample collection, staining and analysis.

There were several potential problems in this study, which could lead to artefacts. Blood was taken from women in labour, which is a rapidly changing physiological condition. Results obtained may vary depending upon which stage of labour the woman was in when the blood sample was taken. This potential for variation was minimised by ensuring that all samples from women in spontaneous labour were only taken once they were in established labour (regular, painful contractions with cervical change, at a cervical dilatation of 4cm or more). Interoperator variability was removed by having one worker collect, process and analyse all samples. All samples were processed within 30 minutes of collection as the optimisation experiments demonstrated that greater delays led to unreliable results. Any samples that were not processed within 30 minutes were discarded.

There was no difference in monocyte MHC Class II expression between pregnant patients and non-pregnant controls. There is disagreement in the limited literature that exists regarding the effect of pregnancy on monocyte MHC Class II expression (172;173;315). Differences between results may reflect disparity in the patients' gestational age or variations in sample handling or techniques. Attempts were made to minimise these potential discrepancies in this work.

The increase in HLA-DR intensity of expression on the monocyte cell surface, seen in the first trimester compared to non-pregnant controls, may play a role in protecting the mother from infection during the first trimester, prior to the establishment of the maternal circulation through the developing placenta (351). Up until 10 –12 weeks of pregnancy, trophoblast plugs the spiral arteries, preventing close contact of maternal and fetal antigens. This limited exposure of the maternal immune system to fetal

antigens is supported by the fact that free fetal DNA has been found in maternal plasma but not in significant quantities until the end of the first trimester (352). It may be that maternal down regulation of antigen presentation is not required until the placental circulation is established. There were no significant changes detected in the cytokine levels in maternal plasma in the first trimester that could explain the increase in HLA-DR expression seen.

There were no significant changes detected in the cytokine levels in maternal plasma or in levels of cytokine production following *in-vitro* LPS stimulation, across the three trimesters of pregnancy. This demonstrates that despite pregnancy being thought of as a state of immune suppression in order to prevent rejection of the fetus, the mother is still capable of mounting a normal pro-inflammatory response to stimulation (174;316;332).

There was evidence of a pro-inflammatory response in term labour, with increases in plasma IL-8 and IL-6 compared to both the 3rd trimester and elective caesarean section groups. IL-8 is known to play an important role in cervical ripening and dilatation, and membrane rupture (206;223;224). This increase in IL-8 with gestational age and during labour has previously been reported (200). A labour-associated rise in serum IL-6 levels has also been reported by other groups (181;183). IL-6 is an important inflammatory cytokine that increases levels of prostaglandins and metalloproteases, as described in chapter 1. It has been postulated that this rise in IL-6 may be due to the physical exertion of labour as prolonged exercise is associated with a monocytosis and increased monocyte production of IL-6 (353;354). It is important

to remember that IL-6 can have anti-inflammatory properties and so could be part of the anti-inflammatory response in term labour.

The source of these cytokines found in maternal blood is unknown, but trophoblasts are capable of producing IL-1β, IL-6, IL-8, IL-10 and TNFα (281). cDNA array analysis has shown up regulation of IL-8 in term labour (210) and IL-1β, IL-6, IL-8 and TNFα have been found in increased amounts in amniotic fluid at the time of labour (181;184;201-203).

There was also evidence of an anti-inflammatory response to term labour. A rise in plasma IL-10 levels was seen in spontaneous labour group compared to the elective caesarean section group. Increases in $TGF\beta_1$ were seen in the elective caesarean section group compared to the 3rd trimester group (median 60.0pg/ml compared to 7.22pg/ml). This may be due to an increase in gestational age as the median age was 32 weeks and 39 weeks for the 3rd trimester group and the elective caesarean section group respectively. Power *et al.* found maternal plasma levels of $TGF\beta_1$ to increase with gestational age but did not investigate changes with labour (189). We did not find any significant differences in $TGF\beta_1$ levels between the elective caesarean section and spontaneous labour groups.

The most interesting finding in this study was the evidence of altered immune response seen at the time of labour, with a significant fall in monocyte MHC Class II and HLA-DR expression, in conjunction with a reduction in LPS stimulated production of TNFα. These changes have been described as *immune paresis*

(299;355). One other group has also described reduced production of TNFα in women in term labour, compared to 3 months post-partuum (356).

This fall in monocyte MHC Class II expression and alteration in response to *in-vitro* LPS stimulation may be a protective measure by the mother because of the vast antigen exposure she will experience peri-partuum. Downregulating her monocyte MHC Class II expression may protect the mother from an excessive pro-inflammatory response after labour, which has been associated with a poor outcome in sepsis (139).

We were unable to determine the timing of this fall in MHC Class II expression from this study. The fall may precede labour or may be an anti-inflammatory response to the pro-inflammatory phase of labour.

A rise in IL-10 levels was also seen in spontaneous labour group. IL-10 has been shown to downregulate monocyte MHC Class II expression through post translational effects, as discussed in chapter 1 (165). Thus this may be the mechanism by which monocyte MHC Class II expression falls at the time of labour.

However circulating IL-10 levels do not directly correlate with monocyte MHC Class II expression. This lack of correlation has previously been described by other groups (161). IL-10 is only one of many factors that control monocyte MHC Class II expression, therefore it is probably not surprising that a direct correlation between IL-10 levels and monocyte MHC Class II expression was not seen here. It may be that a single measurement of circulating IL-10 levels is not sufficient to adequately describe the complex nature of cytokine balance during pregnancy and labour.

This study provides evidence that a reduction in monocyte MHC Class II expression is seen in spontaneous labour at term and these monocytes have altered function in response to LPS stimulation. There was also evidence of an anti-inflammatory response with an increase in IL-10 levels. A pro-inflammatory response to labour was seen with increases in maternal plasma IL-6 and IL-8.

This reduction in monocyte MHC Class II expression may reflect changes in antigen presentation abilities and in the cytokine milieu of the labouring woman. This may alter her susceptibility to infections in the peripartum period. The maternal immune response to preterm labour and PPROM will be investigated in chapter 4.

CHAPTER 4 – MATERNAL CYTOKINE BALANCE IN PRETERM LABOUR

4.1 INTRODUCTION

Infection, and the inflammatory response it induces, has been found to play a role in preterm labour and PPROM, as discussed in chapter 1(22;29;242;357).

Evidence of a pro-inflammatory response can be found in amniotic fluid of women in preterm labour, especially if infection is present (28;208;256). Studies of the pro-inflammatory response in maternal circulation reveal a less consistent story. Some authors have demonstrated a pro-inflammatory response in maternal blood (183;246;247) but others have failed to find this (249;250). The results of studies looking at the maternal anti-inflammatory response are even less consistent (193;226).

This chapter aims to explore monocyte MHC Class II expression, and relate this to maternal plasma cytokine levels and the response of the maternal system to LPS stimulation in preterm labour and in pre-term pre-labour rupture of membranes.

4.2 METHODS

4.2.1 Patient Groups

Two groups of patients were recruited for this study:

1. Preterm labour (PTL). Threatened preterm labour was defined as regular painful contractions with cervical change at less than 32 weeks completed gestation.

2. Preterm pre-labour rupture of the membranes (PPROM) at less than 32 weeks completed gestation. PPROM was diagnosed by a suggestive history together with evidence of liquor draining on speculum examination and /or oligohydramnios on ultrasound scan.

These women were compared to the groups of women described in chapter 3. These included women in term labour. From the healthy pregnant women recruited from antenatal clinic, a reference group (**controls**) was selected, who had similar gestational ages to the PTL and PPROM groups.

4.2.2 Clinical Care

Women admitted with threatened preterm labour were managed in accordance with the hospital protocols. A diagnosis of PPROM was confirmed on speculum examination and with ultrasound assessment of liquor volume.

Routine care included a clinical assessment, including fetal cardiotocography and vaginal examination. Cervical and high vaginal swabs were taken and sent for microscopy, culture and sensitivities, and for PCR for *Chlamydia trachomatis*. A urine sample was tested for evidence of proteinuria or infection. A full blood count and CRP were taken.

An ultrasound examination was performed to assess fetal size and presentation, liquor volume, placental site and cervical length and dilatation. Serial fetal wellbeing scans were performed for those who did not deliver immediately.

A course of erythromycin was given for those with PPROM. The mother was monitored for signs of infection (pyrexia, offensive liquor draining, uterine tenderness, elevated CRP or white cell count). If there were concerns about chorioamnionitis, the pregnancy was delivered as appropriate.

Women were offered 1 course of antenatal betamethasone from 24 weeks onwards. This was given as 2 doses of 12mg 12 hours apart. Any evidence of infection was managed with appropriate antibiotics. Atosiban was given in the absence of antepartuum haemorrhage or sepsis, in order to enable steroids to be given.

4.2.3 Outcome Measures

For the purposes of this study, the following outcome measures were recorded:

- **Maternal history** - demographics, past obstetric history, current obstetric history including date of last menstrual period, early dating by ultrasound, maternal drugs used.
- **Evidence of clinical chorioamnionitis** – details such as maternal pyrexia >38 ^{0}C, uterine tenderness, offensive discharge, maternal white cell count and C reactive protein level were recorded.
- **Evidence of histological chorioamnionitis –** routine histological assessment of placentas in UCLH pathology department was performed. Chorioamnionitis was diagnosed if there was polymorph infiltration of the amnion and chorion. Funisitis was defined as polymorph infiltration of the umbilical cord vessels, through the endothelium and beyond.
- **Microbiological evidence of maternal / fetal infection** – as part of routine clinical management, results from swabs taken from maternal cervix, vagina and placenta and neonatal blood cultures were collected.
- **Pregnancy outcome** – duration of rupture of membranes, spontaneous onset of labour, mode of delivery, birth weight, gestational age, and gender of neonate(s), apgar scores, umbilical cord pH, and admission to neonatal unit, time and cause of neonatal death.

4.2.4 Study Design & Sample Collection

Blood samples were collected on admission from women who consented to take part. Repeat samples were taken when further clinical samples were taken and in labour if possible. Blood collection and handling has been described in 2.5.

4.2.5 Monocyte MHC Class II Expression & Cytokine Assays

The following assays were performed on all blood samples collected:

- Monocyte MHC Class II and HLA-DR expression, as described in 2.6.1.
- Cytokine assays, as described in 2.6.2 and 2.7
- Whole blood LPS stimulation studies, as described in 2.8

4.3 STATISTICS

MHC Class II and HLA-DR expression were expressed as percentages, and not normally distributed. The numbers in each group for the cytokine assays were small as so all comparisons between groups were performed with Mann Whitney test. Correlations were performed with Spearman's rank analysis. Analysis was performed with the Statistical Package for the Social Sciences (version 12.0 SPSS, Chicago, IL.).

4.4 RESULTS

4.4.1 Patient Characteristics

Over the 22-month study period, 24 women with preterm prelabour rupture of the membranes and 14 women in preterm labour at less than 32 completed weeks of gestation were studied. Another 3 women were approached but declined participation.

	PTL	PPROM	Controls
N	14	24	15
Maternal Age in years median (range)	33.5 (20-39)	31.5 (22-43)	31 (21-41)
Parity median (range)	0.5 (0-2)	0 (0-6)	1 (0-2)
Ethnicity % (n)			
White	57.1 (8)	62.5 (15)	53.3 (8)
Black	21.3 (3)	20.9 (5)	13.3 (2)
Asian	7.1 (1)	12.5 (3)	6.7 (1)
Other	14.2 (2)	4.2 (1)	13.3 (2)

Table 13: Patient Demographics

4.4.1.1 Previous Obstetric History

Six women in the PPROM group had a previous history of preterm delivery compared to one in the PTL group. Two women in the PPROM had previously been diagnosed with a history of cervical incompetence and had an elective cervical cerclage inserted in the current pregnancy. Three women in PTL group underwent cervical cerclage, one as a rescue suture at 21 weeks, and two as elective procedures in early pregnancy. One of these was due to a history of preterm delivery and one was inserted due to uterus didelphys.

4.4.2 Outcome

4.4.2.1 Antenatal Steroids

85% of PTL group received one dose of antenatal betamethasone but four delivered prior to the second dose, see Table 14.

MEDICATION	PTL %(n)	PPROM %(n)
Antibiotics	57.1 (8)	95.8 (erythromycin75%)
Tocolysis	14.3 (2)	4.2 (1)
Steroid course completed	57.1 (8)	75 (18)
Bethamethasone	57.1 (8)	66.7 (16)
Dexamethasone	0	8.3 (2)

Table 14: Antenatal Medications

4.4.2.2 Gestational Age At Delivery

The median gestational age at delivery was 28 weeks and 26 weeks for PTL and PPROM groups respectively. The median interval from rupture of membranes to delivery for the PPROM group was 6 days with a range of 2 to 62 days, see Table 15 and Figure 28. There was a high caesarean section rate in the control group; the overall caesarean section rate for UCLH was around 24% over the same time period; the difference is probably because of small numbers in the study. The elective caesarean sections were for breech or for previous caesarean section. The emergency caesarean sections were for failure to progress or fetal distress.

	Preterm Labour % (n)	PPROM % (n)	Controls % (n)
Presentation			
Cephalic	64.3 (9)	62.5 (15)	93.3 (14)
Breech	28.6 (4)	29.3 (7)	6.7 (1)
Transverse	7.1 (1)	8.3 (2)	0
Mode of delivery			
SVD	64.3 (9)	77.3 (17)	33.3 (5)
Instrumental	14.3 (2)	0	20 (3)
Emergency CS	21.4 (3)	22.7 (5)	26.7 (4)
Elective CS	0	0	20 (3)
Median Gestation age at delivery – days (range)	202.5 (157-232)	184 (138-238)	280.5 (274-289)
Multiple births	4 sets DCDA twins	4 sets DCDA twins	0
Male gender % (n)	57.1 (8)	50 (11)	46.7 (7)
Stillbirths	0	2 (1 set of twins)	0
Neonatal deaths	3	5	0
Clinical chorioamnionitis	35.7 (5)	12.5 (3)	0
CRP >10mg/L	28.6 (4)	33.3 (8)	0

Table 15: Patient Delivery Outcomes

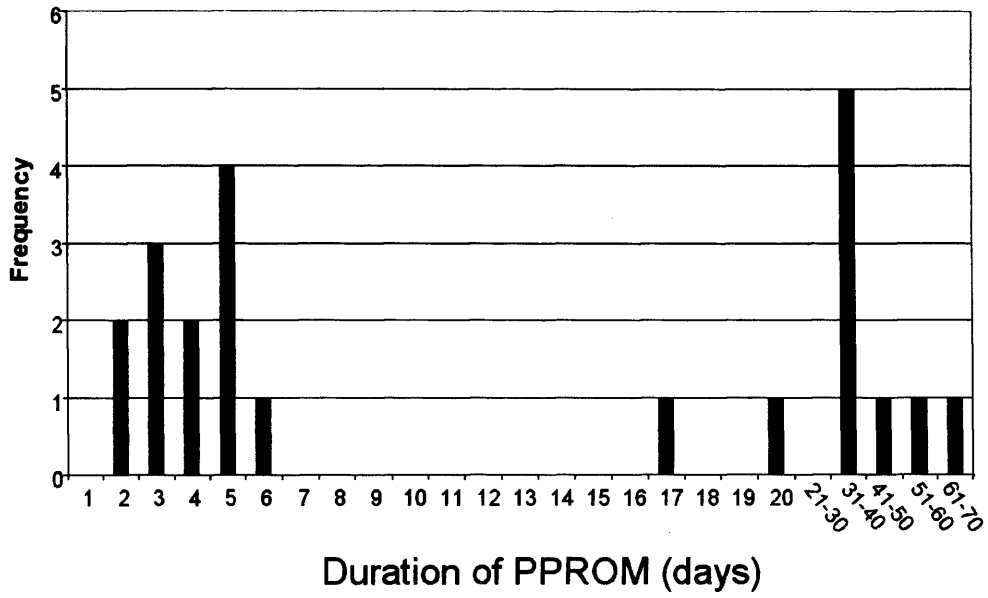

Figure 28: Interval from rupture of membranes to delivery for PPROM group.

4.4.2.3 Placental Histology

Overall 42.8% and 58.3% of the PTL and PPROM groups had histological evidence of chorioamnionitis, and 35.7% and 12.5% had clinical chorioamnionitis, see Table 15 and Table 16. Several different organisms were isolated from placental microbiology swabs, such as Group B *streptococcus*, *Listeria sp.*, *Candida albicans* and *Mycoplasma hominis.* All women with clinical chorioamnionitis were subsequently found to have evidence of histological chorioamnionitis. In contrast, 65% of women with histological chorioamnionitis did not have a clinical diagnosis of chorioamnionitis

PLACENTAL HISTOLOGY	PTL% (n)	PPROM % (n)
Normal	42.9 (6)	16.7 (4)
Chorioamnionitis	14.3 (2)	25 (6)
Funisitis	28.6 (4)	33.3 (8)
Infarction +/- excess fibrin/synctial knots	7.1 (1)	4.2 (1)
Not done	7.1 (1)	20.8 (5)

Table 16: Placental Histology Findings

4.4.3 Monocyte MHC Class II Expression In PTL And PPROM

4.4.3.1 PTL & PPROM Are Associated With Reduced Expression Of Maternal Monocyte MHC Class II

Monocyte MHC Class II and HLA-DR was significantly lower in both PTL and PPROM than controls, see Table 17. A lower percentage of monocytes expressed MHC Class II and HLA-DR in these groups compared to women in labour at term, see Figure 29.

		PTL	PPROM	Controls
MHC Class II	**%**	84.06 *** (69.35-92.86)	91.42 *** (83.09-98.98)	99.69 (99.17-99.89)
	mfi	13.81 * (6.97-25.63)	19.99 ** (11.01-30.23)	32.78 (23.93-43.32)
HLA-DR	**%**	41.65 *** (32.04-68.68)	56.25 * (43.63-76.71)	79.38 (73.45-89.18)
	mfi	5.39 ** (3.34-8.55)	7.90 ** (5.42-10.75)	12.08 (10.46-17.47)

Table 17: Median monocyte MHC Class II and HLA-DR expression (interquartile range) in women in PTL, PPROM and healthy controls.

Key: * = Mann Whitney $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

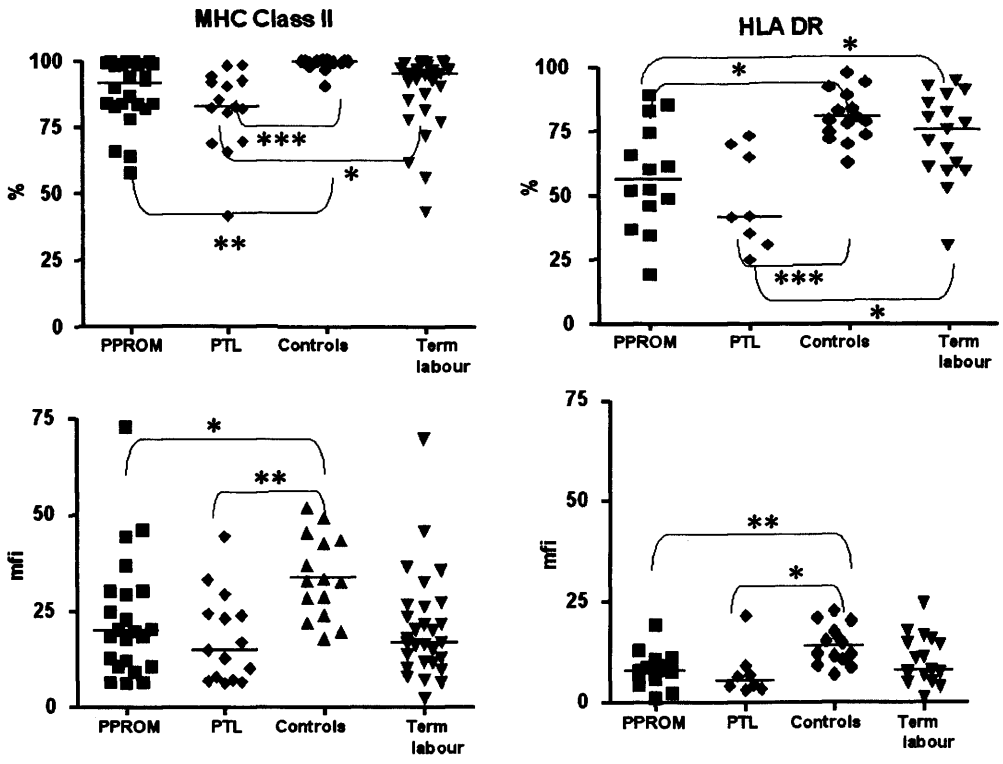

Figure 29: Monocyte MHC Class II and HLA DR expression in women with PTL, PPROM, controls and term labour.

A fall in monocyte MHC Class II and HLA DR was seen in PTL and PPROM compared to controls. The fall in the percentage of monocytes expressing MHC Class II and HLA-DR may be slightly more pronounced than the decrease seen in term labour.

Key: * = Mann Whitney *p<0.05*, ** = *p<0.01*, *** = *p<0.001*.

4.4.3.2 No Correlation Between Gestational Age & Maternal Monocyte MHC Class II Expression

There was no correlation between ethnicity, maternal age or gestational age, and monocyte MHC Class II or HLA-DR expression for any of the groups, see Figure 30.

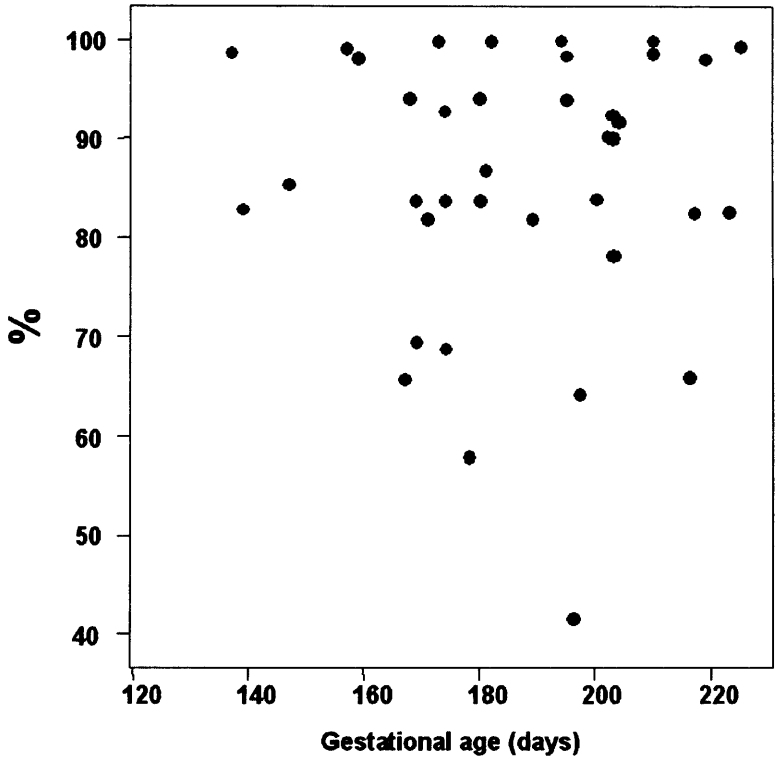

Figure 30: Scatter plot of monocyte MHC Class II expression (%) against gestational age of women in PTL and PPROM groups.

There was no correlation between monocyte MHC Class II expression and gestational age, Spearman's rank coefficient 0.035, p=0.833.

4.4.3.3 Interval To Delivery Is Inversely Related To Monocyte MHC Class II Expression

There was a significant correlation between interval from admission to delivery and monocyte MHC Class II expression for those with PPROM or with threatened PTL, see Figure 31.

The median monocyte MHC Class II expression was 94.04% (interquartile range 85.05-99.16) and 23.92 mfi (16.49-44.92) for those who delivered more than 7 days after admission and 82.80% (69.01-93.77) and 13.86 mfi (6.83-23.36) for those who delivered within 7 days, Mann Whitney $p<0.05$.

There was a trend towards lower monocyte HLA-DR percentage expression in those who delivered within 7 days, median 45.90% (interquartile range 34.33-65.00) compared to 73.19% (48.71-83.05) in those who delivered >7 days after admission, Mann Whitney $p=0.053$.

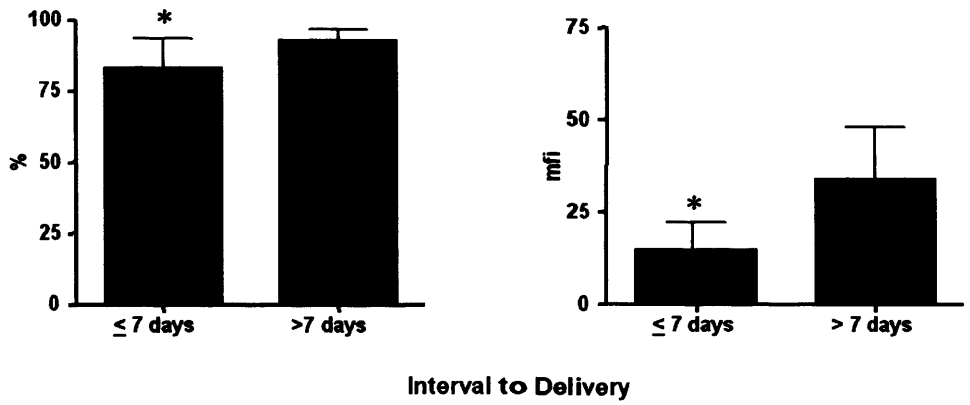

Figure 31: Monocyte MHC Class II expression and interval from admission to delivery in the PTL and PPROM groups.

Median and interquartile range shown.

Key: * = Mann Whitney $p<0.05$

In the PPROM group, those who delivered within 7 days of rupture of membranes also had significantly lower monocyte MHC Class II expression than those who delivered more than 7 days after membrane rupture. Median monocyte MHC Class II expression was 83.73% (interquartile range 72.06-96.09) vs 98.65% (86.89-99.87), Mann Whitney $p<0.05$. The intensity of staining was also reduced, mfi 17.78 (7.92-21.62) vs 30.23 (18.43-46.14), Mann Whitney $p<0.05$.

Those who delivered within 7 days had a significantly higher CRP level than those who did not, median 25mg/L (interquartile range 15.4-60.9) vs 1.6mg/L (0.0-11.9), Mann Whitney $p<0.01$.

4.4.3.4 Maternal MHC Class II Expression & Placental Histology

There was no difference in monocyte MHC Class II or HLA-DR expression between women with histological chorioamnionitis and those without. There was also no correlation between CRP levels and histological chorioamnionitis. However clinical chorioamnionitis was associated with a significantly lower percentage of monocytes expressing MHC Class II, see Figure 32. Median MHC Class II % for those with clinical chorioamnionitis was 76.20% (interquartile range 64.61-89.06) vs 92.16% (82.66-98.66) for those without, Mann Whitney $p<0.05$. CRP correlated positively with clinical chorioamnionitis, Mann Whitney $p<0.05$. There was no difference in monocyte HLA-DR expression between these groups.

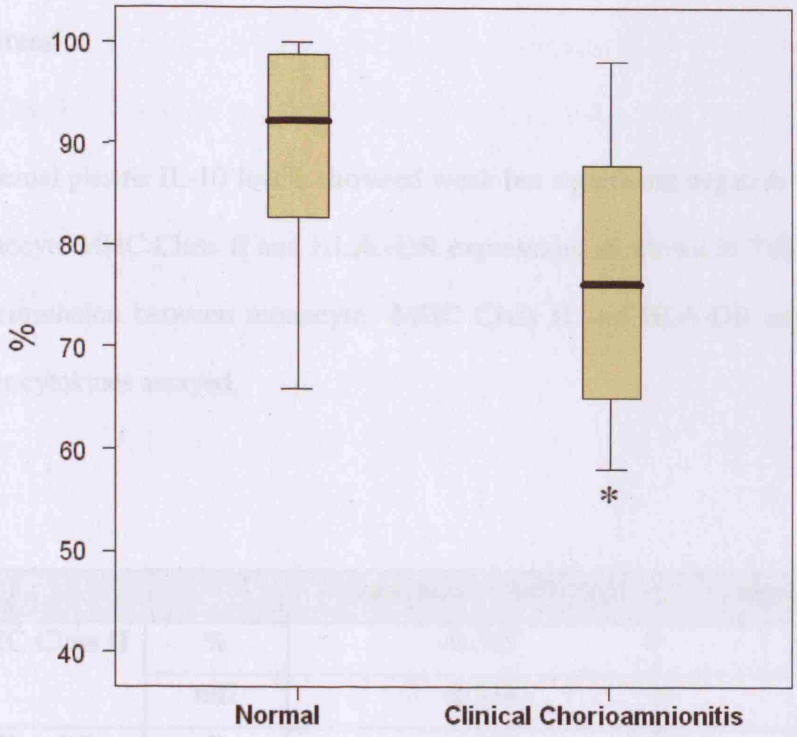

Figure 32: Median monocyte MHC Class II expression (%) in PPROM and PTL groups, with and without clinical chorioamnionitis.

Median, range and interquartile range shown. * Mann Whitney $p<0.05$.

4.4.3.5 CRP Levels & Monocyte MHC Class II Expression

There was a negative correlation between C reactive protein (CRP) level and monocyte MHC Class II expression, Spearman's rank coefficient 0.499, $p<0.05$ for % and –0.726, $p<0.01$ for mfi. There was also a negative correlation between CRP and the intensity of monocyte HLA-DR staining, Spearman's rank coefficient 0.900, $p<0.05$.

4.4.3.6 IL-10 Negatively Correlates With Maternal Monocyte MHC Class II Expression

Maternal plasma IL-10 levels showed weak but significant negative correlations with monocyte MHC Class II and HLA-DR expression, as shown in Table 18. There was no correlation between monocyte MHC Class II and HLA-DR expression and the other cytokines assayed.

		Correlation Coefficient	Significance (p)
MHC Class II	**%**	-0.365	0.026
	mfi	-0.534	0.001
HLA-DR	**%**	-0.520	0.016
	mfi	-0.563	0.008

Table 18: Correlation of maternal plasma IL-10 levels and monocyte MHC Class II and HLA-DR expression in PPROM and PTL groups

4.4.4 Maternal Plasma Cytokine Levels In PTL & PPROM

4.4.4.1 Reduced Pro-Inflammatory Response Compared To Term Labour

There was an increase in levels of IL-6 in PPROM and PTL above that seen in gestational age matched controls, but not reaching the levels seen in term labour, median IL-6 levels 7.1 pg/lm, 3.8pg/ml and 37.2pg/ml in PTL, PPROM and term labour respectively, see Figure 33.

The increase in IL-8 seen in term labour was not found in PTL or PPROM, Mann Whitney $p<0.01$. No significant difference was found between the levels of IL-12, IL-1β and TNFα between the PTL, PPROM and control groups, see Table 19.

Cytokines (pg/ml)	PTL	PPROM	Controls	Term Labour
IL-1β	1.0 (1.0-34.7)	Undetectable	Undetectable	Undetectable
IL-6	7.1 (1.0-28.7)** †	3.8 (1.0-14.1)*** †	Undetectable	37.2 (11.8-70.4)
IL-8	7.4 (4.6-16.1)**	7.8 (4.7-14.0)**	6.7 (4.7-7.8)	17.5 (10.6-24.2)
IL-12	1.0 (1.0-7.3)	1.0 (1.0-3.3)	1.0 (1.0-5.5)	1 (1.0-3.5)
TNFα	1.0 (1.0-3.4)	Undetectable	1.0 (1.0-3.2)	1 (1.0-4.3)
IL-10	1.0 (1.0-5.1)*	Undetectable***	Undetectable	5.5 (3.7-7.7)
TGFβ$_1$	1.0 (1.0-21.6)**	1.0 (1.0 –17.5)**	1.0 (1.0-8.6)	28.6 (1.0-95.0)

Table 19: Median maternal plasma cytokine levels in women in PTL, with PPROM, controls and term labour groups.

Key: * compared to term labour, Mann Whitney $p<0.05$
** compared to term labour, Mann Whitney $p<0.01$
*** compared to term labour, Mann Whitney $p<0.001$
† compared to gestation matched controls, Mann Whitney $p<0.05$

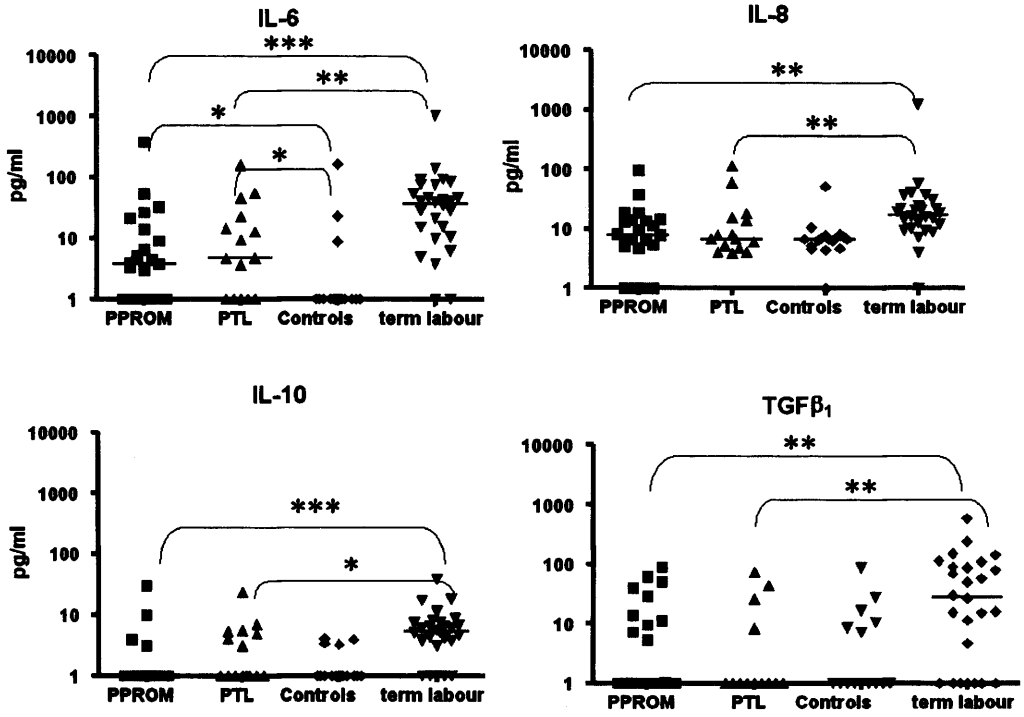

Figure 33: Cytokine levels in maternal blood in patients with PPROM, PTL, Term Labour and Controls.

A pro-inflammatory response was seen in PPROM and PTL, with increases in levels of IL-6 although these did not reach the levels seen in term labour and no increase in IL-8 was observed. No evidence of an anti-inflammatory cytokine response was seen in PTL or PPROM, with no increases in IL-10 or $TGF\beta_1$, in contrast to term labour.

Key: * compared to term labour, Mann Whitney $p < 0.05$
** compared to term labour, Mann Whitney $p < 0.01$
*** compared to term labour, Mann Whitney $p < 0.001$

4.4.4.2 Lack Of Anti-Inflammatory Response In PTL & PPROM

The increase in maternal plasma IL-10 seen in term labour was not found in either the PTL or PPROM groups, Mann Whitney $p<0.05$ for PTL and term labour, $p<0.001$ for PPROM and term labour, see Figure 33. $TGF\beta_1$ levels were also significantly higher in term labour than in PTL and PPROM, Mann Whitney $p<0.01$.

4.4.4.3 Positive Correlation Between IL-10, IL-6 And CRP Levels

There was no correlation between gestational age, clinical or histological chorioamnionitis and cytokine levels for any of the groups. There were positive correlations between IL-12, TNFα, IL-10 and IL-6 and CRP levels, $p<0.05$.

4.4.5 Maternal Cytokine Production With LPS Stimulation

4.4.5.1 Decreased Responsiveness To LPS Stimulation In Preterm Labour

Whole blood stimulation in the PTL group resulted in significantly less IL-6, IL-1β, IL-8 and TNFα production than the Controls or the term labour group, see Figure 34. The PTL group also produced significantly less IL-1β and TNFα than the PPROM group, see Table 20.

Cytokines (pg/ml)	PTL	PPROM	Controls	Term Labour
IL-1β	79.7**†♣ (1.0-403.5)	1380.3 (1.0-3900.4)	1284.0 (1017.0-1484.0)	647.5 (311.8-1232.8)
IL-6	8659.5*†♣ (2523.5-23194.0)	26347.5 (9607.3-55257.3)	48698.0 (42067.0-65456.0)	30125.5 (10000.0-50735.5)
IL-8	10000**† (5080.2-24707.8)	25874.0 (9561.0-67216.5)	57211.0 (38200.0-64326.0)	42307.0 (10000.0-94574.5)
IL-12	1.0 (1.0-4.3)	3.0 (1.0- 21.0)	38.5 (18.0-46.5)	5.3 (1-29.8)
TNFα	324.5*†♣ (36.8-604.8)	1015.1 (239.0 –3082.8)	1425.0 (1124.0-3284.0)	714.2 (187.3-1666.3)
IL-10	259.1 (132.3-537.0)	375.5 (184.3-911.3)	608.0 (533.0-755.0)	416.5 (277.3-657.4)

Table 20: Median maternal cytokine levels following whole blood LPS stimulation of whole blood in the PTL, PPROM, Controls and the Term Labour groups.

Key:
- * compared to term labour, Mann Whitney $p < 0.05$
- ** compared to term labour, Mann Whitney $p < 0.01$
- *** compared to term labour, Mann Whitney $p < 0.001$
- † compared to controls, Mann Whitney $p < 0.05$
- ♣ compared to PPROM, Mann Whitney $p < 0.05$

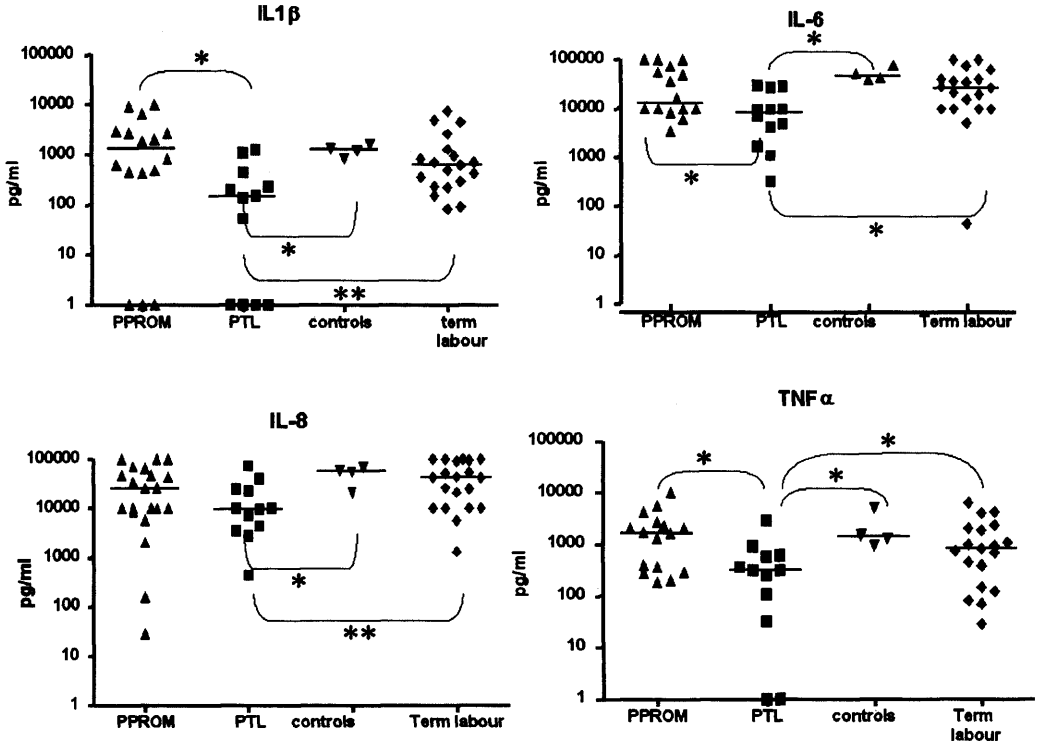

Figure 34: Maternal production of IL-1β, IL-6, IL-8 and TNFα following LPS stimulation of whole blood in the PTL, PPROM, Controls and Term Labour groups.

There was decreased production of IL-1β, IL-6, IL-8 and TNFα in the PTL group compared to the other groups studied,

Key: Mann Whitney * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.4.5.2 Similar Anti-Inflammatory Response To LPS Stimulation In PTL & PPROM

A similar anti-inflammatory response to LPS was seen across all groups with no significant differences in the amounts of IL-10 produced, see Table 20.

4.4.5.3 Lack Of Correlation Between Gestational Age & Cytokine Production

There was no correlation between gestational age, CRP levels, interval to delivery, clinical or histological chorioamnionitis and levels of cytokine production for any of the groups. There was no correlation between plasma IL-10 levels and the level of LPS stimulated TNFα production.

4.4.5.4 Correlation Between Maternal Monocyte MHC Class II Expression & Cytokine Production In Response To LPS Stimulation

There were weak correlations between the amount of cytokine response to whole blood LPS stimulation and maternal monocyte MHC Class II expression, see Table 21.

Cytokine	Spearman's Rank Coefficient	p-value
IL-1β	0.210	0.046
IL-6	0.374	0.0001
IL-8	0.344	0.001
IL-12	0.387	0.0001
TNFα	0.344	0.001

Table 21: Correlation between monocyte MHC Class II (%) and LPS stimulated cytokine production in the PTL, PPROM, gestational age matched controls and the term labour groups.

There were significant positive correlations between LPS stimulated production of IL-12, TNFα, IL-6, IL-1β, IL-8 and monocyte MHC Class II expression (%).

There were no significant differences between the monocyte count in the PTL, PPROM and term labour groups that could account for the variation in cytokine response to whole blood LPS stimulation seen between groups.

4.5 DISCUSSION

The difficulties of blood collection and handling, described in chapter 3, were increased in this study, due to the nature of the patients recruited. Patients with PPROM or in PTL often delivered unexpectedly and rapidly during the night. It was therefore difficult to obtain blood samples and process them within 30 minutes. These factors lead to relatively small numbers of patients due to the large amount of work involved to recruit and collect samples from each case. There were 8 sets of twins in

the PTL and PPROM groups. These pregnancies may differ from singleton pregnancies in the aetiology of PTL or PPROM. In future work it would be ideal to be able to divide the preterm deliveries into singleton and multiple pregnancies for separate analyses. Due to the small numbers in this pilot work, this was not possible in this instance.

The optimisation experiments described in chapter 3 aimed to minimise artefacts in the results. However as preterm labour is a rapidly changing state, the effects of variations in the timing of blood sampling cannot be entirely excluded.

The plasma cytokine assays show evidence of an inflammatory response in the PTL and PPROM groups compared to gestation matched controls, but this was not as pronounced as the response seen in term labour. IL-6 levels were raised, but not IL-8 levels. No elevations in TNFα were seen, in agreement with other authors (249). As IL-6 can have pro- and anti-inflammatory effects, it is difficult to assess whether this data demonstrate a pro-inflammatory or an anti-inflammatory response. No rise in IL-10 levels was seen, in contrast to term labour.

There is some discrepancy in the literature regarding the maternal systemic response in PTL and PPROM. Several groups have demonstrated a pro-inflammatory cytokine response to PTL and PPROM (183;246;247). Others have not reported any increase in maternal serum pro-inflammatory cytokine levels in PTL (249;250). There are very little data regarding anti-inflammatory cytokines and PTL or PPROM, with some conflicting data regarding levels of IL-10 in amniotic fluid (193;226).

The most striking finding of this study was the fall in monocyte MHC Class II expression in PTL and PPROM compared to gestation-matched controls. This fall was even greater than the decrease in monocyte MHC Class II expression seen in term labour, as described in chapter 3.

This fall in monocyte MHC Class II expression correlated with the risk of imminent delivery, as a lower median MHC Class II was seen in those who delivered within 7 days from admission. Therefore changes in the maternal immune system are occurring in the days preceding preterm delivery and may play a role in its aetiology. The drop in MHC Class II expression was independent of gestational age.

As monocyte MHC Class II expression has been shown to decrease in sepsis, a fall in expression may be expected in cases with chorioamnionitis (301;355). No reduction in expression was associated with histological chorioamnionitis. However where there was evidence of maternal systemic illness such as raised CRP and/or clinical chorioamnionitis, a negative correlation with monocyte MHC Class II expression was seen.

Monocyte MHC Class II expression also showed negative correlation with plasma IL-10 levels. IL-10 has been shown to down regulate MHC Class II molecules at monocyte cell surface through post translational effects (165). However no increase in maternal plasma IL-10 levels was seen in PTL or PPROM groups (or the subgroup of those with clinical chorioamnionitis). Plasma levels may not be indicative of levels in other compartments such as the amniotic cavity and a single measurement may miss

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the peak levels of IL-10. There are many other factors besides IL-10 play a role in the regulation of monocyte MHC Class II expression (161).

There is very little work looking at monocyte MHC Class II expression in PPROM or PTL. One group have found no difference between levels of monocytye HLA-DR expression in women with PPROM or PTL and gestation matched controls (358;359). However, we have shown this fall in monocyte MHC Class II in labour occurring both at term and prematurely. The disparity between results may be due to differences in laboratory techniques and the timing of sampling. In this study, all samples were collected, assayed and analysed by a single operator, reducing interoperator error.

A reduced pro-inflammatory cytokine response to *in-vitro* LPS stimulation was seen in PTL and PPROM, compared to gestation-matched controls. No differences in the production of IL-10 following endotoxin stimulation were seen.

Amory *et al.* found that in non-pregnant women with a history of preterm labour, whole blood stimulation with LPS produced more TNFα than women with a history of term delivery (333). We found significantly less TNFα production in response to whole blood stimulation with LPS in the PTL group. This positively correlated with the degree of monocyte MHC Class II expression, and so demonstrates a degree of *immune paresis* in these patients.

These changes in the maternal immune system may be due to neuroendocrine effects involved in the initiation of labour (194). The neuroendocrine system has been shown to alter surface expression of MHC Class II on monocytes in other models (314). This

could therefore be the mechanism underlying the reduction in monocyte MHC Class II expression seen in term and preterm labour in this work.

In summary, a fall in maternal monocyte MHC Class II expression was found in PTL and PPROM, greater than that seen in term labour. There was also some evidence of *immune paresis* in the PTL group, with reduced TNFα response to whole blood LPS stimulation. Evidence of similar changes in the fetal immune response to preterm delivery will be investigated in chapter 6.

CHAPTER 5 – THE FETAL IMMUNE RESPONSE TO TERM LABOUR

5.1 INTRODUCTION

During term labour, a pro-inflammatory response is seen in the maternal system, however there are conflicting reports in the literature as to whether this effect is also seen in the fetus (199;212;342). Rizos *et al.* found increases in IL-6 and IL-1β in cord blood from infants born following term labour compared to elective delivery (199). Others have shown an increase in TNFα but no change in IL-6 levels, compared to adult controls (342).

Similarly, little is known regarding the anti-inflammatory arm of the fetal immune system in response to term labour. Kotiranta-Ainamo *et al.* and Seghaye *et al.* reported low levels of IL-10 in cord plasma, following term labour (212;342). Power *et al.* found no change in IL-10 levels with labour, but did report a fall in $TGF\beta_1$ levels (189).

It is widely reported that neonates have a reduced level of monocyte MHC Class II expression compared to a healthy adult population (322-326). However little is known regarding the effects of labour on monocyte MHC Class II expression. Birle *et al.* found cord monocyte HLA-DR expression did not vary with mode of delivery (322).

There are a small number of studies of term neonatal monocyte responses to LPS stimulation, with conflicting results. Weatherstone *et al.* and Seghaye *et al.* report similar cytokine responses to stimulation with cord blood as adult controls (212;338).

However, Strunk *et al.*, Hodge *et al.* and Sautois *et al.* found some impairment of the pro- inflammatory response to stimulation in neonates, with reduced production of TNFα (212;337;340). There are also quantitatively differing reports of the anti-inflammatory response to stimulation in neonates. Kotiranta-Ainamo *et al.* report a reduction in IL-10 production compared to adult controls (360), whereas Seghaye *et al.* found no difference between adult controls and cord blood in LPS stimulated IL-10 production (212).

This chapter aims to describe the inflammatory response of healthy term neonates during labour or elective delivery.

5.2 METHODS

5.2.1 Study Design & Patient Selection

Three patient groups were recruited for this study:

- **Term labour** - infants born following spontaneous labour and vaginal delivery at term
- **Term elective caesarean section** - infants born following pre-labour elective caesarean section at term
- **Feticides** - fetuses with conditions such as trisomies, major neurological and cardiac malformations undergoing feticide.

Outcome measures such as birth weight, gender, apgar scores, admission to the neonatal unit, concerns regarding sepsis including septic screen and administration of antibiotics, were recorded.

All families were contacted after the neonatal period to ascertain whether the infant was admitted with suspected sepsis during the neonatal period. Any infants with suspected or proven sepsis were excluded from this study.

Blood were also taken from healthy members of staff in the hospital and laboratory to provide adult control data.

5.2.2. Sample Collection

Umbilical cord venous blood was collected within 5 minutes of delivery of the placenta. In the feticide group, fetal blood samples were collected at the time of feticide, prior to intracardiac injection of potassium chloride. Blood collection and handling has been described in 2.5.

5.2.3 Monocyte MHC Class II Expression & Cytokines Assays

The following assays were performed on all blood samples collected:

- Monocyte MHC Class II and HLA-DR expression, as described in 2.6.1.
- Cytokine assays, as described in 2.6.2 and 2.7.
- Whole blood LPS stimulation studies, as described in 2.8.

There was insufficient blood available from the feticide samples to perform the LPS stimulation studies.

5.2.4 Statistics

As MHC Class II and HLA-DR were expressed as percentages, and not normally distributed, comparisons between groups were performed with the Mann Whitney test. Analysis was performed with the Statistical Package for the Social Sciences (version 12.0 SPSS, Chicago, IL.)

5.3 RESULTS

5.3.1 Patient Characteristics

50 infants were studied over the study period. An additional 5 parents were approached but declined participation. 7 feticide samples were also obtained. 24 healthy adult female controls were also recruited. The patient characteristics are shown in Table 22.

Patient Group	n	Median gestational age, days (range)	Male gender (%)
Term labour	26	282 (259-295)	40.7%
Term elective caesarean section	24	272 (259 –290)	60.8%
Feticide	7	175 (146 –231)	n/a

Table 22: Patient Characteristics

5.3.2 Monocyte MHC Class II Expression In Term Neonates

Monocyte MHC Class II expression was significantly lower in neonates than the adult controls as shown in Figure 35, Mann Whitney test $p<0.001$.

There was no statistical difference between monocyte MHC Class II or HLA DR expression in infants delivered by elective caesarean section or by spontaneous vaginal delivery and the feticide samples, see Table 23 and Figure 35.

There was no correlation between maternal and cord MHC Class II or HLA-DR expression in any of the groups. There was also no correlation between monocyte MHC Class II and HLA-DR expression and gestational age, gender, birthweight, or apgar scores.

		Term labour	Term Elective Caesarean	Feticide
MHC Class II	**%**	92.01 (81.40-97.59)	90.91 (77.24-96.28)	89.10 (75.58-92.42)
	mfi	17.15 (12.98-20.72)	15.40 (9.56-17.15)	23.29 (13.58-45.32)
HLA DR	**%**	74.90 (65.31-84.04)	74.41 (58.64-79.74)	66.59 (50.83-90.55)
	mfi	10.80 (7.98-13.68)	10.49 (6.22-13.41)	11.04 (6.32-30.23)

Table 23: Median cord monocyte MHC Class II and HLA-DR expression in feticides, and infants born following term labour or elective caesarean section (interquartile range).

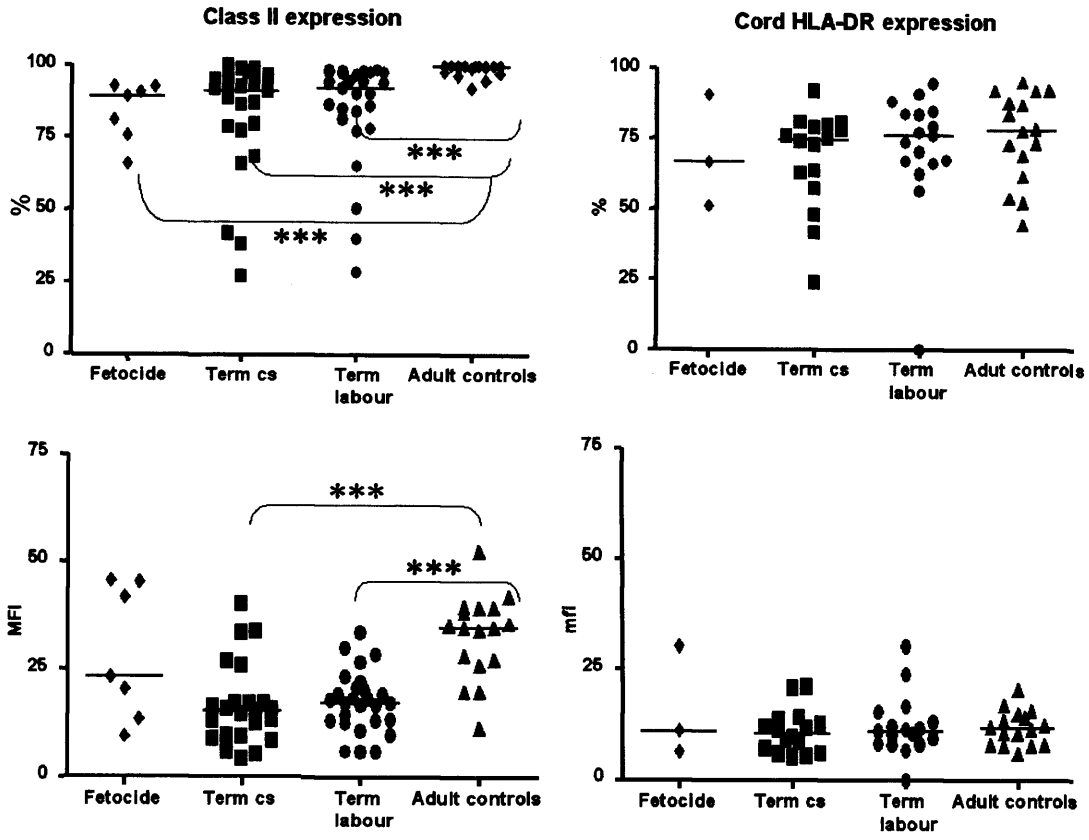

Figure 35: Monocyte MHC Class II and HLA DR expression in feticides, in term infants after spontaneous vaginal delivery or elective caesarean section and in adult controls.

The percentage of monocytes expressing MHC Class II was significantly lower in all fetal groups than the adult controls. There was no difference seen between groups with regard to monocyte HLA-DR expression.

Key: ***Term cs*** **Term elective caesarean section**
Term labour **Term spontaneous labour and delivery**
*** **Mann Whitney test *p<0.001***

5.3.3 Cord Plasma Cytokine Levels

There was evidence of a pro-inflammatory response to labour. There was a significant increase in cord levels of IL-6 and IL-8 in infants born following spontaneous labour, compared to those delivered by elective caesarean section, see Figure 36.

There was a significant fall in $TGF\beta_1$ levels in spontaneous labour, compared to feticide samples, see Table 24. No significant differences were seen in levels of IL-10 between the groups.

Cytokine (pg/ml)	Term Labour	Term Elective Caesarean	Feticide
IL-1β	Undetectable	Undetectable	1.0 (1.0-312.2)
IL-6	16.1 (3.4-54.4) **	3.8 (1.0-7.7)	5.6 (1.0-20.7)
IL-8	27.5 (17.0-56.9)*	13.6 (11.7-21.9)	33.2 (13.0-173.2)
IL-12	Undetectable	1.0 (1.0- 5.3)	3.2 (1.0-32.4)
TNFα	1.0 (1.0-4.0)	1.0 (1.0-3.8)	4.0 (2.2-27.8)
IL-10	3.0 (1.0-5.25)	1.0 (1.0-4.7)	4.0 (3.2-24.0)
$TGF\beta_1$	36.85 (21.6-185.0)†	137.6 (21.2-192.9)	295 (80.9-462.4)

Table 24: Cord plasma cytokine median levels (interquartile range).

IL-8 and IL-6 levels were significantly higher following labour than elective caesarean section. $TGF\beta_1$ levels were significantly lower in the spontaneous labour group than the feticide group.

Key: * compared to term elective caesarean section, Mann Whitney $p < 0.05$
** compared to term elective caesarean section, Mann Whitney $p < 0.01$
† compared to feticide, Mann Whitney $p < 0.05$

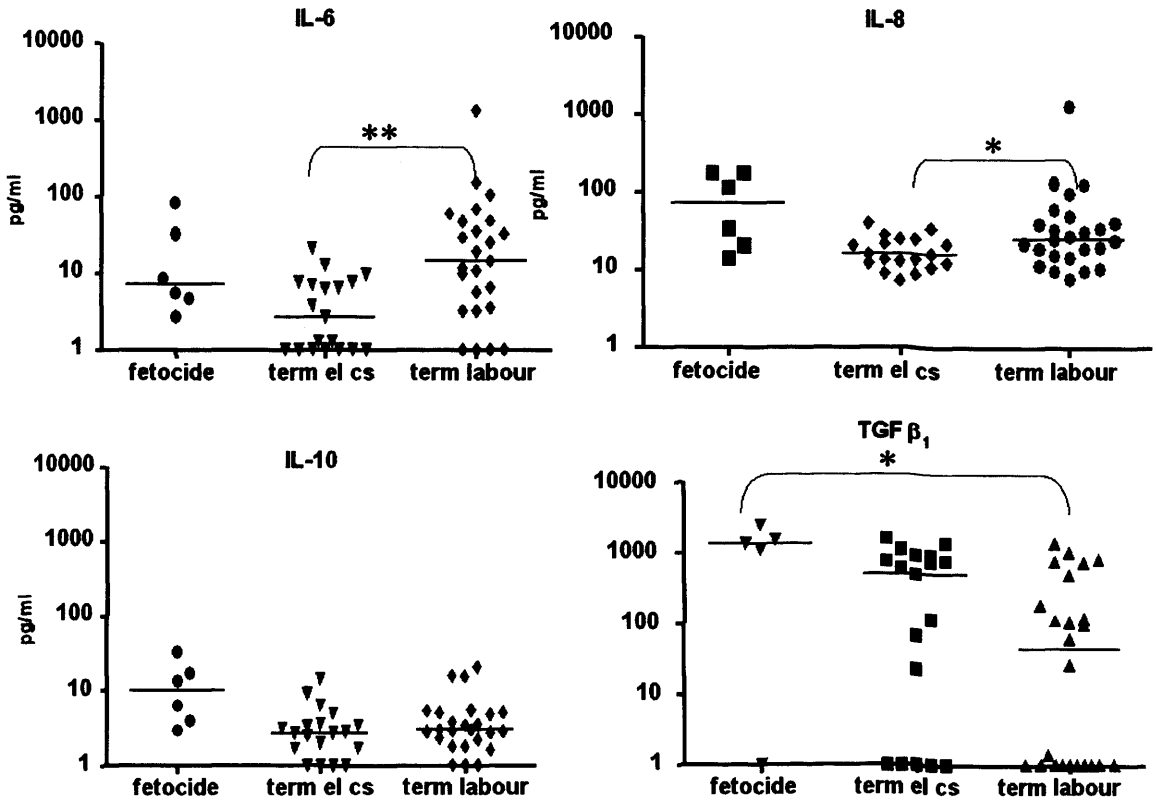

Figure 36: Cord plasma cytokine levels.

IL-8 and IL-6 levels were significantly higher following labour than elective caesarean section. TGFβ_1 levels were significantly lower in the spontaneous labour group than the feticide group.

Key: *term labour* term spontaneous labour
term el cs term elective caesarean section
* Mann Whitney $p < 0.05$
** Mann Whitney $p < 0.01$

There was a correlation between maternal and cord plasma IL-8 levels in the spontaneous labour group, Spearman's rank coefficient 0.722, $p < 0.001$. There were no correlations between any other cytokine levels in mothers and infants.

There was no correlation between cord plasma IL-10 levels and monocyte MHC Class II or HLA-DR expression.

5.3.4 Cytokine Production Following Whole Blood Stimulation

Cord samples from the spontaneous labour group produced less TNFα and more IL-10 than the adult controls. There was no correlation between TNFα and IL-10 production. There were no significant differences between levels of cytokines produced in the spontaneous labour or elective caesarean section groups, see Table 25.

However there was a significant difference between the IL-10 / TNFα ratios in the spontaneous labour group and the elective delivery group, median 17.1 (interquartile range 5.6 – 121.6) compared to 0.7 (0.2 – 63.3) respectively, Mann Whitney $p = 0.047$. The cord samples from the elective caesarean section group produced less IL-6 than the adult controls, see Figure 37.

There was no correlation between cord plasma IL-10 levels or monocyte MHC Class II expression and levels of cytokines produced following LPS stimulation.

Cytokine (pg/ml)	Term Labour	Term Elective Caesarean	Adult controls
IL-1β	3317 (1423-8660)	2916 (284-55618)	1663 (1101-3560)
IL-6	50000 (10000-80835)	10000 (1331-59172)*	50562 (41261-65538)
IL-8	50000 (10000-100000)	20790 (5000-100000)	67079 (47989-100000)
IL-12	4 (1-29)	35 (5-633)	38 (26-58)
TNFα	698 (259-3632)*	781 (148-4887)	2697 (1427-5262)
IL-10	1589 (907- 4426) ***	635 (292-2483)	663 (524-924)

Table 25: Median cytokine levels following 24-hour LPS stimulation (interquartile range).

Cord samples from the spontaneous labour group produced less TNFα, and more IL-10 than the adult controls. The cord samples from the elective caesarean section group produced less IL-6 than the adult controls. There were no significant differences between levels of cytokines produced in the spontaneous labour or elective caesarean section groups.

Key: * compared to adult controls, Mann Whitney $p < 0.05$
*** compared to adult controls, Mann Whitney $p < 0.001$

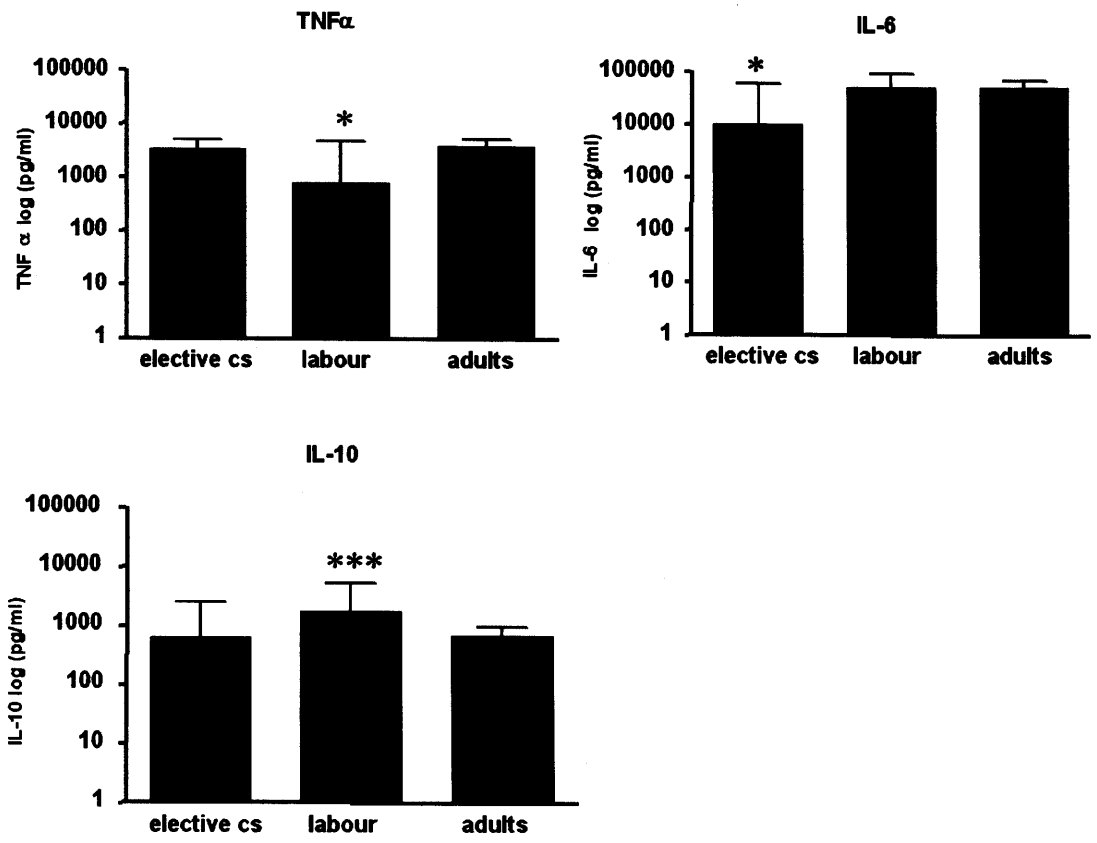

Figure 37: Cytokine production following whole blood LPS stimulation in cord samples from the elective caesarean section group, the spontaneous labour group and adult controls.

Median and interquartile ranges shown. Cord samples from the spontaneous labour group produced less TNFα, and more IL-10 than the adult controls. The cord samples from the elective caesarean section group produced less IL-6 than the adult controls. There were no significant differences between levels of cytokines produced in the spontaneous labour or elective caesarean section groups.

Key: ***elective cs*** Term elective caesarean section
Labour Term labour
Adults Adult controls
* compared to adult controls, Mann Whitney $p < 0.05$
*** compared to adult controls, Mann Whitney $p < 0.001$

5.4 DISCUSSION

Problems inherent in this work include the selection of appropriate control groups for comparison to the infants born following term labour. Those delivered by elective caesarean section have a younger median gestational age (272 days versus 282). The feticide group were not exposed to labour, infection or other insults such as placental insufficiency. However they had major structural or chromosomal defects. These problems would not be expected to affect monocyte MHC Class II expression. It would not be ethically possible to obtain samples from normal fetuses as this would involve an invasive procedure.

The optimisation experiments described in chapter 3 aimed to improve reliability and reproducibility in small volume samples, such as those obtained from cord samples and feticides. However enough blood was not obtained to perform LPS stimulation experiments on the feticide samples, which leaves unanswered questions regarding the normal fetal immune response *in-utero.*

A pro-inflammatory response to labour was seen in term infant samples, with increased levels of IL-6 and IL-8 in cord samples compared to elective delivery. It is not possible to determine from this study whether these cytokines are of fetal origin, or if they originate from the maternal pro-inflammatory response to labour, as described in chapter 3, and have crossed the placenta to the fetal circulation. Alternatively, trophoblasts have also been shown to produce pro-inflammatory cytokines (281). Several other groups have found increased levels of pro-

inflammatory cytokines in cord blood of infants born by spontaneous vaginal delivery compared to elective caesarean section or maternal blood (199;212).

No evidence of a marked anti-inflammatory response to term labour in cord blood was seen. Little or no IL-10 was detected in neonates delivered by either mode. This is in concordance with other groups (192;212). We also observed a significant fall in $TGF\beta_1$ levels in the cord samples of infants delivered following labour, compared to the feticide controls. Power *et al.* also described a reduction in cord $TGF\beta_1$ levels with term labour (189). Chegini *et al.* showed that $TGF\beta_1$ inhibits production of $TNF\alpha$, which increases MMP-1 and MMP-3 (235). They suggest that lower $TGF\beta_1$ expression in labour may lead to increased MMP production, leading to the extracellular matrix degradation seen in membrane rupture.

The main finding of this study was that there was no fall in neonatal monocyte MHC Class II expression during term labour, in contrast to the reduction in maternal monocyte MHC Class II expression during labour, described in chapter 3. Lower levels of monocyte MHC Class II expression were seen in fetal samples compared to healthy adult controls. However, levels of HLA-DR in the cord samples were similar to adult controls.

Despite the low levels of monocyte MHC Class II expression, cells within the neonatal blood samples were capable of mounting a response to an LPS challenge. However they produced less pro-inflammatory cytokines than the adult controls. Those infants exposed to labour were capable of producing more IL-10 in response to stimulation than the adult controls.

There are a small number of studies looking at monocyte MHC Class II expression in cord blood samples. Lower levels of monocyte MHC Class II expression in neonates compared to healthy adult controls have been reported by several groups (322-326). No other studies in the literature have investigated the effect of term labour versus elective delivery on cord monocyte MHC Class II expression. Birle *et al.* found no difference in monocyte HLA DR levels in the first day of life, between infants delivered vaginally or by caesarean section (322). Their population differed to ours as all the infants in their study were admitted to the neonatal unit and included a range of gestational ages.

Jones *et al.* found that monocyte MHC Class II expression increased with gestational age. Blood was sampled from neonates born at various gestation ages. (324). This association was not seen in the feticides studied here. This is interesting as although of similar gestational age, it would appear from this data that exposure to labour, infection or other insults such as placental insufficiency may influence MHC Class II expression at birth.

In this study we found that whole blood stimulated with LPS from all groups produced high levels of IL-6. However, blood from neonates born by spontaneous labour showed a reduced capacity to generate TNFα on stimulation and an enhanced capacity to produce IL-10 when compared to elective delivery. Indeed the ratio of these two cytokines was much higher in the spontaneous labour group than in the caesarean section group. This contrasts with Kotiranta-Ainamo *et al.* who found that the anti-inflammatory response of term cord blood to LPS stimulation was lower than adult controls (360). Hodge *et al.* used a similar whole blood stimulation technique

with the same dose of LPS (335). They found similar levels of IL-6 to adult controls but reduced levels of TNFα and IL-1β production in infants born following term labour. Another group using 30ng/ml of LPS (compared to 100ng/ml in this study) and a 5 hour incubation period found a reduced production of IL-6 and TNFα from term cord blood (340). The difference in the results between these studies may be because of the different LPS doses used.

This whole blood LPS stimulation study illustrates that the term neonate is capable of producing both pro- and anti-inflammatory cytokines. However it may be a reflection of the general immaturity of the fetal immune system that less pro-inflammatory cytokines were produced than the adult controls. In contrast, cord samples following term labour produced more IL-10 with LPS stimulation than adult controls. This may suggest that there is some shift towards an anti-inflammatory response during labour. Although this is not obviously reflected in monocyte MHC Class II expression at birth, it is unclear if MHC Class II expression falls in the following days. Such a fall was seen in preterm neonates, as will be described in chapter 6.

This study provides evidence of inflammation at birth when babies are delivered by spontaneous delivery as compared to a caesarean section, but a reduced pro-inflammatory response to LPS *in-vitro*. This may reflect the anti-inflammatory milieu within the fetus at birth as the levels of IL-10 produced in response to LPS stimulation were higher than that seen in babies born by caesarean section.

CHAPTER 6 - CYTOKINE BALANCE IN THE PRETERM INFANT

6.1 INTRODUCTION

As shown in the previous chapters, inflammatory mediators are present in both the mother and fetus at birth. While the role of inflammation in term delivery is unclear, recent work (discussed in chapter 1) has revealed a potentially important role for inflammation in preterm delivery. Indeed the neonatal inflammatory response to preterm delivery may be, at least in part, responsible for subsequent mortality and morbidity associated with prematurity.

There is a substantial body of work investigating the pro-inflammatory response of the preterm infant (275;276;361). Levels of IL-1β, IL-6, IL-8 and TNFα have been found to be elevated in cord blood following preterm delivery, especially in the presence of chorioamnionitis (265;266;271). However there is relatively little knowledge of the anti-inflammatory arm of the immune response in the preterm infant. Blanco-Quiros *et al.* reported elevated levels of IL-10 in preterm neonates compared to term infants (192).

Several groups have found a reduction in the cytokine response to *in-vitro* LPS stimulation of whole blood in preterm infants. Yachie *et al.* reported decreased production of IL-6 compared to term infants (336). Schibler *et al.* described reduced production of IL-8 (339). Dembinski *et al.* reported reduced production of TNFα and IL-1β compared to term infants (279). In contrast, Schultz *et al.* found increased numbers of cells staining for IL-8 and IL-6 following LPS stimulation, compared to

adult controls (278). Dembinski *et al.* also reported an increase in anti-inflammatory response to LPS stimulation, with higher levels of IL-10 and $TGF\beta_1$ than adult controls (279).

Several studies have found monocyte MHC Class II expression in preterm neonates to be lower than healthy adult and term neonatal controls. Birle *et al.* and Jones *et al.* found a correlation between monocyte MHC Class II expression and gestational age in preterm infants, however these studies have not accounted for the reason for preterm delivery (322;324).

Therefore there is relatively little known about the balance between the pro- and anti-inflammatory arms of the fetal immune response to preterm labour. This provides the basis for this study. This chapter sets out to describe the effects of preterm delivery on the cytokine balance of the neonate, by measuring monocyte MHC Class II expression, cytokine assays and whole blood LPS stimulation studies.

6.2 METHODS

6.2.1 Patient Groups

Three main groups of preterm infants were recruited:

- **Preterm labour (PTL)** - the infants of women in preterm labour at less than 32 weeks completed gestation, recruited for the studies presented in chapter 4.

- **Pre-term pre-labour rupture of membranes (PPROM)** - the infants of women with PPROM at less than 32 weeks completed gestation, recruited for the studies presented in chapter 4.
- **Intrauterine growth restriction (IUGR)** – infants born following elective delivery at less than 32 weeks, who were estimated to be <3rd centile on fetal medicine unit ultrasound. The reason for delivery was severe growth restriction +/-pre-eclampsia.

All patients had had a dating scan, confirming the gestational age of the pregnancy.

Results from the above groups were compared with the term labour and feticide groups as well as the adult controls, as described in chapter 5.

6.2.2. Clinical Care

6.2.2.1 Antenatal Care of PTL and PPROM

Antenatal care of women in preterm labour or with PPROM has been described in 4.2.2.

6.2.2.2 Antenatal Management Of IUGR Group

These were fetuses that were estimated to be <3rd centile on fetal medicine unit ultrasound. Some women had pre-eclampsia and therefore were managed in accordance with the unit's pre-eclampsia protocol. Other women were well in

themselves but electively delivered due to concerns about fetal size and well-being. All women were offered one course of antenatal betamethasone from 24 weeks onwards. Details regarding the unit's policy on caesarean section have been described in 3.2.6.

6.2.2.3 Neonatal Care

Routine care of extremely preterm infants on UCLH neonatal unit included use of surfactant and antibiotic cover from birth until negative cultures were received.

6.2.3 Outcome Measures

Mode of delivery, gestational age, birthweight, sex, apgar scores and cord gas results were recorded. Placentas were sent for routine histology. A histological diagnosis of placental insufficiency was made if infarctions, excess syncytial knots, and/or excess fibrin were present.

6.2.4 Study Design

Blood was sampled from the umbilical vein of the placenta at delivery. Neonatal blood samples were taken on day 1, 2 and 7 of life if possible.

6.2.5 Blood Collection & Handling

In the feticide group, fetal blood samples were collected at the time of feticide, prior to intracardiac injection of potassium chloride. Cord blood was collected from the

placenta at delivery. Neonatal blood samples were collected at the same time as routine clinical blood sampling. 0.5ml was collected if possible. Blood collection and handling has been described in section 2.5.

6.2.6 Monocyte MHC Class II Expression & Cytokine Assays

The following assays were performed on all blood samples collected, if there was sufficient sample volume:

- Monocyte MHC Class II and HLA-DR expression, as described in 2.6.1.
- Cytokine assays, as described in 2.6.2 and 2.7.
- Whole blood LPS stimulation studies, as described in 2.8.

There was insufficient blood available from the feticide samples to perform the LPS stimulation studies.

6.2.7 Statistics

MHC Class II and HLA-DR expression were expressed as percentages, and not normally distributed. The numbers in each group for the cytokine assays were small as so all comparisons between groups were performed with the Mann Whitney test. Correlations were performed with Spearman's rank analysis. Analysis was performed with the Statistical Package for the Social Sciences (version 12.0 SPSS, Chicago, IL.)

6.3 RESULTS

54 infants and 7 feticides were studied from October 2003 to July 2005. Three sets of parents declined study participation. It was not possible to obtain blood samples for all infants at all time points. In addition, not all assays could be performed on every sample, as sufficient blood could not always be collected due to the clinical status of the neonate. The raw data for each of the groups are summarised in appendices 2, 3 and 4.

6.3.1 Patient Characteristics

Table 26 describes the patient characteristics of the study population. The feticides were performed for chromosomal and major cardiac anomalies and had a median gestational age of 175 (interquartile range 146 – 231) at blood collection. 14 fetuses were recruited antenatally in the PPROM group but 5 were stillborn and so are not included in the table. 17 fetuses were recruited antenatally to the PTL group, one of which was stillborn.

	PTL	PPROM	IUGR	Feticides
N	16	9	13	7
Maternal Ethnicity	56.3% white 25% black 18.7% asian	55.6% white 33.3% black 11.1% asian	61.5% white 15.4% black 23% asian	71.4% white 28.6% black
Twins	1 set MCDA 3 sets DCDA	1 set DCDA	1 set DCDA	0

Table 26: Patient Characteristics

6.3.2. Outcome

Delivery details are described in Table 27. In the PPROM group, the median duration of rupture of membranes was 6 days, with a range of 2 to 43 days. The perinatal mortality rate was 27.2% and the neonatal mortality rate was 15.8%. In the IUGR group, all infants weighed less than the 5th centile on neonatal growth charts at birth.

	PTL % (n)	PPROM % (n)	IUGR % (n)
Presentation			
Cephalic	56.3 (9)	77.8 (7)	84.6 (11)
Breech	37.5 (6)	22.2 (2)	15.4 (2)
Transverse	6.3 (1)		0
Mode of delivery			
SVD	50.0 (8)	77.8 (7)	0
Instrumental	31.3 (5)	0	0
Emergency CS	18.8 (3)	22.2 (2)	0
Elective CS	0	0	100 (13)
Median gestation age at delivery – days (interquartile range)	196 (170.3-203)	186.0 (177.5-205.5)	201 (190-212.5)
Median birthweight (interquartile range)	1073.5g (726.3-1448.3	892.5g (602.3- 1198.0)	863g (615-1030)
Male gender % (n)	50 (8)	66.7 (6)	61.5 (8)
Interval from admission to delivery			
< 7days	87.5 (14)	66.7 (6)	100 (12)
≥ 7days	12.5 (2)	33.3 (3)	
Neonatal and infant deaths	2	1	3

Table 27: Delivery details of patient groups

6.3.3 Monocyte MHC Class II Expression Is Reduced In PTL & PPROM

Monocyte MHC Class II expression was lower in infants born following PTL or PPROM, compared to both term labour and gestation-matched feticide controls, see Table 28, Figure 38 and Figure 39. A similar trend was seen in the expression of HLA-DR on monocytes from these groups but there were only a small number of samples available. Monocyte MHC Class II expression was particularly low in infants exposed to PPROM.

		PTL	PPROM	IUGR	Feticide	Term Labour
IHC lass II	%	74.65*† (49.69-82.28)	63.55*♣ (59.92-78.34)	72. 92♣ (53.57-87.51)	89.10 (75.58-92.42)	92.01 (81.40-97.59)
	mfi	9.65* (8.20-19.36)	12.99*‡ (10.12-14.24)	16.70 (8.74-27.17)	23.39 (13.58-45.32)	17.15 (12.98-20.72)
LA-DR	%	42.48‡ (22.05-51.42)	33.38*‡ (24.46-37.25)	58.59 (28.58-72.24)	66.59 (50.83-90.55)	74.90 (65.31-84.04)
	mfi	5.38† (3.62-7.26)	3.34*‡ (3.05-4.26)	9.22 (4.53-14.33)	11.04 (6.32-30.23)	10.80 (7.98-13.68)

Table 28: Median monocyte MHC Class II and HLA-DR expression (% and mfi) with interquartile range.

Monocyte MHC Class II expression was lower in PTL and PPROM than term labour or feticide groups.

Key: * compared to feticides, Mann Whitney $p < 0.05$
‡ compared to term labour, Mann Whitney $p < 0.05$
♣ compared to term labour, Mann Whitney $p < 0.01$
† compared to term labour, Mann Whitney $p < 0.001$

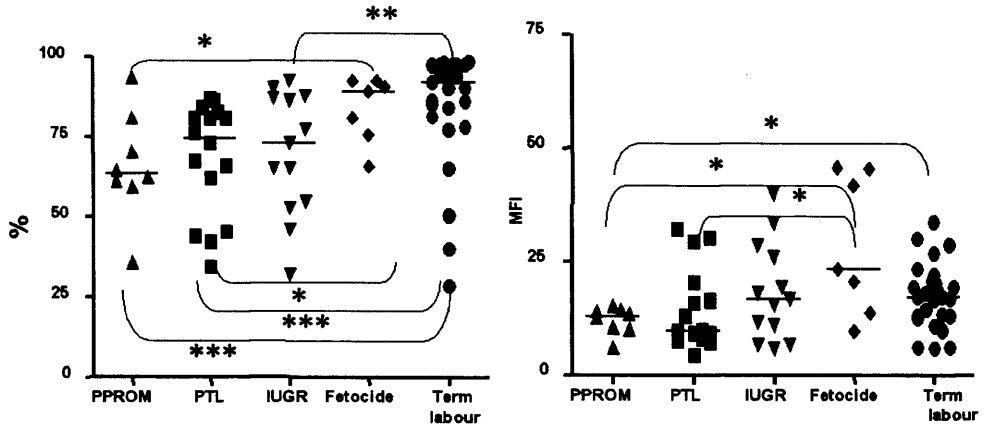

Figure 38: Monocyte MHC Class II expression in cord blood of infants born after PPROM, PTL, preterm elective delivery, feticides and term normal delivery.

These graphs demonstrate that monocyte MHC Class II expression, both in terms of percentage and mfi, was lower in the PPROM and PTL groups compared to feticides and term labour. The IUGR group also had lower percentage of monocytes expressing MHC Class II than the term labour group.

Key: * Mann Whitney $p < 0.05$
** Mann Whitney $p < 0.01$
*** Mann Whitney $p < 0.001$

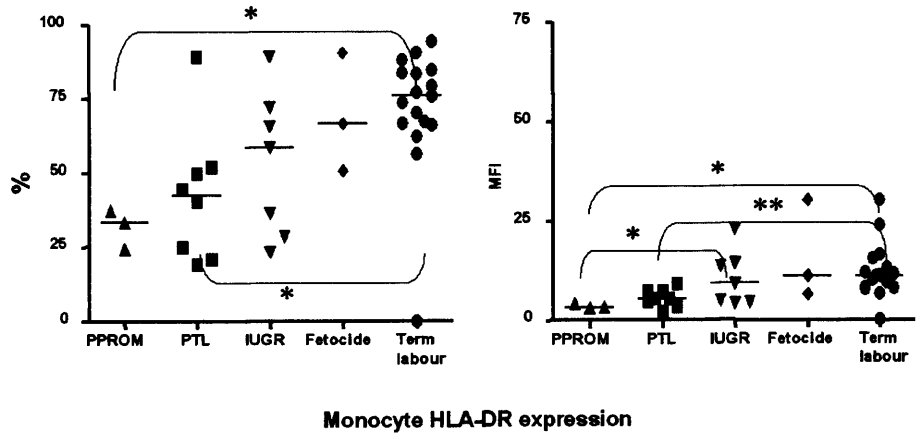

Figure 39: Monocyte HLA-DR expression in cord blood of infants born after PPROM, PTL, preterm elective delivery, feticides and term normal delivery

These graphs show that despite the small numbers of patients, monocyte HLA-DR was lower in the PPROM and PTL groups compared to term labour.

Key: * Mann Whitney $p < 0.05$
** Mann Whitney $p < 0.01$

Infants delivered by elective preterm delivery (IUGR group) had a significantly lower percentage of monocytes expressing MHC Class II than those infants in the term labour group, $p<0.01$.

6.3.4 Lack Of Correlation Between Monocyte MHC Class II Expression & Gestational Age

Changes seen in levels of monocyte MHC Class II or HLA-DR expression at birth do not seem to be due to an effect of gestational age, as there were no correlations between gestational age and monocyte MHC Class II or HLA-DR expression. There were no significant differences between levels of monocyte MHC Class II or HLA-DR expression in the feticide samples compared to more mature infants delivered after term labour, see Table 28.

There was no significant difference between cord MHC Class II expression between neonates who had received antenatal steroids and those who had not.

6.3.5 Lack Of Correlation Between Monocyte MHC Class II Expression & Interval From Admission To Delivery

The majority of women in the PTL and PPROM groups delivered within 7 days of admission, 85.2% and 60% respectively Table 27. In chapter 4, a relationship was seen between maternal monocyte MHC Class II expression and interval to delivery. However, no such correlation was seen in the fetal samples.

6.3.6 Serial Monocyte MHC Class II Measurements Over First Week of Life

Figures 40, 41 and 42 show median monocyte MHC Class II expression over the first week of life. The PPROM and IUGR groups show a continued fall in median monocyte MHC Class II expression, both in percentage and mfi, over the first week of life. In the PTL group, there appears to be some recovery of monocyte MHC Class II expression levels (%) by day 7, but this was not back to umbilical cord levels.

The number of infants for whom serial samples were available was small, so statistical analyses were not performed.

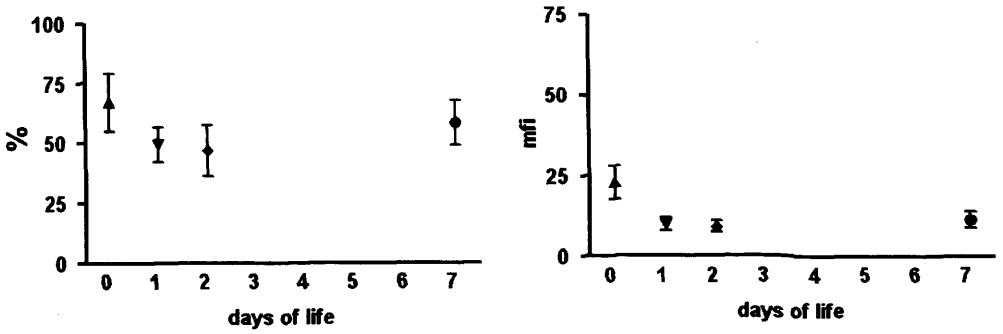

Figure 40: Serial monocyte MHC Class II expression over first week of life in infants born following PTL (% and mfi) n=8.

Medians and standard error bars are plotted.

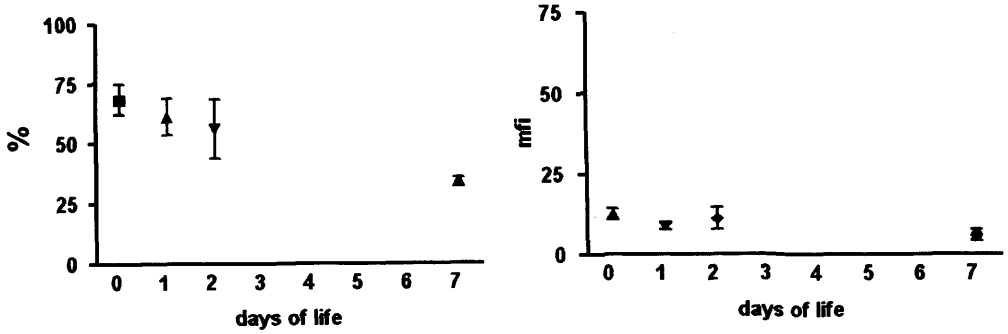

Figure 41: Serial monocyte MHC Class II expression over first week of life in infants born following PPROM (% and mfi) n=5.

Medians and standard error bars are plotted.

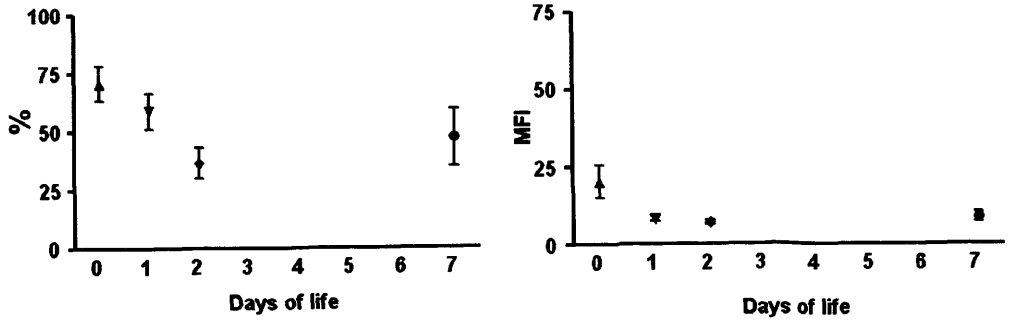

Figure 42: Serial monocyte MHC Class II expression over first week of life in infants born following elective delivery for IUGR (% and mfi) n=9.

Medians and standard error bars are plotted.

6.3.7 Plasma Cytokine Response to PTL & PPROM

A pro-inflammatory response was seen in PTL and PPROM, with increases in IL-8 and IL-6 above the levels seen in term labour, see Table 29 and Figure 43. There was no correlation between maternal and cord IL-8 and IL-6 levels.

TNFα, IL-12 and IL-1β were undetectable or present in very low levels in all groups. There were positive correlations between maternal and cord plasma levels of TNFα, IL-1 β and IL-12, see Figure 45.

There was little evidence of a anti-inflammatory response in PPROM or PTL with low levels of IL-10 across all groups, see Figure 44. There was a wide range of $TGF\beta_1$ levels seen in all the groups and no significant differences between the groups.

Cytokines (pg/ml)	PTL	PPROM	IUGR	Feticides	Term Labour
IL-1β	Undetectable	1.0 (1.0-184.4)	1.0 (1.0-151.2)	1.0 (1.0-312.2)	Undetectable
IL-6	19.6 (6.5-192.6)	192.8*† (35.9-299.1)	8.3 (4,3-151.0)	5.6 (1.0-20.70	16.1 (3.4-54.4)
IL-8	36.8‡ (20.3-309.1)	324.3 ‡ (33.0-960.9)	67.1† (47.0-151.2)	33.2 (13.0-173.2)	27.5 (17.0-56.9)
IL-12	3.7 (1.0-5.7)	1.0 (1.0-21.5)	4.7 (2.1-9.20	3.2 (1.0-32.4)	Undetectable
TNFα	1.0 (1.0-3.6)	3.1 (1.0-11.0)	4.3 (1.0-6.4)	4.0 (2.2-27.8)	1.0 (1.0-4.0)
IL-10	1.0 (1.0-5.7)	3.0 (1.0-35.8)	5.2 (2.1-12.3)	4.0 (3.2-24.0)	3.0 (1.0-5.25)
TGFβ$_1$	1.0 (1.0-5.9)	60.6 (12.9-131.5)	160.0 (34.2-252.0)	295.0 (80.9-462.4)	36.85 (216.1-185.0)

Table 29:Median cord plasma cytokine levels (interquartile range) for preterm labour, PPROM, IUGR, feticide and term labour

* Compared to IUGR - Mann Whitney $p < 0.05$
† Compared to term labour - Mann Whitney $p < 0.05$
‡Compared to term labour - Mann Whitney $p < 0.001$

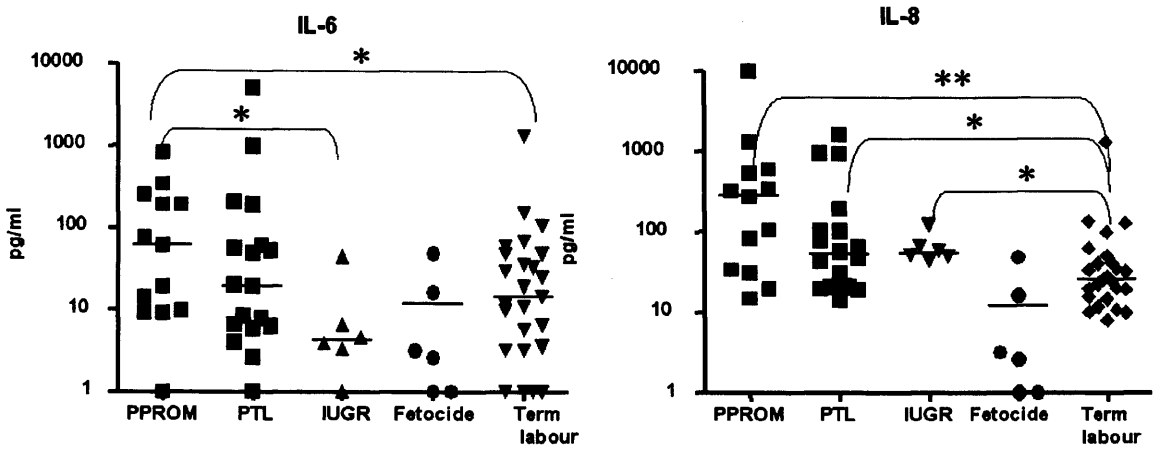

Figure 43: IL-6 and IL-8 levels in cord plasma samples.

IL-6 levels were significantly higher than term labour levels in the PPROM group, but not in the PTL group. IL-8 levels were significantly higher in PTL, PPROM and IUGR than the term labour group.

Key: * Mann Whitney $p<0.05$
** Mann Whitney $p<0.01$

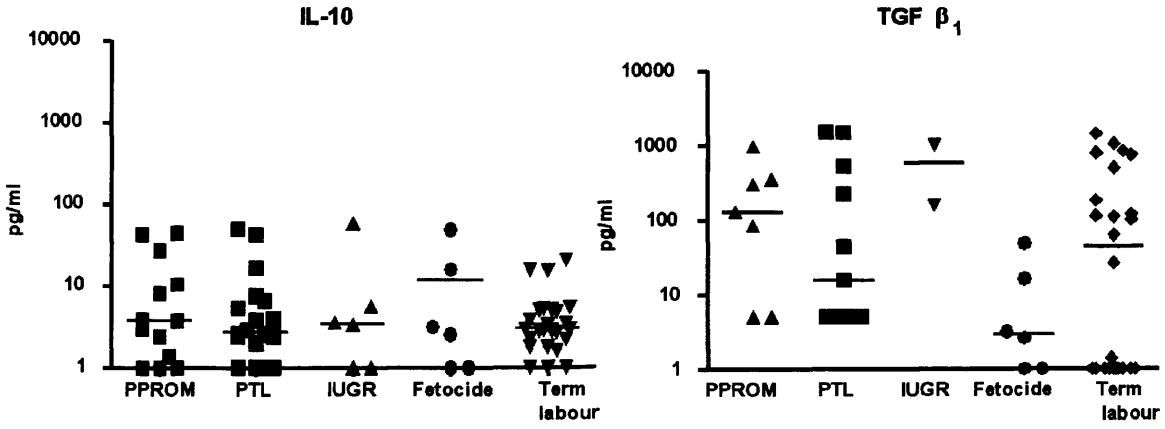

Figure 44: IL-10 and TGFb1 cord plasma levels (pg/ml).

There was no significant difference in IL-10 levels across the groups. There was a wide range of TGFb1 levels seen in all groups and no significant differences between the individual groups.

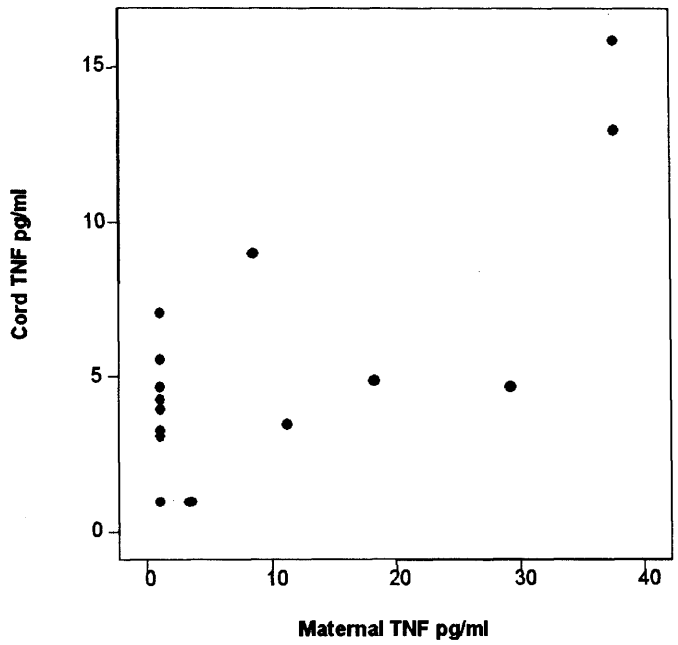

Figure 45: Correlation between maternal and cord plasma TNF levels, Spearman's rank correlation 0.463, p = 0.015

6.3.8 Lack of Correlation between Monocyte MHC Class II & Plasma IL-10 Levels

There was no evidence of correlation between monocyte MHC Class II levels and plasma IL-10 levels in PTL, PPROM or IUGR (overall Spearman's rank -0.069, *p= 0.666*). There was also no evidence of correlation between the pro-inflammatory cytokines and monocyte MHC Class II levels.

6.3.9 Intrauterine Growth Restriction Is Associated With Elevated Levels Of IL-8

Fetal plasma IL-8 levels were significantly increased in the IUGR group, compared to term labour samples. There was also a trend towards elevated IL-8 levels compared to the feticide samples, see Figure 43. No increases in the plasma levels IL-6, TNFα or IL-10 were seen in this group.

6.3.10 Serial Cytokine Assays over the First Week of Life

Serial cytokine measurements, made if samples were available, show a tendency for a fall in the pro-inflammatory cytokines IL-6 and IL-8, n=18 see Figure 46. Serial TNFα and IL-1β levels remained fairly steady over the first week.

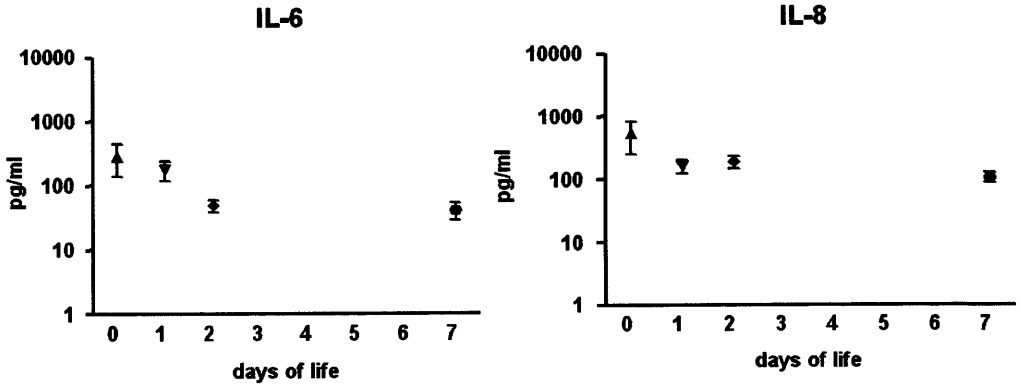

Figure 46: Serial plasma IL-6 and IL-8 levels over the first week of life in all preterm infants (pg/ml), medians and standard errors shown, n=18.

A small decrease in IL-6 and IL-8 levels was seen over the first week of life.

IL-10 was present at low levels in plasma over the first week, whereas TGFβ_1 levels rose over the first 2 days to reach a plateau that was maintained until day 7, see Figure 47.

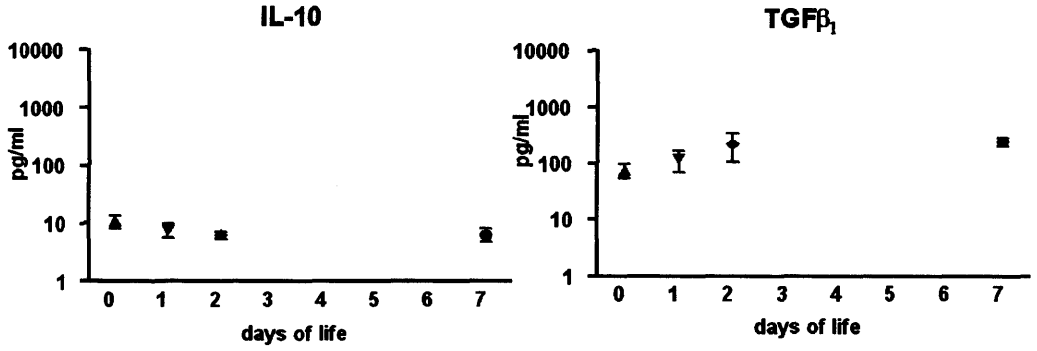

Figure 47: Serial plasma IL-10 and TGFβ_1 levels over the first week of life in all preterm infants (pg/ml), medians and standard errors shown.

No change in median IL-10 levels was seen over the first week whereas an increase in TGFβ_1 was seen, which appeared to plateau at day 2 and remain elevated until day 7.

6.3.11 Reduced Responsiveness To LPS In Preterm Infants

Significantly lower levels of pro-inflammatory cytokines, TNFα, IL-1β, IL-6 and IL-8 were produced by all preterm infants following LPS stimulation, compared to adult controls, see Figure 48 and Table 30. Reduced levels of IL-10 production were seen in the IUGR group, compared to adult controls, see Figure 49.

There was no significant difference in monocyte count across the groups that could explain these results.

Cytokine (pg/ml)	PTL	PPROM	IUGR	Term Labour	Adult controls
IL-1β	617**♦ (160-1082)	10000**♦ (1596-10365)	76**♦ (1-1247)	3317 (1423-8660)	1663 (1101-3560)
IL-6	10000*‡ (4693-10000)	10001***‡ (10000-70401)	4252*** (1-9776)	50000 (10000-80835)	50562 (41261-65538)
IL-8	10000** ‡ (7504-100000)	10001*** (10000-100000)	29*** ‡ (1-17286)	50000 (10000-100000)	67079 (47989-100000)
IL-12	1** (1-10)	1 (1-36)	4** (1-21)	4 (1-29)	38 (26-58)
TNFα	40*** (25-1092)	204* (168-5182)	63*** (1-112)	698 (259-3632)	2697 (1427-5262)
IL-10	218† (61-1078)	739‡ (617-1317)	30***♦ (1-153)	907 (1589-4426)	663 (524-924)

Table 30: Median cytokine production (interquartile range) following 24 hour LPS stimulation of whole blood.

Key:
Compared to adult controls, Mann Whitney * *p<0.05*, ** *p<0.01*, *** *p<0.001*.
Compared to term labour controls, Mann Whitney † *p<0.05*, ‡ *p<0.01*, ♦ *p<0.001*

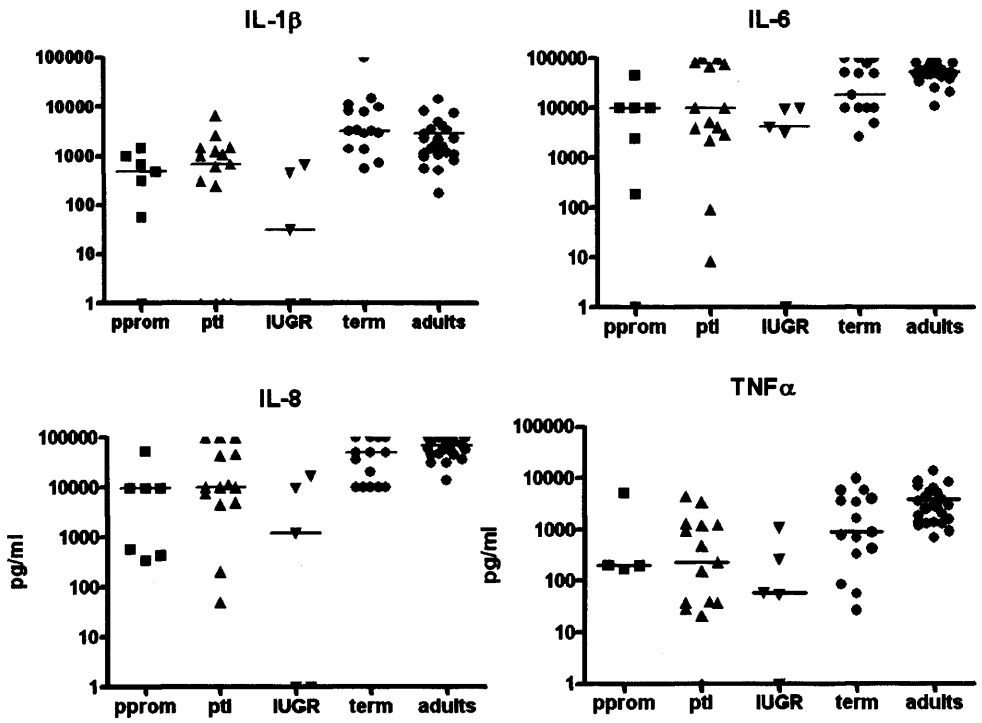

Figure 48: Cord pro-inflammatory cytokine production following whole blood LPS stimulation compared to term cord controls and adults controls.

LPS stimulation resulted in lower levels of production of several pro-inflammatory cytokines in the PPROM, PTL and IUGR groups compared to controls.

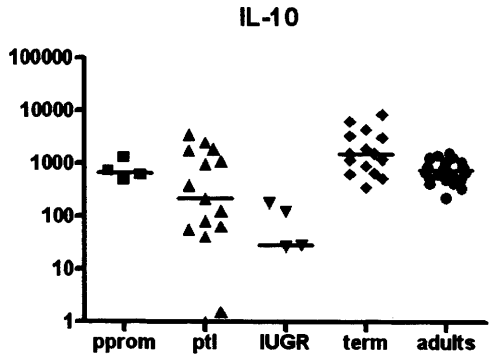

Figure 49: Cord IL-10 production following whole blood LPS stimulation, compared to term cord controls and adult controls.

There were no significant differences between IL-10/ TNFα ratios between the IUGR, PTL, PPROM and term elective caesarean section groups. All were significantly lower than the IL-10/ TNFα ratios seen in term labour, as discussed in chapter 5. There were no correlations between levels of cytokines produced in response to LPS and monocyte MHC Class II expression.

6.3.12 Placental Histology

Table 31 describes the frequency of various placental histological diagnoses in the different groups. Chorioamnionitis was present in all cases with funisitis. Overall 73.3% of the PPROM group had chorioamnionitis +/- funisitis, compared to 29.6% of the PTL group. Over 80% of the IUGR group had a histological diagnosis of placental insufficiency.

	PTL % (n)	PPROM % (n)	IUGR % (n)
Normal	33.3 (9)	26.7 (4)	16.7 (2)
Chorioamnionitis	11.1 (3)	20 (3)	0
Funisitis	18.5 (5)	53.3 (8)	0
Placental insufficency	11.1 (3)	0	83.3 (10)
Not done	25.9 (7)	0	0

Table 31: Placental histology and patient group

6.3.13 Placental inflammation and neonatal immune response

In infants who had evidence of chorioamnionitis, the percentage of monocytes expressing MHC Class II was significantly lower than those with normal placental histology, see Figure 50. These infants also had significantly higher levels of IL-6, IL-1β and IL-8 in their cord blood samples, see Table 32. There was no significant difference between the groups in IL-10 or $TGF\beta_1$ levels.

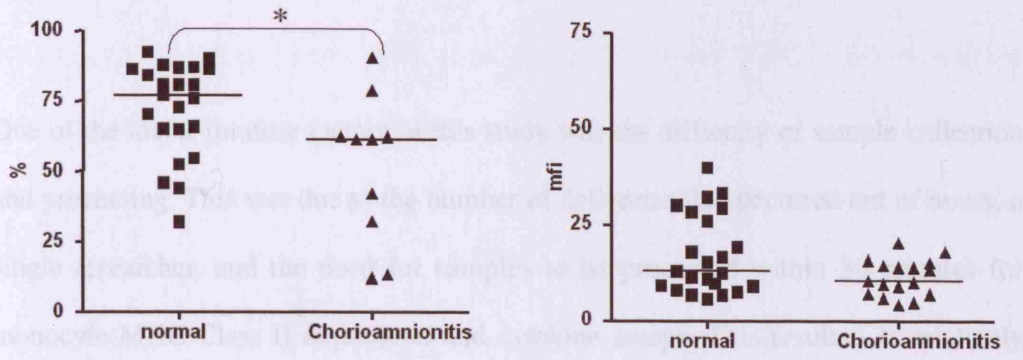

Figure 50: Monocyte MHC Class II expression (% and mfi) and placental histology.

There was a significant reduction in MHC class II expression (%) in those infants with chorioamnionitis.

Key: * Mann Whitney $p<0.05$

Assay	Histology		p-value
	Normal	Chorioamnionitis	
Monocyte Class II %	77.21 (32.06 – 92.66)	62.29 (34.58 – 93.59)	*0.05*
Monocyte HLA DR %	50.90 (23.30 – 89.26)	28.92 (19.18 – 44.52)	*0.031*
IL-6 (pg/ml)	8.3 (1- 658.9)	192.8 (1- 5171)	*0.005*
IL-1β (pg/ml)	1 (1- 608.9)	104.1 (1- 5532.4)	*0.032*
IL-8 (pg/ml)	50.5 (14.2 – 944.6)	541.5 (19.5 – 10000)	*0.009*

Table 32: Inflammatory parameters in cord blood samples of infants with chorioamnionitis.

Infants with evidence of chorioamnionitis on placental histology had significantly lower levels of monocyte MHC Class II and HLA-DR expression than those with normal placental histology. They also had significantly higher levels of IL-6, IL-1β and IL-8.

6.4 DISCUSSION

One of the major limiting factors in this study was the difficulty of sample collection and processing. This was due to the number of deliveries that occurred out of hours, a single researcher, and the need for samples to be processed within 30 minutes for monocyte MHC Class II expression and cytokine assays. This resulted in relatively small numbers of infants and samples to be analysed. The optimisation experiments described in chapter 3 aimed to improve reliability and reproducibility in small volume samples, such as those obtained from cord samples and feticides.

There were 6 sets of twins in the preterm infant groups. These pregnancies may have different aetiologies for PTL, IUGR and PPROM. Therefore it is not ideal to include them in the same groups as singleton pregnancies. However due to the numbers of infants recruited in this pilot study, it was not feasible to analyse the results from these multiple pregnancies separately as the numbers would be too small. In future work twin pregnancies should be appraised separately to identify differences to singletons.

A pro-inflammatory cytokine response was seen in the PTL and PPROM groups compared to controls, with increased levels of IL-6 and IL-8. However, TNFα and IL-1β levels were not raised. There was a tendency for levels of IL-6 and IL-8 to fall over the first week of life.

Other authors have also seen increased levels of pro-inflammatory cytokines in umbilical cord blood from preterm infants compared to term infants (265;266). Very little is reported regarding levels of anti-inflammatory cytokines in preterm infants.

No marked anti-inflammatory cytokine response (IL-10 and TGFβ_1) was seen in the PTL and PPROM groups. TGFβ_1 increased postnatally to peak at day 2 of life. In preterm infants there was no correlation between plasma IL-10 levels and monocyte MHC Class II expression.

Dammann *et al.* reported similar levels of IL-10 in term and preterm infants (266). They also reported a fall in pro-inflammatory cytokines over the first few days of life, in contrast to studies of term infants that show an increase over the first 4 days (362;363).

The main finding of this work was that monocyte MHC Class II expression was reduced in the PTL and PPROM groups, compared to the term labour controls. The gestation matched control group of feticides illustrates that this phenomenon is not purely related to gestational age. Monocyte MHC Class II expression continued to fall over first week of life.

In agreement with our findings, other groups have also shown lower levels of surface MHC Class II expression, compared to adult populations and term infants (322-326). Jones *et al.* found an inverse relationship between monocyte MHC class II expression and gestational age at delivery, but factors such as mode of delivery, sepsis, chorioamnionitis etc, were not taken into account (229;322).

Birle *et al.* also found a positive correlation between monocyte MHC Class II expression and gestational age (322). We have demonstrated this fall in monocyte

MHC Class II expression is not solely due to an effect of gestational age as monocyte MHC Class II expression levels were higher in the gestation matched feticide controls. Corticosteroids, such as dexamethasone, reduce monocyte MHC Class II expression (364). However we found no difference in levels of expression between infants who had received antenatal steroids and those who had not.

The reasons for the low levels of monocyte MHC Class II expression seen in infants born prematurely are unclear. The level of monocyte MHC Class II expression may reflect the severity of the antenatal insult that has led to delivery, such as the degree of chorioamnionitis or the extent of placental insufficiency. Over 80% of the PPROM group had evidence of histological chorioamnionitis, compared to approximately 1/3rd of the PTL group. Those infants with chorioamnionitis had reduced levels of monocyte MHC Class II expression and raised levels of pro-inflammatory cytokines, compared to infants with normal placental histology. Rates of histological chorioamnionitis in published data are broadly similar to our findings (between 50% to 70% in cases of PTL and PPROM) (22;365). Increased levels of pro-inflammatory cytokines in umbilical cord blood in the presence of chorioamnionitis have also previously been reported (252).

However not all the infants with histological chorioamnionitis had low levels of monocyte MHC Class II expression and low levels of cytokine response to LPS stimulation.

A *fetal inflammatory response syndrome* (*FIRS*) has been described in preterm infants, as discussed in 1.8.1.5. Evidence of such a state has been demonstrated in this

study, with raised levels of IL-6 in infants born following PTL and PPROM. Other pro-inflammatory cytokines such as TNFα and IL-1β levels were not raised. This may be due to the rapid rise and fall of TNFα, which has been described both *in vitro* and *in vivo* (139).

MHC Class II expression may be reflective of this immune dysregulation or *FIRS* occurring in the fetus at the time of preterm delivery. The fall in MHC Class II may represent a reactive anti-inflammatory phase after an initial pro-inflammatory insult leading to preterm delivery. Chorioamnionitis can be chronic and thus expose the fetus to inflammation for days or weeks (366). Chronic hypoxia due to placental insufficiency in the IUGR group may also lead to inflammation (367;368). Thus there could be a common pathway of chronic exposure to inflammation in the fetus, leading to immune paresis, which leaves the neonate at risk of subsequent infection.

An animal model of intrauterine infection has illustrated a fall in fetal monocyte MHC Class II expression following LPS exposure and evidence of a subsequent immune paresis (369). Our findings appear to suggest the same processes may be occurring in preterm infants.

IL-10 is known to downregulate monocyte MHC Class II cell surface expression, as discussed in 1.13.5.7. However in this work, no marked anti-inflammatory cytokine response (IL-10 and $TGF\beta_1$) was seen in the PTL and PPROM groups. In the preterm infants, there was no correlation between plasma IL-10 levels and monocyte MHC Class II expression. This may imply that other mechanisms may be of more importance in the regulation of MHC Class II expression in preterm infants, such as

the neuroendocrine system. Alternatively, plasma IL-10 levels may not be reflective of local concentrations.

Low levels of monocyte MHC Class II cell surface expression may reflect one of the mechanisms by which an initial insult, such as chorioamnionitis +/- preterm delivery, may attenuate the subsequent pro-inflammatory response to a second insult, so called *immune paresis,* discussed in 1.13.8 (298). Cord blood from preterm infants produced less TNFα in response to *in-vitro* LPS stimulation than controls, demonstrating evidence of *immune paresis.* Other studies have found conflicting results (341;360). Differences in results may be due to different experimental protocols, endotoxins and patient groups.

It may be that such a state could affect the preterm neonate's response to life on the neonatal unit, and explain their susceptibility to nosocomial infections (370). However anti-inflammatory cytokines have been found to be neuroprotective in animal models (108). Therefore the long-term consequences of an alteration in the cytokine balance of a preterm infant are unclear. This will be further investigated in chapter 7.

This study demonstrates a fall in monocyte MHC Class II cell surface expression in preterm neonates. This was also associated with a pro-inflammatory cytokine response and reduced TNFα production following whole blood LPS stimulation. The relationship between these effects on the neonatal immune system and short tem neonatal outcome will be investigated in chapter 7.

CHAPTER 7 - NEONATAL OUTCOME AND CYTOKINE BALANCE

7.1 INTRODUCTION

In the preceding chapters, expression of monocyte MHC Class II expression has been measured in mothers and neonates and found to correlate with changes in cytokine balance. In a range of clinical conditions such as surgery, trauma and sepsis, monocyte MHC Class II expression has been observed to fall (306;371-375). Further work has shown that this reduction in monocyte MHC Class II expression can identify a subgroup of patients at increased risk of poor outcome, such as mortality and morbidity (301;355).

In chapter 6 preterm delivery was found to be associated with a reduction in cord blood monocyte MHC Class II expression. However, I was interested to discover if the heterogeneity in outcome of infants was related to the levels of MHC Class II expression in term and preterm infants. I was also interested to see if monocyte MHC Class II expression was related to short-term neurological outcome.

7.2 METHODS

7.2.1 Patient Selection

Several cohorts of neonates were recruited as described in chapters 5 and 6. These included:

- **Term infants** born following vaginal delivery or elective caesarean section.
- **PTL and PPROM** - Preterm infants born following preterm labour (PTL) or preterm pre-labour rupture of membranes (PPROM) at less than 32 completed weeks of gestation.
- **IUGR** - Preterm infants delivered by caesarean section at less than 32 completed weeks of gestation for intrauterine growth restriction.

7.2.2 Study design

Blood was sampled from the umbilical vein of the placenta at delivery. Neonatal blood samples were taken on day 1, 2 and 7 of life if possible.

7.2.3 Blood Collection & Handling

Blood collection and handling has been described in section 2.5

7.2.4 Monocyte MHC Class II Expression & Cytokine Assays

The following assays were performed on all blood samples collected, if there was sufficient sample volume:

- Monocyte MHC Class II and HLA-DR expression, as described in 2.6.1.
- Cytokine assays, as described in 2.6.2 and 2.7.
- Whole blood LPS stimulation studies, as described in 2.8.

7.2.5 Neonatal Outcomes

The performance of reduced monocyte MHC Class II expression as a predictor of neonatal morbidity and mortality was examined. In previous work by our group, monocyte MHC Class II expression of less than 60% was associated with greatly increased risk of later sepsis in paediatric ITU patients, calculated with receiver operating curves (301;376). Therefore the predictive value of monocyte MHC Class II expression of less than 60% was assessed in this study in relation to neonatal sepsis and mortality.

7.2.5.1 Episodes Of Sepsis In Term Infants

For the term infant cohort, any admissions to the neonatal unit, episodes of definite or presumed sepsis, and use of antibiotics were recorded. All parents were contacted to ascertain whether there were any admissions for sepsis during the neonatal period, subsequent to their initial discharge following delivery. Definite sepsis was defined as clinical findings suggestive of sepsis together with positive blood cultures, see Table 33. Presumed sepsis was defined as clinical findings suggestive of sepsis together with raised C-reactive protein (CRP) but no positive blood cultures.

7.2.5.2 Neonatal Mortality In Preterm Infants

Any neonatal deaths (death within the first 28 days of life) were recorded. Cause of death recorded on the death certificate was noted.

7.2.5.3 Episodes Of Sepsis in Preterm Infants

Episodes of sepsis were recorded. Sepsis is defined in Table 33. Early onset sepsis was defined as sepsis within the first 7 days of life. The length of the initial course of antibiotics for each infant was recorded. Neonates are frequently started on antibiotics for suspected sepsis. However if all the investigations to search for a causative organism are negative, the neonate improves clinically and the inflammatory markers such as CRP and white count are not supportive of an infection antibiotics will be stopped. In contrast if an organism is isolated, the CRP and white cell count alter significantly or the neonates clinical condition is compatible with sepsis, most authorities would recommend a minimum of 10 days of antibiotics. This was the policy of the UCLH neonatal unit. As such we chose this as a cutoff in our studies.

Diagnosis	Blood Cultures	Other findings
Definite Sepsis	Positive	CRP >10ng/L **AND** clinical signs
Presumed Sepsis	Negative	CRP >10ng/L **AND** clinical signs
Suspected Sepsis	Negative	CRP >10ng/L **OR** clinical signs

Table 33: Diagnosing sepsis, taken from criteria used for neonatal studies of sepsis (377).

Clinical signs were defined as increased apnoeas or bradycardias, increased oxygen requirements, glucose intolerance, acidosis, prolonged capillary refill time, hypotension or irritability.

7.2.5.4 Short Term Neurological Outcome

Cranial ultrasound examinations were performed with a 7MHz transducer (Acuson Corporation, Charleston Rd, Mountain View, CA94043, USA). All infants were scanned as soon as possible after birth and scans repeated daily for the first week of life and then weekly until discharge.

A consultant neonatologist, who was blinded to the assay results, reviewed all images. Periventricular leucomalacia was graded according to the system used by De Vries *et al.* (71).

7.2.5.5 Other Short Term Outcome Measures

Several aspects of each infant's course on the neonatal unit were recorded, see appendix 1. Where infants were transferred to another unit, information was obtained where possible by writing to that unit.

Data was collected regarding medications and inotrope use, complications such as necrotising entercolitis and persistent ductus arteriosus, including the need for medical or surgical intervention. The total number of days and type of ventilation, total number of days of continuous positive airway pressure ventilation (CPAP) and the length of time the infant required inspired oxygen was recorded.

7.2.6 Statistics

MHC Class II and HLA-DR expression were expressed as percentages, and not normally distributed. The numbers in each group for the cytokine assays were small as so all comparisons between groups were performed with the Mann Whitney test. Correlations were performed with Spearman's rank analysis. Multivariable logistic regression analysis was used to evaluate individual variables and the best combinations of variables predicting outcome. Analysis was performed with the Statistical Package for the Social Sciences (version 12.0 SPSS, Chicago, IL.)

7.3 RESULTS

7.3.1 Term infants, Monocyte MHC Class II Expression & Episodes of Sepsis

Outcome data was obtained on fifty infants. Four of these infants developed definite or presumed sepsis in the first week of life. These infants were admitted to the neonatal unit and treated with intravenous antibiotics. All made a good recovery.

Those infants with definite or presumed sepsis (*septic*) had significantly lower levels of monocyte MHC Class II expression in cord blood samples than those infants with no concerns regarding sepsis (*not septic*), see Table 33 and Figure 51.

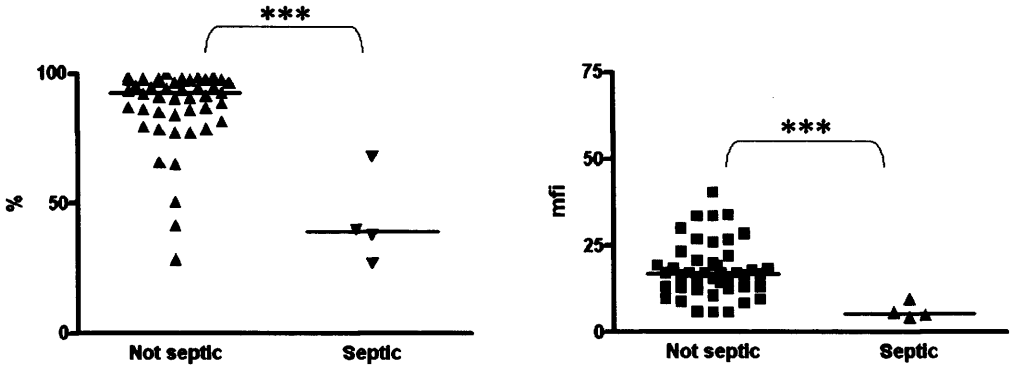

Figure 51: Monocyte MHC Class II expression (% and mfi) in healthy term infants (not septic), and those with definite or presumed sepsis (septic).

Key: *** Mann Whitney *p<0.001*

7.3.1.1 Reduced Monocyte MHC Class II Expression Predicts Sepsis in Term Infants

The performance of reduced monocyte MHC Class II expression as a predictor of neonatal sepsis was examined.

An odds ratio for neonatal sepsis was calculated using a threshold of <60% surface expression of monocyte MHC Class II, see Table 34. Significant odds of sepsis of 43 were found for term infants with a cord monocyte MHC Class II expression of <60%, *p<0.01*.

Cord monocyte MHC Class II expression	Sepsis	Not septic
<60%	**3**	3
≥60%	1	43
	OR 43 (95%CI 8.2 to 225.3)	

Table 34: The odds ratio of sepsis in term infants if cord monocyte MHC Class II expression was <60% was 43 (95%CI 8.2 to 225.3) by Fisher exact test, *p = 0.00389.*

There were no differences between the two groups in terms of cord plasma cytokine levels or cytokine response to whole blood LPS stimulation.

7.3.2 Monocyte MHC Class II Expression & Neonatal Mortality In Preterm Infants

38 infants were studied. The neonatal mortality rate was 15.8% and the perinatal mortality rate was 27.2%. There were six neonatal deaths. In the PPROM group, there were two neonatal deaths, both attributed to pulmonary hypoplasia. There were two neonatal deaths in the PTL group, both attributed to extreme prematurity. There were two neonatal deaths in the IUGR group, one due to sepsis and one due to complications from necrotising enterocolitis.

7.3.2.1 Low Monocyte MHC Class II Expression Is Associated With Neonatal Mortality

The percentage of monocytes expressing MHC Class II was significantly associated with neonatal death, as shown in Figure 52. In those infants who died during the neonatal period, 44.8% of monocytes expressed MHC Class II, compared to 73.0% in those who survived, Mann Whitney *p=0.026*.

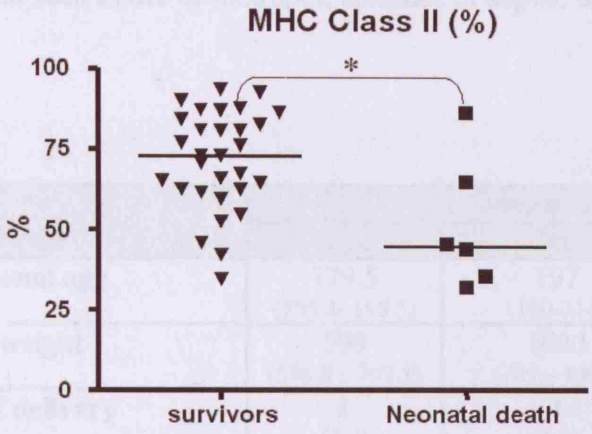

Figure 52: Cord monocyte MHC Class II expression in preterm survivors and neonatal deaths.

Key: * Mann Whitney *p<0.05*

7.3.2.2 Factors Associated With Neonatal Mortality

The most obvious question is whether the reduction in MHC Class II expression seen with neonatal mortality is an independent factor or merely reflects other variables.

MHC Class II expression did not correlate with gestational age at delivery, birthweight, mode of delivery, placental histology coding or duration of rupture of membranes.

The variables that were significantly associated with neonatal mortality are shown in Table 35. These included gestational age and birthweight, as well as cord monocyte MHC Class II expression. No associations were found between neonatal mortality and maternal MHC Class II expression, maternal CRP, placental histology, gender, duration of rupture of membranes, mode of delivery, apgar scores, cord pH, or events on the neonatal unit such as use of inotropes, episodes of sepsis, or cranial ultrasound findings.

Variables	Neonatal Death		
	Yes	No	p value
Gestational age	179.5 (165.3- 195.5)	197 (180-214)	***0.007***
Birthweight	590 (538.8 – 797.5)	1030 (789 – 1402)	***0.002***
Mode of delivery	1 (1-3)	1 (1-2)	0.818
Placental histology	4 (2.5- 6)	2 (1 –4)	0.725
Duration of ROM	0 (0 – 6.8)	0 (0 – 3.8)	0.674
Cord MHC Class II expression %	44.8 (34.8 – 70.4)	73.0 (61.6 – 83.9)	***0.026***

Table 35: Variables and neonatal mortality.

Medians and interquartile ranges (IQR) are shown. Comparisons are made with Mann Whitney test. Mode of delivery groups and placental histology coding are listed in appendix 1.

7.3.2.3 Reduced Monocyte MHC Class II Expression Remains Associated With Neonatal Mortality After Correcting For Other Factors.

In order to further investigate the contribution of MHC Class II expression to neonatal mortality, a logistic regression model was constructed to identify the factors that were independently associated with it.

On univariate logistic regression analysis birthweight and cord monocyte MHC Class II expression are significantly associated with neonatal death, see Table 36.

Variables	Neonatal Death	
	Significance	OR (95% CI)
Gestational age	0.087	0.97 (0.93-1.01)
Birthweight	***0.026***	0.99 (0.992- 0.999)
Mode of delivery	0.829	0.95 (0.61 – 1.48)
Placental histology	0.200	1.24 (0.89-1.71)
Duration of ROM	0.863	1.01 (0.94 – 1.08)
Cord MHC Class II expression %	***0.024***	0.93 (0.88 – 0.99)

Table 36: Univariate logistic regression analysis was performed to assess the contribution of each variable to neonatal mortality.

Birthweight and cord MHC Class II expression (%) significantly contributed to neonatal death. A significance level of <0.1 was chosen to determine which variable would be analysed in the multivariate analysis.

A multivariate logistic regression model was constructed, see Table 37. This identified cord monocyte MHC Class II expression as remaining significantly associated with neonatal mortality, after adjustment for other variables, $p= 0.05$. This indicates that cord monocyte MHC Class II expression is independently associated with neonatal mortality.

Variables	Neonatal Death	
	Significance	OR (95% CI)
Gestational age	0.42	1.04 (0.95-1.14)
Birthweight	0.16	0.99 (0.99- 1.00)
Cord MHC Class II expression %	***0.05***	0.94 (0.88 – 0.99)

Table 37: Multivariate logistic regression analysis which shows that MHC Class II expression remains significantly associated with neonatal mortality.
Variables with a significance of <0.1 on univariate analysis were combined in a logistic regression model in order to control for multiple influences.

There were no correlations between neonatal death and cord plasma cytokine levels, or LPS stimulation results. There were also no correlations between neonatal mortality and maternal monocyte MHC Class II expression, plasma cytokine levels, or LPS stimulation results.

7.3.2.4 Reduced Monocyte MHC Class II Expression Predicts Neonatal Mortality

The performance of reduced monocyte MHC Class II expression as a predictor of neonatal mortality was examined. In previous work by our group, monocyte MHC

Class II expression of less than 60% was associated with greatly increased risk of later sepsis in paediatric ITU patients (301;376).

An odds ratio for neonatal mortality was calculated using a threshold of <60% surface expression of monocyte MHC Class II, see Table 38. Significant odds of neonatal mortality of 7.1 were found for infants with a cord monocyte MHC Class II expression of <60%, *p<0.05*.

Cord monocyte MHC Class II expression	Neonatal Death	Survivors
<60%	**4**	7
≥60%	2	25
	OR 7.1 (95%CI 2.9 to 17.8)	

Table 38: The odds ratio of neonatal mortality if cord monocyte MHC Class II expression was <60% was 7.1 (95% CI 2.9 to 17.8) by Fisher exact test, *p = 0.0466.*

7.3.3. Monocyte MHC Class II Expression & Sepsis In Preterm Neonates

There was a negative correlation between cord monocyte MHC Class II expression and the length of initial course of antibiotic therapy used, see Figure 53. The majority of infants with low cord monocyte MHC Class II expression (<60% or mfi <10) had 10 days or more of antibiotics.

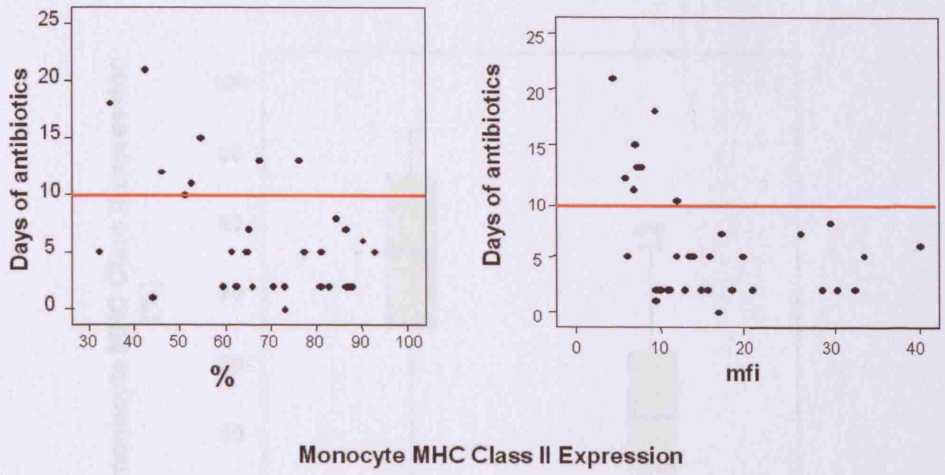

Figure 53: Cord monocyte MHC Class II expression (% and mfi) and number of days of antibiotic treatment.

There was a weak negative correlation between the number of days of antibiotic treatment and cord monocyte MHC Class II expression, Spearman's rank correlation –0.318, *p=0.066* for percentage and –0.359, *p=0.037* for mfi. The solid red line shows that the majority infants with low cord monocyte MHC Class II expression (<60% or <10 mfi) had 10 days or more of antibiotics.

Infants who received 10 days or more of antibiotics had a significantly lower level of cord monocyte MHC Class II expression than those who received less than a 10 day course, Mann Whitney test *p=0.004* see Figure 54.

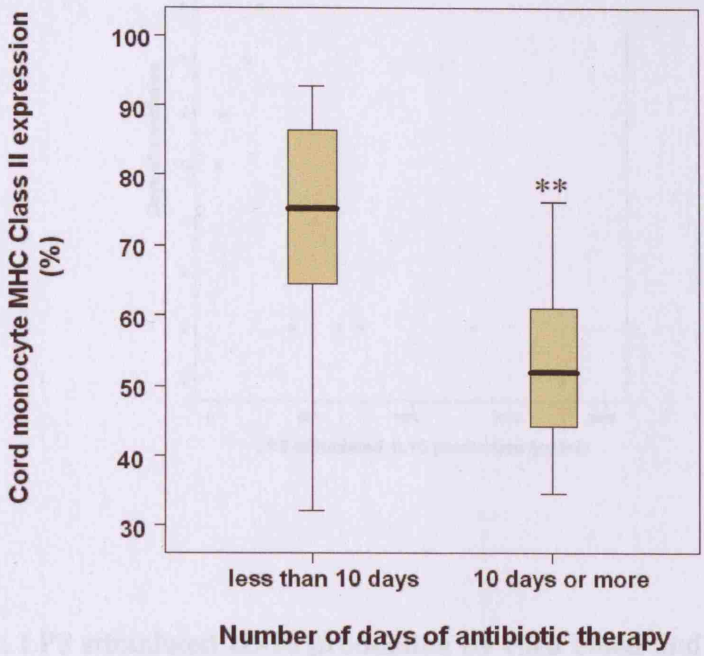

Figure 54: Cord monocyte MHC Class II expression and length of initial antibiotic course.

Median and interquartile range shown. Those infants who received 10 days or more of antibiotics had significantly lower percentage of cord monocytes expressing MHC Class II.

Key: * Mann Whitney *p=0.004*

There was a strong negative correlation between the length of initial course of antibiotic therapy used and the amount of IL-10 produced following LPS stimulation of cord blood, Spearman's rank –0.783 *p= 0.013*, see Figure 55.

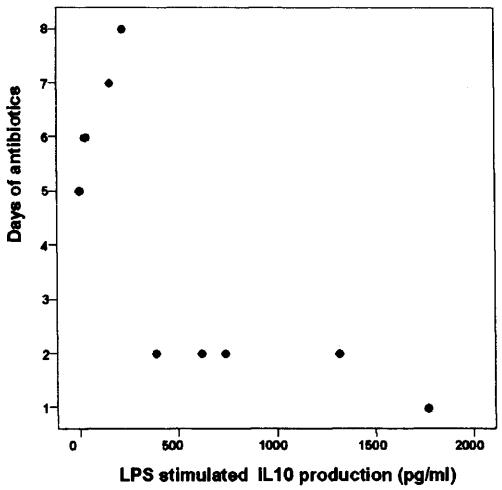

Figure 55: LPS stimulated IL-10 production by cord blood and number of days of antibiotic treatment.

There was a negative correlation between the length of antibiotics given and the amount of IL-10 produced by LPS stimulation, Spearman's rank –0.783 *p= 0.013.*

No clear patterns were seen in monocyte MHC Class II or HLA DR expression in neonates who developed sepsis and those who did not, see Figure 56. There were similarly no trends seen in cytokine levels, response to LPS stimulation or levels of CRP in cord blood between those who developed sepsis and those who did not.

Cord and neonatal CRP levels did not correlate with levels of monocyte MHC Class II or HLA DR expression. However on day two of life, CRP levels did correlate with sepsis. Median CRP levels in those with definite, suspected or presumed sepsis were 11.4g/dL (interquartile range 10.5-12.4) compared to 1.4g/dL (0.6 – 2.2) in those without sepsis, Mann Whitney *p= 0.033.*

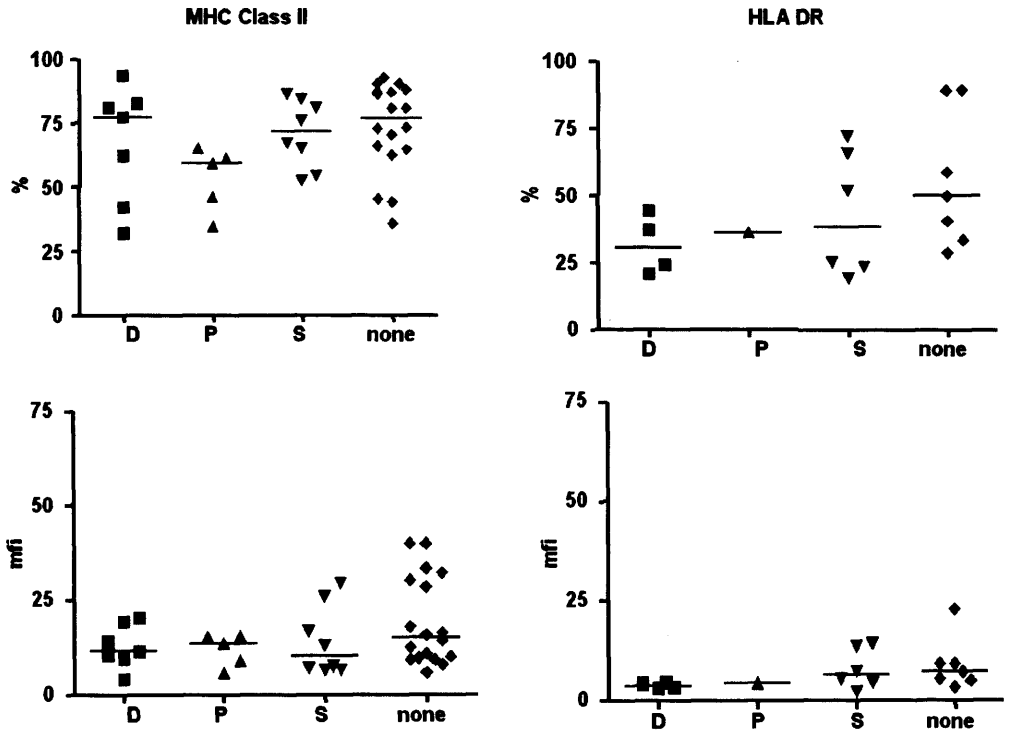

Figure 56: Cord monocyte MHC Class II and HLA-DR expression and episodes of sepsis within the first 7 day of life.

Key: ***D****= definite sepsis,* ***P*** *= presumed sepsis,* ***S****= suspected sepsis,* ***none*** *= no episodes of sepsis.*

There were only 7 positive blood cultures recorded, 4 of which were coagulase negative *Staphylococcus,* which could possibly be containments, one positive culture for *Candida albicans,* one for *Listeria monocytogenes* and one for Group B *streptococcus.*

7.3.3.1 Factors Associated With Sepsis

Correlations between several variables and the length of antibiotic course were investigated. No significant associations were found for variables such as clinical chorioamnionitis, placental histology, gestational age, birthweight, duration of rupture

of membranes, mode of delivery, apgar scores or cord pH, see Table 39. There was a significant association with cord monocyte MHC Class II expression and the length of antibiotic course.

Variables	10 days or more of antibiotics		
	Yes	No	p value
Gestational age	196 (169 –208)	200 (185-216)	0.138
Birthweight	806 (624 – 1242)	1048 (821 – 1469)	0.086
Mode of delivery	4 (2- 5)	3 (1-4)	0.266
Placental histology	4 (1-6)	2 (1-6)	0.162
Duration of ROM	1 (0-2)	0 (0-3.5)	0.863
Cord MHC Class II expression %	51.8 (43.2- 64.2)	75.14 (64.1-86.4)	***0.003***

Table 39: Variables and length of antibiotic course.

Medians and interquartile ranges (IQR) are shown. Comparisons are made with Mann Whitney test.

7.3.3.2 Reduced Monocyte MHC Class II Expression Remains Strongly Associated With Sepsis After Correcting For Other Factors

In order to further investigate the contribution of MHC Class II expression to length of antibiotic course, a logistic regression model was constructed to identify the factors that were independently associated with 10 days or more of antibiotic use.

On univariate logistic regression analysis cord monocyte MHC Class II expression was significantly associated with 10 days or more of antibiotic use, see Table 40.

Variables	10 days or more of antibiotics	
	Significance	OR (95% CI)
Gestational age	0.184	0.98 (0.94- 1.01)
Birthweight	0.096	0.998 (0.996- 1.00)
Mode of delivery	0.197	1.35 (0.86-2.15)
Placental histology	0.848	1.03 (0.77- 0.17)
Duration of ROM	0.444	0.94 (0.80 – 1.10)
Cord MHC Class II expression %	***0.010***	0.92 (0.86- 0.98)

Table 40: Univariate logistic regression analysis was performed to assess the contribution of each variable to the outcome measure of 10 days or more of antibiotics.

Cord MHC Class II expression (%) significantly contributed to neonatal death. A significance level of <0.1 was chosen to determine which variable would be analysed in the multivariate analysis.

A multivariate logistic regression model was constructed, see Table 41. This identified cord monocyte MHC Class II expression as remaining significantly associated with 10 days or more of antibiotic use, after adjustment for other variables, *p= 0.013.* This indicates that cord monocyte MHC Class II expression is independently associated with 10 days or more of antibiotic use.

Variables	10 days or more of antibiotics	
	Significance	OR (95% CI)
Birthweight	0.646	0.999 (0.997- 1.00)
Cord MHC Class II expression %	***0.013***	0.92 (0.86 – 0.98)

Table 41: Multivariate logistic regression analysis that shows that MHC Class II expression remains significantly associated with 10 days or more of antibiotics. Variables with a significance of <0.1 on univariate analysis were combined in a logistic regression model in order to control for multiple influences.

7.3.3.3 Reduced Monocyte MHC Class II Expression Predicts Sepsis

The performance of reduced monocyte MHC Class II expression as a predictor of the use of 10 days or more of antibiotics was examined. In previous work by our group, a threshold of monocyte MHC Class II expression of less than 60% has been found to predict adverse outcome (376).

An odds ratio for 10 days or more of antibiotic use was calculated using a threshold of <60% surface expression of monocyte MHC Class II, see Table 42. Significant odds of 10 days or more of antibiotic use of 23 were found for infants with a cord monocyte MHC Class II expression of <60%, $p<0.01$.

Cord monocyte MHC Class II expression	≥10 days of antibiotic use	<10 days of antibiotic use
<60%	**6**	3
≥60%	2	23
	OR 23 (95% CI 8.3 to 63.9)	

Table 42: The odds ratio of 10 days or more of antibiotic use if cord monocyte MHC Class II expression was <60% was 23 (95% CI 8.3 to 63.9)by Fisher exact test, $p = 0.001438$.

7.3.3.4 Short Term Neurological Outcome

29 infants had their cranial ultrasound images reviewed and scored by one consultant neonatologist from the unit. Nine sets of notes and images were unavailable. The findings of these scans are summarised in Table 43.

12 infants had normal cranial ultrasound images (41.3%). Of the six infants who had an intraventricular haemorrhage, four also developed ventricular dilatation.

There was no statistically significant difference between those infants with lesions such as intraventricular haemorrhage, periventricular leucomalacia or ventricular dilatation and monocyte MHC Class II expression, cytokine levels or response to LPS stimulation.

Cranial Ultrasound Finding		
Intraventricular Haemorrhage	**N**	**%**
No haemorrhage	23	60.5
GLH	3	7.6
Grade II	2	5.3
Grade III	1	2.6
Ventricular Dilatation	**N**	**%**
No dilatation	20	52.6
<97th centile	2	5.3
97th centile to 4mm above	6	15.8
>4mm above 97th centile	1	2.6
Periventricular Leucomalacia	**N**	**%**
None	25	89.5
Grade I	4	10.5
Grade II	0	0
Grade III	0	0
Grade IV	0	0

Table 43: Frequency of lesions seen on cranial ultrasound studies during neonatal period.

GLH = germinal layer haemorrhage

7.3.3.5 Other Short Term Outcome Measures

There were no correlations seen between cord MHC Class II expression, cytokine levels or response to LPS stimulation with a number of outcome measures recorded, including apgar scores, cord pH, the number of days of ventilation, length of time requiring oxygen, episodes of necrotising enterocolitis, hypotension requiring inotropes.

7.4 DISCUSSION

There were a number of problems with the data available for use in this part of the study. The healthy term infants did not have any clinical blood sampling performed so no comparison of CRP levels could be made between those with suspected sepsis and those without. This also means no evaluation of monocyte MHC Class II expression as a marker of sepsis compared to CRP could be made for this group. The diagnosis of sepsis for this group was that of presumed sepsis due to clinical findings rather than a gold standard diagnosis of a positive blood culture together with the clinical picture.

There are also several shortcomings with the follow up data of the preterm cohort. There were relatively small numbers of infants studied, including only six neonatal deaths. Infants were often transferred out to other neonate units as they were weaned off ventilatory support and did not require further intensive care. This made data collection difficult and also led to variation in clinical care received by individual infants in the study group. This was a pilot study and it is planned to continue to

recruit patients to the study and collect longer term follow up data. This will enable singleton and multiple pregnancies to be analysed separately.

Again the diagnosis of sepsis in the preterm neonate is difficult. Few infants had positive blood cultures and over 50% of these were coagulase negative *Staphylococcus,* a possible containment. We used a surrogate marker, the number of days of antibiotic use. This gives an indication of how concerned the attending clinicians were regarding the possibility of sepsis in the infant but is not a standardised measure of sepsis.

A further limitation of this study is the length of follow up. Cranial ultrasound imaging in the early neonatal period is not the most accurate predictor of long-term neurodevelopmental outcome (378) and perhaps it is not surprising that we did not see any correlations between cytokine balance and cranial ultrasound findings. It is our intention to follow up this cohort of infants at one year corrected gestational age with an MRI study and a Griffiths neurodevelopmental assessment.

In spite of these shortcomings, there were a number of findings that indicate the potential significance of detecting a low monocyte MHC Class II level in neonates. The most striking finding of this study was the association of low levels of MHC Class II expression on cord monocytes and poor neonatal outcome in the preterm cohort, with odds ratios of 7.1 for neonatal mortality and 23 for the need for an antibiotic course of 10 or more days.

This association of low levels of MHC Class II expression on cord monocytes and sepsis was also seen in the term neonates, with an odds ratio of 43 for presumed sepsis.

No relationship between levels of monocyte MHC Class II expression and cranial ultrasound findings were seen in this cohort of preterm infants.

There is some work demonstrating low levels of expression of MHC Class II on monocytes in neonates but this has not been correlated to outcome or reason for delivery (322).

The reasons why MHC Class II expression is associated with the subsequent development of sepsis and neonatal mortality are unclear. Mortality is higher in the more extremely preterm infant but monocyte MHC Class II expression was independent of birthweight and gestational age. In fact monocyte MHC Class II expression was the only significant factor in the multivariate analyses for length of antibiotic therapy and neonatal mortality.

Lowered monocyte MHC Class II expression may be a mechanism by which an initial inflammatory insult (possibly chorioamnionitis or placental insufficiency) attenuates the subsequent pro-inflammatory response to a second insult, so called *immune paresis.* This adaptive response may be designed to protect the individual from excessive pro-inflammatory immune stimulation. However in the preterm neonate in the foreign environment of the neonatal unit, it may render the host susceptible to nosocomial infections and even death. This theory of *immune paresis* has lead to

adult trials of administration of substances such as IFNγ in an attempt to restore the host's ability to mount a pro-inflammatory response with some success (298). In the neonatal population a trial of GM-CSF reduced episodes of sepsis (377).

This does not address what the effect of low monocyte MHC Class II expression in cord blood may have on long-term outcome. There is some evidence that anti-inflammatory cytokines may have a neuroprotective effect on the perinatal brain (108). Therefore low levels of monocyte MHC Class II expression may reflect an anti-inflammatory cytokine milieu, which may have adverse short-term neonatal consequences (such as increased risk of sepsis) but may provide some neuroprotection to the developing brain. Long term follow up of these preterm infants is required to address this issue.

CHAPTER 8 – GENERAL DISCUSSION

8.1 PRETERM DELIVERY & PERINATAL OUTCOME

Preterm delivery accounts for 70% of all perinatal mortality and nearly half of long-term neurologic morbidity (3;379). These complications are concentrated in the 1-2 % of infants born at less than 32 weeks gestation or weighing <1500g (6).

Evidence points towards materno-fetal inflammatory pathways with release of pro-inflammatory cytokines, such as IL-6 and TNF-α, as key in the initiation of preterm labour as well as contributing to neonatal complications such as PVL. However, the direct relationship between maternal-fetal infection/inflammation and neonatal outcome remains unclear as histological chorioamnionitis is found in up to 60% pregnancies delivering prematurely, but significant white matter damage or bronchopulmonary dysplasia is only seen in a minority of these neonates. Some studies have found an association between chorioamnionitis and cerebral palsy whereas others have not (73;88). These conflicting data contribute to one of the major dilemmas facing obstetricians and neonatologists – when is the appropriate time to deliver the fetus of a mother with preterm pre-labour preterm rupture of membranes?

The work undertaken in this thesis contributes to the understanding of the inflammatory processes occurring in these preterm infants and their mothers, and highlights areas for future research.

8.2 THE MATERNAL INFLAMMATORY RESPONSE TO LABOUR, AT TERM AND PRETERM

This thesis has demonstrated that labour is an inflammatory event with an anti-inflammatory response as well as the pro-inflammatory response previously described.

8.2.1 Alteration of Monocyte MHC Class II Expression During Pregnancy & Labour

Free fetal DNA has been found in maternal plasma at significant quantities by the end of the first trimester (352). However in this study a reduction in maternal MHC class II was not seen until the onset of labour.

While the fall in maternal MHC Class II expression seen in term and preterm labour may be a protective measure to fetal antigen exposure as described above, it could just reflect her inflammatory status during the peri-partuum period.

Normal parturition involves a pro-inflammatory phase, which is thought to initiate labour. In this study evidence for pro-inflammation came from elevated levels of IL-6 and IL-8. From this work it is now also clear that there is also evidence of an anti-inflammatory process. Monocyte MHC Class II levels were reduced, plasma IL-10 levels were elevated, plasma TNFα was undetectable and upon *in-vitro* stimulation TNFα production was reduced. It would appear that by the time labour is established, the maternal immune system could be entering the anti-inflammatory phase of its

response. This relative immune paresis may indicate that the mother is unable to mount an adequate response to any secondary insults that she may encounter, and thus may be susceptible to infection in the peripartum period. In contrast to the descriptions of obstetric care in the time of Semmelweis (380) puerperal sepsis is not a major problem in the developed world, probably due to the availability of antibiotics and aseptic techniques. However it is interesting to speculate that the high rate of infection in the peripartum period in the past and in the developing world may in part be due to immune paresis.

The neuroendocrine system plays an important role in the onset of labour, and also in the response to insults such as infection (194;381). It affects immune response through the hypothalamic pituitary adrenal (HPA) axis and the autonomic nervous system. Hormones such as glucocorticoids and both the parasympathetic and sympathetic nervous systems have been shown to regulate the pro-inflammatory response to insults and also to increase production of anti-inflammatory cytokines such as IL-10 (161). There also appears to be feedback on the HPA axis and autonomic nervous system by cytokines such as TNFα and IL-10 (382;383). Therefore the neuroendocrine system may play a role in the initiation of preterm labour and the immune response of mother and fetus.

Women in preterm labour showed even more evidence of *immune paresis*. The monocytes from these women had even lower MHC Class II expression and more pronounced suppression of pro-inflammatory cytokine production in response to LPS stimulation. Interestingly, levels of IL-10, which were detected in the circulation of mothers of term deliveries, were undetectable in the mothers of preterms. This may

reflect a more chronic inflammatory process, which in turn may be play a role in the aetiology of preterm labour and PPROM itself (29;34).

8.2.2 Low Levels of Expression Of MHC Class II on Maternal Monocytes Predicts Preterm Delivery

The majority of mothers who delivered at term had monocyte MHC Class II expression levels of greater than 90% through out pregnancy, with levels dropping only during labour. In contrast, some mothers who subsequently delivered prematurely had reduced levels of monocyte MHC Class II on admission. Futhermore the levels dropped prior to the onset of labour.

The reasons for this fall in monocyte MHC Class II expression are unknown. However, there was a strong correlation with chorioamnionitis, which indicates maternal MHC Class II expression may be reflecting events occurring in the uterine environment.

8.3 THE RESPONSE OF THE TERM & PRETERM FETUS TO LABOUR AND DELIVERY

MHC Class II expression on monocytes in cord blood was significantly lower after PTL and PPROM than term labour. This may merely reflect the gestational age of the fetus. This appears unlikely as in a small number of fetuses obtained during 2nd trimester, (undergoing feticide for structural abnormality), levels of monocyte MHC Class II expression were comparable to cord blood from term infants. It is more likely

that the low levels in the preterm delivery groups were related to inflammatory activation, rather than being gestation related.

In support of this concept are the data showing negative correlations between monocyte MHC Class II expression and chorioamnionitis. Furthermore circulating levels of the pro-inflammatory cytokines, IL-6 and IL-8, were much higher in pre-terms than in term deliveries. Consistent with the concept that more "severe" inflammation *in utero* results in low MHC Class II expression are the data showing that cytokine production in response to an *in-vitro* endotoxin challenge is reduced in the preterm neonate. This has been described as *immune paresis*, where prior exposure to an inflammatory challenge renders monocytes tolerant to endotoxin (384).

8.3.1 Significance Of These Findings To The Preterm Infant

The preterm neonate may not be able to mount an appropriate inflammatory response to any further insults received in the perinatal period. During this period there are particular risks to the preterm neonate such as hypoxia-ischaemia during delivery, infection from indwelling long lines and arterial lines, translocation of bacteria from gut, barotrauma from ventilation etc (370;385). These insults may be sufficient to propagate an excessive anti-inflammatory state, increasing the risk of sepsis and death. This multi-hit hypothesis where chorioamnionitis primes the patient's immune response *(endotoxin tolerance)* but a second insult is required to drive the loss of homeostasis would fit in well with these data. This would be consistent with the findings of Kaukola *et al.* who found that isolated features such as raised pro-inflammatory cytokines or chorioamnionitis alone were not risk factors for poor

neurological outcome in preterm infants, but several factors in combination were (386).

Much of the research presented in this thesis is observational and as such these data should be interpreted with caution. However the findings that term infants with low levels of monocyte MHC Class II expression were 43 times more likely to be admitted for treatment of presumed sepsis, that preterm infants with low levels of monocyte MHC Class II expression were 23 times more likely to need 10 days or more of antibiotics and 7 times more likely to die, supports a view that the processes involved in reducing monocyte MHC Class II expression are important.

8.4 LIMITATIONS OF THIS WORK

There are many limitations of the work presented here. Some of these are inevitable in clinical studies involving preterm neonates. There are many potential confounders and unlike animal models, patients will be exposed to a range of insults over a varying time course.

The data presented in this thesis regarding monocyte hyporesponsiveness arises from studies performed in whole blood. An assumption has been made that the changes observed are reflecting changes in monocytes. However monocytes are only one group of cells operating in a complex inflammatory environment.

Maternal peripheral blood was used as it is easy to obtain but is distal from the site of interest, the uterus. The results presented here may not reflect the *in-utero* levels of

inflammatory mediators or cytokine balance. Similarly umbilical cord blood may not reflect the environment in the fetal tissues such as the brain or lung. However it would be difficult to design a study acceptable to an ethics committee directly examining the *in-utero* or fetal environment.

Monocytes have a short life span in the circulation ($t_{1/2}$ = 17 hours). Therefore a possible explanation for the changes in MHC Class II seen could be due to changes in the circulating population of monocytes. If the inflammatory stimulus of PTL resulted in all the monocytes expressing high levels of MHC Class II leaving the circulation, one could expect to see a significant fall in the number of circulating monocytes. This did not occur.

Feticide samples were collected in order to provide gestation-matched controls for the preterm neonates. These were obviously not normal fetuses as they were undergoing feticide for major structural anomalies, but were not exposed to the inflammatory stimulus of labour. It would not be possible to gain ethical approval for a study performing cordocentesis on normal fetuses.

The major limitations of the work presented here were that this is a pilot study with small patient numbers, and the restriction in the volume of blood available for analysis. Only short-term neonatal outcome measures were available, leaving many questions unanswered regarding the effects of immune paresis in the perinatal period on long-term neurodevelopmental outcome. The aim is to continue recruiting patients in order to obtain larger numbers for analysis, enabling multiple and singleton pregnancies to be analysed separately.

8.5 FUTURE RESEARCH

There are four main areas where the work initiated in this project should be continued:

- When & why does the fall in monocyte MHC Class II expression occur?
- Considering the role of genetic polymorphisms in variation of neonatal outcome
- Investigating the role of *in-utero* infection in cytokine balance in preterm neonate
- Investigating the effects of variation in cytokine balance and long-term outcome.

8.5.1 When Does The Fall In Monocyte MHC Class II Expression Occur?

Accepting the limitations described above, the work presented in this thesis has consistently shown a fall in maternal monocyte expression of MHC Class II expression in labour, whether at term or preterm. What is not known is when this fall occurs in relation to the process of labour and how long it takes to recover postpartum. As suggested above, this fall may be part of an adaptive response designed to protect the mother from excessive pro-inflammatory activation, which can be deleterious. There is some evidence that neuroendocrine changes, such as an increase in CRH levels, occur several days, if not weeks, prior to labour (194). Changes in MHC Class II expression may alter in response to this neuroendocrine signalling and therefore may be a marker of the risk of preterm labour. The studies presented here could be supplemented by longitudinal studies of maternal MHC Class

II expression to assess when levels fall and how long they remain suppressed for in the postpartum period.

The current studies illustrated that a fall in MHC Class II expression was seen in the preterm neonate. Again it is unclear when this fall occurs but it would be very difficult to address this in human pregnancies, as it would involve cordocentesis. An animal model such as the fetal sheep would be a more appropriate tool to address this issue.

8.5.2 Why Does The Decrease In Surface Expression Of MHC Class II Expression On Monocytes Occur?

The mechanism by which the reduction in monocyte surface expression of MHC Class II occurs is not addressed by this study. In children undergoing cardiopulmonary bypass, the postoperative fall in monocyte MHC Class II expression was associated with a depletion of intracellular MHC Class II stores and a subsequent increase in transcription of genes required for production. It has been hypothesised that the fall may be due to cleavage or secretion of MHC Class II (376). In contrast, in sepsis there is an accumulation of intracellular MHC Class II in monocytes, due to problems with recycling and exocytosis of MHC Class II molecules (307).

The mechanisms leading to monocyte surface expression of MHC Class II could be investigated using intracellular staining techniques, confocal microscopy and semi-quantitative assessment of monocyte MHC Class II related mRNA levels.

8.5.3 Considering The Role Of Genetic Polymorphisms In Variation Of Neonatal Outcome

In the current work presented, one striking finding is the wide variability in monocyte MHC Class II expression seen in the preterm and term infants. Potential factors include: 1) the presence of genetic polymorphisms that favour either a pro or anti-inflammatory response 2) the stage of the inflammatory process - following an inflammatory stimulus, the balance between pro- and anti-inflammation may vary with time and 3) the nature of the insult – infection, hypoxia-ischaemia, trauma and labour can all trigger a cytokine response but may have different effects on cytokine balance.

Several studies have suggested that cytokine responses are under significant genetic control (293;387). Studies of polymorphisms in cytokine and MHC Class II related genes could assess whether an individual is genetically predisposed to respond to an inflammatory insult in a particular way. This could lead to the stratification of mothers and fetuses according to their risk of immune dysregulation and aid the targeting of immune modulating treatment in the future. DNA samples have already been collected from patients recruited for this work so that genotyping for MHC Class II and cytokine polymorphisms can be undertaken.

8.5.4 Investigating The Role Of *In-Utero* Infection In Cytokine Balance In Preterm Neonate

Bacterial components, including endotoxins, are potent activators of the inflammatory response. Chorioamnionitis has been associated with a fetal inflammatory response

and morbidity such as white matter injury and chronic lung disease. However not all studies demonstrate this link. Histological chorioamnionitis is presumed to represent bacterial infection, but often microbiological investigations are inconclusive.

The 16S rDNA gene is found in all bacteria and contains highly conserved regions as well as areas with species-specific sequence. Using appropriate PCR primers it is theoretically possible to detect the presence of bacterial DNA from any species in any specimen and then to identify it by sequencing the PCR product. Samples of placental tissue have been collected from the patients in this work in order to investigate the hypothesis that an important factor in determining the level of MHC Class II expression on monocytes in cord blood is fetal exposure to bacteria *in utero*.

8.5.5 Investigating The Effects Of Variation In Cytokine Balance & Long-Term Outcome

The work presented here has described the cytokine balance in preterm infants. An obvious question is whether the wide variability in monocyte MHC Class II expression seen is predictive for long term neurodevelopmental outcome. It is planned to offer the patients recruited for this work follow up with magnetic resonance imaging (MRI) and formal neurodevelopmental assessment at 1 year of age corrected for prematurity using the Griffiths Developmental Scales.

The MRI will use new techniques such as diffusion tensor imaging enabling measurement of several aspects of brain development such as white matter

myelination, cortical folding and analysis of grey and white matter volumes, all factors known to be affected by perinatal brain damage.

These outcome measures can then be correlated with cord plasma cytokine levels, monocyte MHC Class II expression and response to whole blood LPS stimulation. This may provide more information regarding pathogenesis of the neurodevelopmental problems seen in infants born prematurely.

8.6 CURRENT CONCEPTUAL MODEL

From the results obtained in this thesis together with existing reports in the literature, it is possible to map out the potential inflammatory sequences occurring in the mother and fetus around the time of delivery.

Labour elicits a systemic inflammatory response in the mother, which is even more marked in preterm labour. There is a pro-inflammatory wave of cytokines and also an anti-inflammatory response, as measured by the reduction in monocyte surface expression of MHC Class II. In the neonate, there is little evidence of imbalance of the immune response during term labour but a marked response to preterm labour. A pro- and anti-inflammatory response is seen, together with evidence of altered monocyte function. This *immune paresis* appears to be associated with neonatal mortality and morbidity.

APPENDIX 1: DATA COLLECTION SHEET

Data collected	Details
Maternal demographics	Age Ethnicity Smoking status
Obstetric History	Parity Miscarriages Terminations History of PTL, PPROM, cervical incompetence or surgery
Current Pregnancy	Conception LMP EDD
Problems	Antepartum haemorrhage, placenta praevia, cervical cerclage, pre-eclampsia, preterm labour, rupture of membranes, clinical chorioamnionitis
Investigations	Ultrasound scans, amniocentesis, TORCH screen, microbiology and laboratory investigations
Medications	Administration of corticosteroids, tocolysis, antibiotics etc
Delivery	Gestation, onset of labour, duration of rupture of membranes, mode of delivery, cord gases, birthweight, apgar scores, sex
Placenta	Histology and microbiology results
Admission to neonatal unit	Ventilatory and oxygen requirements, blood transfusions complications, medications, use of antibiotics, episodes of sepsis and necrotising enterocolitis, discharge details and weight
Neonatal investigations	Cranial ultrasound reports, hearing & retinopathy of prematurity screening, laboratory and microbiology results

study id	Gestational age (days)	MHC Class II		HLA-DR		IL-12 (pg/ml)	TNF (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)	IL-1 (pg/ml)	IL-8 (pg/ml)	TGF (pg/ml)	LPS IL-12 (pg/ml)	L T (pg
		%	MFI	%	MFI									
1003	175	35.70	10.00	.	.	1	1	27.7	61.9	1	541.5	.	.	
1005	175	59.43	15.12	.	.	1	1	1	254	1	34.4	192.98	1	16
1008	197	62.50	14.33	.	.	6	9	8.2	77.4	142.4	1319	.	1	20
1013	186	61.38	13.46	.	.	15.6	5.6	43.9	840.7	144.6	324.3	.	.	
1019	238	70.49	12.52	.	.	1	3.1	1	9.3	1	19.8	60.55	36	5
1046	203	64.61	5.83	33.38	3.34	1	1	1	344.1	1	278.1	70	.	
1048A	180	80.95	10.46	37.25	4.26	30	15.9	44.7	192.8	5532.4	10000	0	.	
1048B	180	93.59	13.95	24.46	3.05	27.4	13	3	192.8	224.1	602.8	25.73	.	
1058	208	51.12	11.65	17.54	6.21	1	1	1	9.8	1	31.5	.	.	

APPENDIX 2: CORD BLOOD RESULTS FOR PPROM GROUP

Study ID	Gestational age (days)	Class II		HLA-DR		IL-12 (pg/ml)	TNF (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)	IL-1 (pg/ml)	IL-8 (pg/ml)	TGF (pg/ml)	LPS IL-12 (pg/ml)	L T (pg
		%	MFI	%	mfi									
1001	157	45.49	15.75	.	.	1	1	1	993.3	215.2	1640	1	1	3
1006	203	84.26	29.43	.	.	3.1	1	1	55.8	1	20.5	1	1	2
1010	203	80.84	30.23	.	.	5.2	3.1	49.9	8.3	1	30.2	.	.	
1010	203	86.92	32.20	.	.	1	1	1	1	1	20.5	.	1	9
1011	217	44.00	9.14	.	.	6	4	3.9	7.8	1	14.2	1	1	12
1014	263	73.07	16.40	.	.	5.1	4.7	3.8	188.2	1	102.3	.	.	
1015	174	62.29	20.35	.	.	7.6	7.1	6.6	20	104.1	47.7	.	.	
1025	169	34.58	9.06	.	.	.	.	.	.	.	.	.	.	
1030	176	76.23	7.50	24.97	5.38	4.3	1	5.4	205.8	1	944.6	.	.	
1039A	168	67.43	7.04	19.18	2.21	1	1	1	1	1	19.5	.	.	
1039B	168	82.72	9.56	44.52	4.74	.	.	.	.	.	.	8.68	.	
1043	223	80.87	7.91	49.87	5.38	5.6	3.5	1	52.4	1	64.1	.	19	
1053A	196	86.40	9.73	89.2	9.22	1	1	1	6.2	1	21.5	.	.	
1053B	196	65.94	9.22	40.44	7.23	1	1	1	6.6	1	18.8	1	.	
1054	202	42.29	4.24	21.07	3.25	1	3.3	42.5	5171.6	1	929.3	1	.	
1059	174	80.95	12.98	51.93	7.27	15.1	1	1	19.1	1	43.3	3.07	.	

APPENDIX 3: DATA FOR CORD BLOOD FROM PTL GROUP

stational age (days)	Class II %	Class II MFI	HLA-DR %	HLA-DR mfi	IL-12 (pg/ml)	TNF (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)	IL-1 (pg/ml)	IL-8 (pg/ml)	TGF (pg/ml)	LPS IL-12 (pg/ml)	LPS TNF (pg/ml)	LPS IL-10 (pg/ml)	LPS IL-6 (pg/ml)	LPS IL-1 (pg/ml)	LI IL (pg
194	32.06	11.55	.	.	1	1	1	1	1	51.5	.	1	1	1	1	1	
184	65.00	15.40	.	.	5.3	4.9	59.1	44.6	1	123.4	.	.	.	.	.	.	.
213	90.20	39.95	.	.	5.4	4.3	5.7	4.6	106.5	43.5	31.63	4.3	63.1	30.1	4251.7	76.1	
201	77.21	19.28	.	.	3.1	1	3.4	3.3	1	50.5	.	.	.	.	.	.	.
186	65.12	16.70	.	.	3.1	1	3.6	6.6	1	67.1	203.96	21	1112	153	9776	1247	17
183	72.92	10.75	58.59	9.22	1	3.2	1	3.9	1	59.4	.	.	.	.	.	.	.
225	87.82	17.94	28.58	4.96	28.9	4.3	5.2	25.9	133.3	34.5	36.77	.	.	.	.	.	.
212	92.66	33.38	.	.	4.7	5.6	10.8	253.8	1	179	40.98	.	.	.	.	.	.
196	86.39	25.95	65.73	14.33	89.7	4.7	3.2	64.4	608.9	84.6	0	.	.	.	.	.	.
208	52.50	6.61	23.30	4.53	9.9	11.9	15.7	237.6	133	245.3	200	.	.	.	.	.	.
208	45.93	5.67	36.34	4.37	8.5	11.9	13.8	658.9	101.9	464	300	.	.	.	.	.	.
214	87.20	28.39	89.26	22.98	3.7	7.1	5.2	5.6	1	38.2	160	.	.	.	.	.	.
196	54.63	6.73	72.24	13.58	1	1	1	8.3	1	90.2	312	.	.	.	.	.	.

APPENDIX 4: CORD BLOOD RESULTS FOR IUGR GROUP

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