Developmental Innate Immunoinsufficiency: Comparison of Term Neonatal Neutrophil Proteinases and Complement Component levels relative to Adults

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Summary of thesis

Despite current improvement in newborn care, infection is still a common cause of neonatal morbidity and mortality. Innate immunity is the first line of defence against pathogens particularly in newborn infants. Quantitative and functional deficit in non-cellular (complement system) and cellular (neutrophils) arms of innate immune system is believed to contribute to neonatal susceptibility to infection.

Neutrophil granule subsets contain a variety of proteases, including elastase, cathepsins, MMP-9 and proteinase 3 along with different granule markers and receptors. This thesis demonstrated that normal term neonatal neutrophils express more proteinase 3 and CD177 on their surface while no differences were found in expression of markers CD35, CD66b and CD63 (representing the secretory, secondary and primary granule subsets, respectively). Cord neutrophils contain more PR3 than adult cells but the proportion of PR3 released by cord and adult neutrophils was similar. In contrast, neonatal neutrophils contained only half of the cathepsin G and elastase functional activity of adult neutrophils.

Bronchoalveolar lavage fluid studied from preterm infants ventilated for respiratory distress demonstrated higher proteinase 3 concentrations in lavage samples from infants who went on to develop chronic lung disease than in infants with resolved respiratory distress syndrome. Concentration of proteinase 3 in lavage samples was significantly higher than MMP-9 and elastase levels, suggesting that it may have an important role in disease pathogenesis.

Complement is an equally important component of the innate immune system that plays a central role in recruiting and activating neutrophils, as well as being directly bactericidal through the terminal lytic pathway. Analysis of neonatal complement system revealed that Complement function and terminal components levels particularly C9 are deficient (except C7) in healthy term newborn infants compared to normal adults. Bactericidal capacity of a selection of neonatal sera was tested along with adult sera against four serovars of *Ureaplasma parvum,* a potentially important perinatal pathogen. Results showed impaired bactericidal capacity of neonatal serum compared to adult serum especially against SV1. *Ureaplasma* SV3 was the most serum sensitive serovar whereas killing of the resistant serovars SV6 and 14 could not be induced by supplementation of the deficient components C6, C8 and C9.

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Abbreviations

AAT	Alpha-1-antitrypsin
AAT-E	Alpha-1 anti-trypsin- elastase
Ab	Antibody
ACT	Alpha-1-antichymotrpsin
AGA	Appropriate for gestational age
Ag	Antigen
ANCA	Anti- neutrophil cytoplasmic autoantibodies are
AP	Alternative pathway
BAL	Bronchoalveolar lavage
BPD	Bronchopulmonary displasia
BPI	Bactericidal paermeability increasing protein
BSA	Bovine serum albumin
CCU	Colour changing units
cGA	Corrected gestational age
CH50	Complement haemolytic activity
C1INH	C1 inhibitory
CLD	Chronic lung disease
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitors
CMV	Cytomegalo virus
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CP	Classical pathway
CR1	Complement receptor 1

CRP	C Reactive Protein
CSF	Cerebro- spinal fluid
CVF	Cobra venom factor
C5aR	Complement receptor 5a
C3aR	Complement receptor 3a
DAF	Decay accelerating factor
DCs	Dendritic cells
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FH	Factor H
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GA	Gestational age
Cat G	Cathepsin G
G-CSF	Granulocyte-colony stimulating factor
GM-CFU	Granulocyte- macrophage colony forming unit
GPI	Glycosyl-phosphatidylinositol
HAE	Hereditary angioedema
HBP	Heparin binding protein
HBSS	Hanks buffered saline solution
hCAP 18	Human Cathelicidin Antimicrobial pro-peptide
HIE	Hypoxic ischaemic encephalopathy
HI-NHS	Inactivated normal human serum
HLE	Human leukocyte elastase
HNP 1-3	Human neutrophil peptides 1-3
HPA	Health Protection Agency
HRPO	Horseradish peroxidase

HUS	Haemolytic uremic syndrome
ICAM-1	Intercellular adhesion molecule-1
ICs	Immune complexes
IL-8	Interleukin-8
IL-6	Interleukin-6
IUGR	Intra-uterine growth retardation
LPS	Lipopolysaccharides
mAb	monoclonal antibodies
MAC	Membrane attack complex
MAS	Meconium aspiration syndrome
MASP-2	MBL-associated serine protease -2
MASPs	MBL-associated serine proteases
MBL	Mannan-binding lectin
MCF	Mean cellular fluorescence
MCP	Membrane cofactor protein
MFI	Mean fluorescence intensity
MMP	Matrix metalloproteinase
MNEI	Monocyte neutrophil elastase inhibitor
MPO	Myeloperoxidase
mPR3	Membrane proteinase 3
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NCPAP	Nasal continuous positive airway pressure
NEC	Necrotising enterocolitis
NETs	Neutrophil extracellular traps
NF-KB	Nuclear factor KB
NGAL	Neutrophil gelatinase associated lipocalin

NHLBI	National Heart, Lung and Blood Institute
NHS	Normal human serum
NICHD	National Institute of Child Health and Human Development
NICU	Neonatal intensive care unit
NPP	Neutrophil Proliferative Pool
NSP	Neutrophil Storage Pool
NSPs	Neutrophil serine proteases
OPD	Ortho-Phenylenediamine
OR	Odds ratio
PAD4	Peptidylarginine deiminase 4
PAF	Platelet activation factor
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween 20
PCR	Polymerase chain Reaction
PDA	Patent Ductus arteriosus
PGRN	progranulin
PI	Isoelectric point
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear cells
PMSF	Phenylmethanesulphonyl fluoride
PPP	Platelet poor plasma
PPV	positive-pressure ventilation
PROM	Premature rupture of membrane
PRRs	Pattern recognition receptors
PR3	Proteinase3
PRV-1	Polycythemia rubra vera-1
PT	Preterm

PVP	Polyvinylpyarrolidone
RA	Room air
RCA	Regulators of Complement Activation
RDS	Respiratory distress syndrome
ROS	Reactive oxygen radicals
RPE	R- phycoerythrin
RT	Room temperature
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SGA	Small for gestational age
SLE	Systemic Lupus Erythematosus
SLPI	Secretory Leukoprotease Inhibitor
SP-A	Surfactant protein A
SP-D	Surfactant protein D
SRBCs	Sheep erythrocytes
SV	Serovars
TAF	Tracheal aspirate fluid
TCC	Terminal complement component
TIMP	Tissue Inhibitors of Matrix Metalloproteases
TLR	Toll-like receptor
TNF-α	Tumour Necrosis Factor alpha
USM	*Ureaplasma* specific medium
VBS	Veronal buffer saline
VLBW	Very low birth weight

Chapter One

Introduction

1.1 Innate immunity. Overview

The innate immune system is the first line of defence against pathogens and several mechanisms contribute to its function. The cellular components of innate immunity comprise a variety of cells that first recognize microbes by "foreign" pattern-recognition receptors (PRR) and then respond to them by launching defensive measures. Immediately after microbial invasion, these cells are recruited in increasing numbers to sites of infection. The innate immune system also informs and directs the response of the adaptive immune response through the release of bioactive peptides (complement fragments, chemokines, etc.) that leads to the evolution and maturation of a primary humoral and cellular immune response as well as the capacity for immune memory, for faster and more specific responses to repeat microbial exposure. The adaptive response is also important for clearing microbes that evade or overcome the innate immune system. While the innate immune response is quick and can act without delay, the adaptive immune response takes days or weeks to develop (Kindt *et al*., 2007; Murphy *et al*., 2008).

Cells of the innate and adaptive immune systems are derived from common pluripotent haematopoetic progenitors called stem cells. These stem cells give rise to i) common lymphoid progenitors (CLP) which further differentiate into all cells of the lymphoid lineage, including T and B lymphocytes, and ii) common myeloid progenitors (CMP), that in turn give rise to granulocytes, monocytes, mast cells, and dendritic cells (DCs), all of which are involved in innate immune responses (Kindt *et al*., 2007; Murphy *et al*., 2008). However, serum proteins that make up the non-cellular components of the innate system are usually made by non-haemopoetic organs such as the liver (complement) or lung (surfactant proteins SP-A and SP-D).

Complement is a serum protein cascade integral to the innate immune response, and its role in combating invasive pathogens is more prominent in newborns owing to their immature adaptive immunity. Neutrophils represent the most abundant cellular component of the innate immune response. Infection remains a leading cause of neonatal morbidity and mortality and is linked to several quantitative and functional defects in complement as well as other humoral and cellular immune components especially neutrophils.

In this introduction I aim to review the current literature about functional capacity of neutrophils and complement system in innate immunity of newborn infants with emphasis on neutrophil proteases in combating infection and their untoward effect implicated in pathogenesis of chronic lung disease. This review is also focused on serum levels and function of terminal complement components in full term newborn infants.

1.2 Neutrophils

1.2.1 Neutrophil origin and structure

Neutrophils are key components of innate immunity and the most effective killing phagocytes in the arsenal of cellular host defence. They were first described by Metchnikoff in 1892 who named them microphagocytes in comparison with macrophages. Neutrophils are the most abundant phagocyte in blood with 40-80 % of circulating leukocytes being neutrophils. There are 1.2 to 7.6 $x10^9$ neutrophils per litre of blood in healthy person which can instantly accumulate in large numbers at the site of infection reaching over 20 $x10^9$/L within 24 hours in diseases such as pneumonia and septicaemia (Friedland *et al*., 1992; Braun *et al*., 1997). The neutrophil has a short life span in circulation 8-20 h compared with 1-4 days in tissue (Ogura, 1999). The transfer of neutrophils from blood to tissue is considered to be 'one way only' with fewer than 5% of the total number of neutrophils in the body normally present in the circulation (Dale and Liles 1998).

They are large cells with a size of 9-16 μm in diameter and they have multi-lobed nuclei hence they are also known as polymonuclear neutrophils (PMNs), with number of lobes increased in more mature cells. Neutrophils are formed in bone marrow from a common progenitor cells to monocyte's referred to as the granulocyte- macrophage colony forming unit (GM-CFU). The regulation of granulopoiesis is by a homeostatic balance between stimulatory and inhibitory humoral factors, in which colony stimulating factors play a major stimulatory role (Dale *et al*., 1995; Molineux, 2002). Neutrophils evolve from the myeloblast differentiating through the stages of premyelocyte, myelocyte, metamyelocyte, band cell and eventually mature neutrophil. Myelocytes do not proliferate and so there is no mitosis after this stage of neutrophil development (figure 1.1).

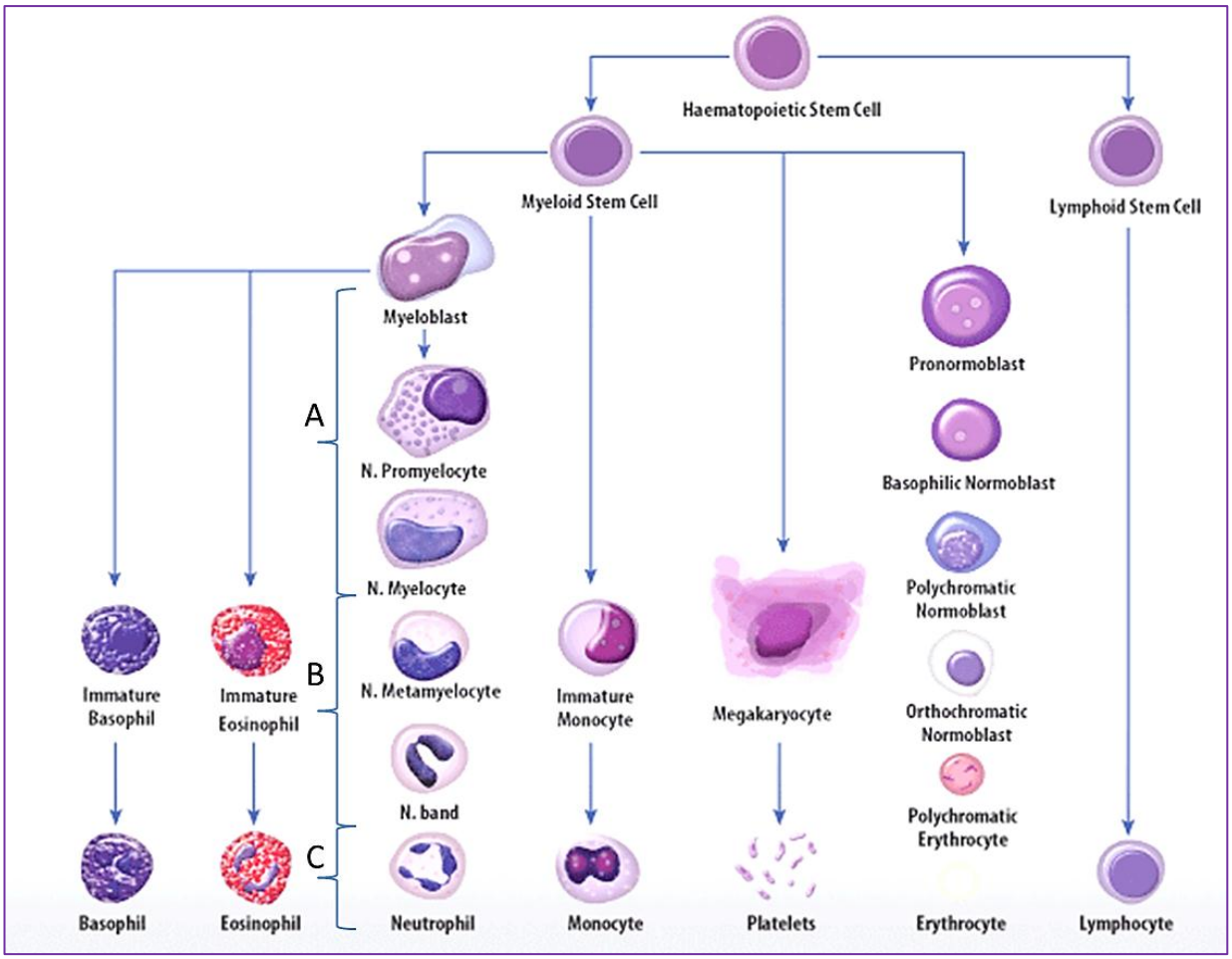


Figure1.1 Neutrophil myelopoiesis. Granulocytes and erythrocytes are derived from the Myeloid Stem Cell. Neutrophil cell line started from stem cells which differentiate into Myeloblasts, Promyelocytes, and then Myelocytes. These together form the Neutrophil Proliferative Pool (NPP) (A), Post-mitotic maturation then takes place, forming Metamyelocytes and Band cells which make the Neutrophil Storage Pool (NSP) (B) in bone marrow which can be released into the bloodstream before being fully mature. (C) Finally mature neutrophils are released into the circulation.Reproduced with permission from bpac[nz]. Available from: www.bpac.org.nz/resources/campaign/cbc/cbc_poem.asp.

1.2.2 Neutrophil granules

Neutrophils contain four distinct types of granules: primary, also known as azurophilic granules, secondary or specific granules, tertiary or gelatinase-rich granules and secretory vesicles (Borregaard and Cowland 1997; Faurschou and Borregaard 2003) (Figure1.2). Proteomic studies have identified over 280 proteins which shed light on the complex structure of these organelles and the diverse functions of their contents. Granule proteins could be classified according to function into receptors and cytoskeletal membrane anchors, channels and transporters, cytoskeletal and actin binding proteins, luminal and host defense proteins, redox proteins, kinases and phosphatases membrane traffic and other proteins (Lominadze *et al*., 2005). The allocation of these proteins to the corresponding granule subset is determined by the timing of protein synthesis during neutrophil myelopoiesis in bone marrow and not by granule specific targeting (Borregaard *et al*., 2001). Subsequently, granule protein synthesised at a given stage of differentiation will be localized to the granule subset synthesised at the same time. However, overlap in the timing of protein synthesis may lead to localization in other granule types. It is estimated that approximately 50% of granule proteins are present in more than one granule subset (Lominadze *et al.,* 2005).

While the primary distinction between different granule types is the order in which they are synthesised during maturation, they can be distinguished by proteinase or membrane-associated markers. Azurophilic granules contain all of the elastase and CD63, while secondary granules contain all the intracellular CD66b, and tertiary granules contain 75-80% of the MMP-9 (Kjeldsen *et al*., 1992).

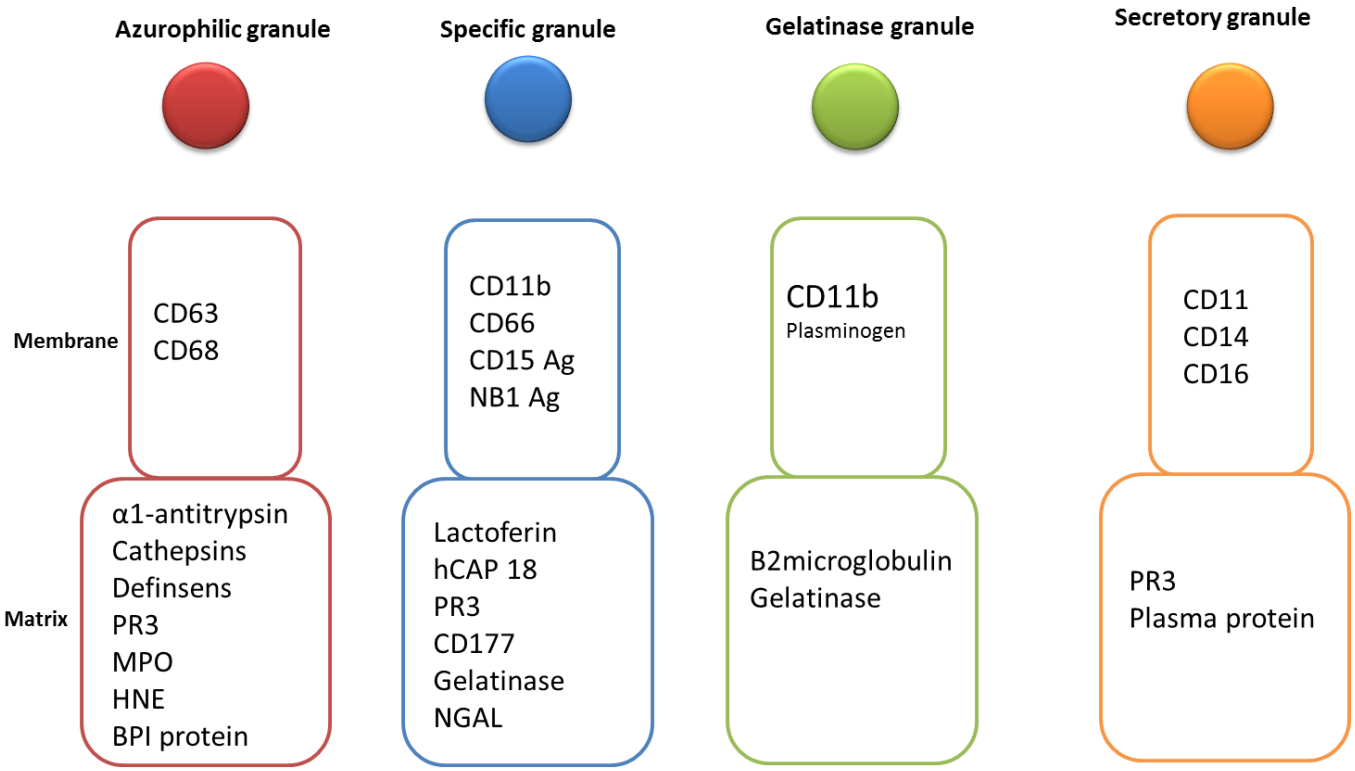


Figure 1.2 Selected granule content. Bactericidal permeability increasing (BPI), Neutrophil gelatinase associated lipocalin (NGAL), = Human leukocyte elastase (HLE). Proteinase3, (PR3). Myeloperoxidase (MPO) = Human cationic antimicrobial protein-18 (hCAP-18).

Azurophilic granules are formed in the promyelocytic stage followed by secondary granule formation in myelocytes. At the metamyelocyte stage tertiary granules are produced whereas secretory vesicles are believed to be formed by endocytosis in circulating neutrophils (Borregaard *et al*., 1987). Similarly, the stimulation required to mobilize these granules to the surface requires an increasing gradient: Secretory vesicles are easily mobilised upon neutrophil contact with endothelial cells while degranulation of tertiary granules coincides with transmigration of neutrophils. Secondary granule contents are subsequently released at the site of inflammation, while azurophilic granules are only released externally when inflammation or infection cannot be overcome by engulfing the organisms.

Differential mobilization of neutrophil granules subsets helps to co-ordinate the multi-step inflammatory response components which include adhesion to activated endothelium that is followed by extravasation and migration of neutrophils towards the site of infection and culmination in elimination of the invading micro-organisms. All granule subsets have common structural features such as phospholipid bi-layer membranes and a matrix containing proteins for delivery into the phagosomes or for release outside the cell by exocytosis.

1.2.2.1 Azurophilic (Primary) granules

These are packed with acid hydrolases and antimicrobial proteins including myeloperoxidase, human neutrophil peptides 1-3 (HNP 1-3) and serine proteases: proteinase 3, cathepsin G and elastase.

The three serine proteases found in azurophil granules have a major role in regulation of immune responses and in control of cell signalling through modulation of the cytokine network, processing of chemokines and activation of specific cell surface receptors. Together, they contribute to non-oxidative killing of pathogens inside the neutrophils. In addition, they

have bactericidal function on cell walls and they are involved in degradation of extracellular matrix in the process of inflammation (Korkmaz *et al.,* 2008).

Azurophilic granules are present in three forms large/spherical, nucleated and small granules. Myeloperoxidase, the defining protein of azurophil granules (Introne *et al.,* 1999), reacts with hydrogen peroxide which is produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and this reaction results in formation of hypochlorus acid which is highly toxic for pathogens. Myeloperoxidase is released to the phagosome or to the exterior of the cell upon neutrophil activation (Gallin, 1985). Myeloperoxidase has a peripheral distribution in large azurophilic granules whereas its distribution in nucleated granules is uniform. Similar overall distribution was described for elastase, cathepsin G as well as for proteinase 3 which is additionally found in crystalloid structure in nucleated azurophilic granules. Bactericidal/ permeability increasing protein (BPI) which has bactericidal and bacteriostatic effect on Gram negative bacteria is localised to the membrane in all types of azurophilic granules (Eqesten *et al*., 1994).

CD63, a member of the tetraspanin superfamily, is present only in azurophilic granules along with myeloperoxidase (MPO) and serine proteinases; therefore it has been described as a suitable marker for azurophilic granule fusion with the plasma membrane (Kuijpers *et al*., 1991). It is expressed on the cell surface following neutrophil activation (Cham *et al*., 1994). Lopez *et al*. found that CD63 expression increased on synovial fluid neutrophils of rheumatoid arthritis patients (Lopez *et al*., 1995). It has been hypothesized that CD63 may be associated with signal transduction although its role in leuckocyte function is not completely understood.

1.2.2.2 Secondary (Specific) granules

Secondary granules are smaller and more numerous than azurophilic granules. They appear at a later stage in myelopoisis and are mobilised more readily than azurophilic granules upon stimulation. They are de-granulated *in vitro* by low concentrations of phorbol myristate acetate (PMA) or formyl Met-Leu-Phe (fMLP) (Cham *et al*., 1994). CD66b is an exclusive marker present in membrane of specific granules (Molinedo *et al*., 1999; Lominadze *et al*., 2005). Lactoferrin is a member of transferrin family of iron binding protein which has a broad spectrum antimicrobial activity. Iron sequestration by lactoferrin deprives bacteria of essential iron for bacterial growth. It has also a direct bactericidal activity as a result of its irreversible binding to bacterial cell walls (Chapple *et al*., 1998; Farnaud and Evans 2003). Lysozyme is present in all granules types but secondary granules have the highest concentration (Lollike *et al*., 1995; Cowland and Boregaard 1999), and it is most noted for its intracellular role in the lysosome and phagolysosome.

Secondary granules contain human cathelicidin antimicrobial pro-peptide (hCAP 18) which is converted to the active peptide LL-37. Cathelicidins are a group of antimicrobial and endotoxin binding proteins found in neutrophil granules of vertebrates (Zannetti *et al*., 1995) and hCAP is only found in humans (Cowland *et al*., 1995). Proteolytic processing of LL-37 results in shorter peptides which have immune-stimulatory effect on certain cell types, for example, inducing production of interleukin-8 (IL-8) by keratinocytes (Braff *et al*., 2005). In addition to its antimicrobial action, LL-37 is thought to have a role in regulation of tissue neutrophil life span as it was demonstrated to induce neutrophil secondary necrosis *in vitro* (Zhang *et al*., 2008).

Secondary granules also contain a peptide known as neutrophil gelatinase –associated lipocalin (NGAL) which has antimicrobial properties. Most NGAL is not associated with gelatinase but found as monomer or homo-dimers in specific granules colocalising with lactoferrin (Kjeldsen *et al*., 1994). Its antibacterial action is thought to be by iron depletion through binding to bacterial ferric siderophores (Goetz *et al*., 2002).

1.2.2.3 Gelatinase (tertiary) granules

This subset of neutrophil granules contains 75 -80% of the cellular metalloproteases gelatinase (MMP-9) content and leukolysin (MMP-25) which are stored as proforms and activated only after exocytosis (Kjelsden *et al.,* 1992; Pei, 1999; Kang *et al*., 2001). They are probably of central importance in the degradation of vascular basement membranes during neutrophil extravasation due to degradation of extracellular matrix components (Borregaard and Cowland 1997; Owen and Campbell 1999; Kang *et al*., 2001).

1.2.2.4 Secretory vesicles

This intracellular compartment formed by endocytosis represents an important store for membrane associated receptors which will be incorporated into the plasma membrane upon their release. This enables the neutrophil, not only to interact with endothelial cells, monocytes and dendritic cells, but also to receive inflammatory signals from the environment (Boregaard and Cowland 1997). These receptors include ß2 integrins, CR1 or complement receptor 1 (CD35), formyl peptide (such as fMLP) receptors, CD14 and CD16 (FcγRIII) that mediate the binding of immunoglobulin opsonized particles to neutrophils. Secretory vesicles in addition contain the metalloproteinase leukolysin as well as azurocidin, also known as heparin binding protein (HBP) (Sengeløv *et al*., 1993; Tapper *et al*., 2002). Azurocidin release facilitates extravasation of neutrophils by increasing vascular permeability and it

brings about enhancement of monocytes adhesions to endothelium (Gautam *et al*., 2001; Lee *et al*., 2003; Soehnlein *et al*., 2005).

1.2. 3 Serine Proteinases

1.2.3.1 Neutrophil Elastase (NE) and Cathepsin G (Cat G)

Neutrophil elastase and cathepsin G belong to the same subfamily of chymotrypsin-like serine proteinases. They are both synthesised as inactive zymogens in the promyelocytic and myelocytic neutrophils (Fouret *et al*., 1989) and processed post-translationally to mature active enzymes in azurophilic granules when they achieve a relatively high concentration of 1-2 pg/cell (Wiedow *et al*., 1996). Both are very basic with isoelectric point (pI) of > 9 for elastase and approximately 12 for Cat G, which likely facilitates their anchorage to the heparin and chondroitin sulphate proteoglycan matrix of the granules, probably through arginine residues. Cat G has a chymotrypsin-like specificity and it comprises up to 18 % of azurophilc granule protein (Sun *et al*., 2004). Surface expression of elastase and cathepsin G on neutrophil has been reported following exposure to chemoattractants such as fMLP (Owen *et al*., 1995).

Moreover, evidence from mouse model suggested that Cat G microbicidal activity could be redundant. Cat G knock-out mice have no obvious defect in neutrophil function and they probably use other proteases to compensate for Cat G absence (Maclover *et al*., 1999).

While neutrophil morphology and function were essentially normal in Cat G deficient mice, recruitment to the site of injury was affected which suggests that it probably plays a role in inactivation of pro-inflammatory molecules (Maclvor *et al*., 1999). Anecdotal evidence for the importance of these proteinases comes from Chediak-Higashi syndrome patients, who are

believed to have an inhibitor of Cat G and elastase that contributes to the susceptibility of these patients to infection (Takeuchi and Swank 1989). Cat G is also required for activation of neutrophil collagenase (Capodici and Berg 1989; Capodici *et al*., 1989) and it has been suggested to have anticoagulant activity through inactivation of factor VIII. Nevertheless, this latter observation was disputed by another group that showed Cat G pro-coagulant effect by activation of factor VIII (Gale and Rozenshteyn 2008). In addition, Cat G has been found to be a platelet agonist that binds to specific platelet receptors. Cat G induced platelet activation is further enhanced by elastase (Selak, 1992; Renesto and Chignard., 1993).

At sites of inflammation, neutrophil degranulation results in release of elastase and Cat G primarily into the phagocytic vacuole and to lesser extent into the extracellular environment. The enzymes are either membrane-bound, incorporated in neutrophil extracellular traps (NETs) formation (Brinkmann *et al*., 2004) or freely released in the extracellular milieu (figure 1.3).

Elastase is able to digest almost all components of the extracellular matrix including collagens I-IV, fibronectin, laminin and proteoglycans. In addition, neutrophil elastase is present in cystic fibrosis airways (Delacourt *et al*., 2002). It also induces excessive bronchial mucus production in chronic obstructive pulmonary disease (COPD) (Shapiro, 2002).

1.2.3.2 Proteinase 3 (PR3)

PR3 is a neutrophil serine proteinase, also called myeloblastin, found in the azurophilic granules. It is also present in secondary granules, secretory vesicles and, in a subset of neutrophils, on the plasma cell membrane (Csernok *et al*., 1990; Witiko-Sarsat *et al*., 1999). PR3 in azurophilic granules co-localises with MPO, elastase, and Cat G. Resting neutrophils have a bimodal distribution with two neutrophil subsets, one presenting very low levels of

PR3 ($mPR3^{low}$) and the other presenting high levels of PR3 ($mPR3^{high}$) (Halbwachs-Mecarelli *et al* ., 1995). Despite the wide range of variation in the proportion of the high expression subset (0-100%), the proportion is stable in a given individual over long period of time suggesting genetic control of mPR3 expression (Abdgawad *et al.,* 2010). This was supported by twin studies demonstrating that the proportion of mPR3 expressing neutrophils in monozygotic twins is highly concordant (Schreiber *et al*., 2003). Expression of PR3 on the membrane of neutrophils is upregulated by multiple proinflammatory mediators such as: TNF-α, PMA, LPS, IL-8, PAF, fMLP and GM-CSF (Campbell *et al*., 2000; Hellmich *et al*., 2000; Brachemi *et al*., 2007). The binding of PR3 to the neutrophil cell membrane is still in debate, but widely suggested that the majority is bound hydrophobically to CD177, a glycosyl-phosphatidylinositol (GPI)-anchored surface receptor that is exclusive to neutrophils. Cell confocal microscopy reveals that PR3 and CD177 colocalise on neutrophil membranes (Von Vietinghoff *et al*., 2007). However, CD177 negative individuals are still able to display mPR3 upon activation, (Hu *et al*., 2009) suggesting that alternative receptors other than CD177 can act as partners for membrane PR3 (mPR3). Other protein partner's receptors for PR3 are CD11b/CD18, FcγRIIIb and phospholipase scramblase 1 found in lipid rafts (David *et al*., 2003; Fridlich *et al*., 2006; Kantari *et al*., 2007). Campbell *et al* showed that PR3 can be eluted from the membrane of neutrophils following cellular activation, and that ionic interactions are important in the binding of PR3 to the plasma membrane. PR3 can bind stably to anionic and neutral membranes, but binds strongly to negatively-charged bilayers.

Proteinase 3 activity in elastin degradation is less than that of elastase at pH of 7.4 or 8.9, but it is slightly more active than elastase at the acidic pH of 6.5 (Kao *et al*., 1988). Other functions of PR3 include regulation of the inflammatory response via modulating cytokine

and chemokine signalling, cleaving pro-inflammatory cytokines such as TNFα, IL-1 and IL-8 to their bioactive forms, activating platelets, and inducing IL-8 synthesis by endothelial cells (Renesto *et al*., 1994; Berger *et al*., 1996; Wiedow and Meyer-Hoffert 2005). Many of these proinflammatory activities are orchestrated with the action of the elastase and Cat G (figure 1.3). Moreover, proteinase 3 cleaves the human cathalecidin hCAP-18 to produce the active antimicrobial peptide LL-37 after the cathelecidin release from specific granules (Sørensen *et al*., 2001). Many functions have been reported for mPR3 which can degrade fibronectin, elastin, laminin, collagen type IV and heparan sulfate proteoglycans in the subendothelial matrix.

Activation of neutrophils by immune complexes (ICs) was reduced in mice lacking PR3 and elastase. ICs-mediated neutrophil infiltration was reduced *in vivo* in these mice. Similarly, impaired activation in response to ICs was demonstrated in neutrophils isolated from the combined PR3/elastase knock- out mice. Inflammation suppressing mediator progranulin (PGRN) is cleaved by PR3 and elastase and so its accumulation in these mice has an anti-inflammatory effect (Kessenbrock *et al*., 2008).

Experimental studies on hamsters showed that intra-tracheal proteinase 3 administration resulted in more dramatic bullous emphysema than combined intratracheal cathepsin G and elastase; therefore, the authors concluded that proteinase 3 may be implicated in the pathogenesis of emphysema in humans (Kao *et al*., 1988). PR3 specific anti neutrophil cytoplasmic autoantibodies are (ANCA) are important seromarkers found in a systemic autoimmune vasculitis known as Wegner's granulomatosis. Its characteristic features include vasculitis, necrotizing inflammation of the respiratory tract and pauci-immune glomerulonephritis.

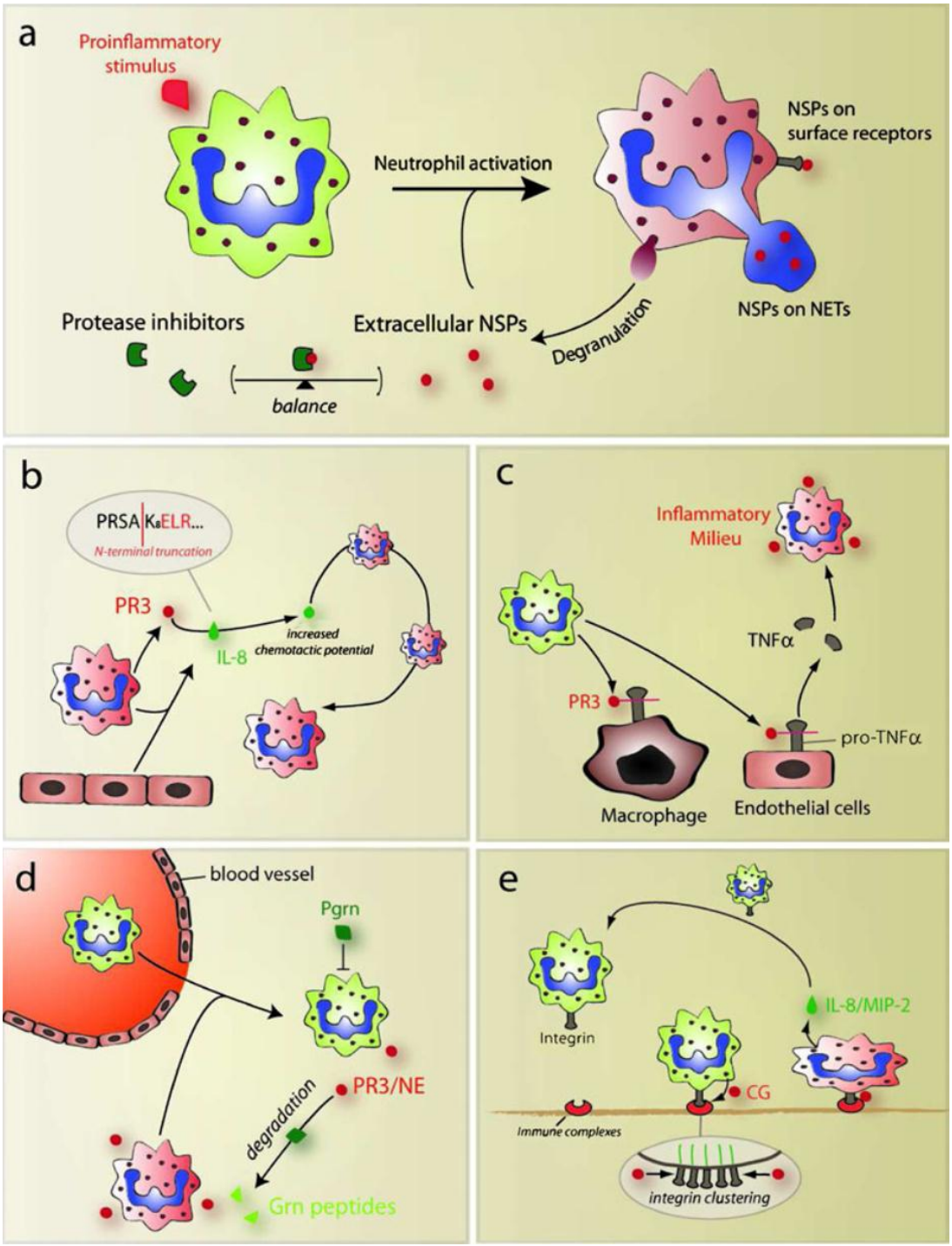


Figure 1.3 Neutrophil serine proteases (NSPs) and regulation of inflammation (a) Activated neutrophils (red) release NSPs by degranulation, or formation of NETs. Part of proteases is expressed on the neutrophil surface and remains in the pericellular environment. Serine proteases are controlled by protease inhibitors circulating in blood and interstitial fluids. (b) Extracellular PR3 converts IL-8 released from neutrophils and endothelial cells by N-terminal truncation into a more bioactive chemokine. This may potently enhance the recruitment of further neutrophils to the site of inflammation. (c) PR3 converts the membrane bound precursor pro-TNFα to the potent inflammatory cytokine TNFα. (d) Neutrophils extravasating from the vasculature are initially controlled by anti-inflammatory progranulin (PGRN). PR3 and elastase cooperatively enhance neutrophil activation by degrading inflammation suppressing PGRN which control neutrophil extravasation. (e) Cat G interacts with surface integrins during neutrophil adhesion to immobilised ICs, where it promotes integrin clustering, cytoskeletal rearrangements, and chemokine release. Reproduced with permission from Kessenbrock et al., 2011.

1.2.3.3 CD177

Human CD177, neutrophil membrane PR3 receptor, is a GPI linked 58-64 KDa membrane protein that belongs to the Leukocyte Antigen 6 (Ly- 6) gene family as its gene is located on chromosome 19q13.2. Ly-6 genes encode proteins implicated in proliferation, differentiation and homing of haemopoietic cells and lymphocytes since these proteins are overexpressed in rapidly proliferating and malignant cells such as in polycythaemia vera (Katz *et al*., 1994; Eshel *et al*., 1995).

CD177 neutrophil-specific glycoprotein is found on the plasma membrane and secondary granules of neutrophils. It is expressed by neutrophils and their precursors but not on other blood cells (Goldschmeding *et al*., 1992). CD177 heterogenous expression is uniquely found on a subpopulation of neutrophils. In the same individual, two subsets of neutrophils in terms of expression, CD177negative as well as CD177 positive could be observed. The reason for this phenomenon is not completely understood but a genetic polymorphism is thought to cause CD177 non expression in a neutrophil subset (Wolff *et al*., 2003).

CD177 overexpression was reported in neutrophils from patients with polycythaemia vera. The commonest allele of CD 177 gene is PRV-1 (Polycythaemia rubra vera-1) (Caruccio *et al.,* 2006). Other studies found that CD177 expression was up-regulated on positive cells when neutrophils were stimulated with granulocyte-colony stimulating factor (G-CSF), but the fraction of CD177 negative neutrophils did not increase after G-CSF stimulation (Temerinac *et al*., 2000; Wolff *et al*., 2003). Also the expression of CD177 antigen increases during pregnancy which suggests that the variability of CD177 expression among individuals may be caused by differences in gene regulation or in post-translational protein modification (Caruccio *et al*., 2003). Absence of CD177 was described in approximately 3% of Caucasians, 5% of African American and 11% of Japanese who have CD177 deficient

neutrophils (Matsuo *et al*., 2000; Stroncek *et al*., 2004). One cause of this deficiency is mRNA splicing defect (Kiessel *et al*., 2002). The role of CD177 in the immune system remains to be defined but it is believed to be involved in adhesion of neutrophil to endothelial cells and in transendothelial migration (Goldschmeding *et al*., 1992; Sachs *et al*., 2007).

1.2.3.4 Protease inhibitors

To avoid digestion of healthy tissue neutrophil elastase and Cat G levels are tightly controlled by physiological inhibitors (Table-1). These plasma- derived inhibitors are synthesized in the liver and they belong to the family of serpins (serine proteinase inhibitors), which are highly abundant extracellular proteins (Shapiro, 2002). Elastase and Cat G are inhibited by α1-antitrypsin (AAT) and α1-antichymotrypsin (ACT), respectively (Beatty *et al*., 1980). They can inhibit free, but not membrane-bound elastase and Cat G (Owen *et al*., 1995). Hereditary deficiency in AAT causes destruction of alveolar walls eventually leading to development of pulmonary emphysema (Gadek *et al*., 1981; Eriksson and Elzouki 1998; DeMeo and Silverman 2004). Similarly, deficiency of ACT predisposes patients to chronic lung disease (Faber *et al*., 1993).

Cat G binds heparin and this binding protects it from inhibition by ACT and AAT. Cat G affinity to bind heparin is identical to that of elastase but heparin binding has little effect on inhibition of elastase with AAT (Ermolieff *et al*., 1994). In addition, DNA binding has similar effect on these enzymes making Cat G resistant to inhibition by AAT and ACT whereas it only marginally affects the rate of elastase inhibition by AAT. Other studies have demonstrated that DNA binding to elastase impairs the inhibitory action of AAT and Secretory Leukoprotease Inhibitor (SLPI) (Belorgy and Bieth 1995; Belorgey and Bieth

1998). PR3 inhibition by AAT was not affected by nucleotide binding (Duranton *et al*., 2000).

PR3 is inhibited by serpins, mainly, AAT. Membrane PR3 is active and quite resistant to inhibition by proteinase inhibitors including AAT, possibly due to steric interference of the membrane-embedded protease. However, when exposed to exogenous AAT, activated neutrophils mPR3 was completely inhibited (Hu *et al*., 2009). AAT binds covalently to PR3 associated with CD177, resulting in a conformational change that promotes PR3 dissociation from CD177 forming a soluble neutralised complex with AAT (Korkmaz *et al*., 2008).

Serpin B1 also known as monocyte neutrophil elastase inhibitor (MNEI) primarily inhibits neutrophil elastase, cathepsin G and proteinase 3. SerpinB1 has been identified in a number of other tissues such as the bone marrow, spleen, pancreas, glandular epithelial cells and conducting airways of the lung (Yasumatsu et *al*., 2006). The cytoplasmic location of Serpin B1 suggests that its role would be the prevention of the intracellular damage by serine proteases.

Secretory Leukoprotease Inhibitor (SLPI) is present at the highest concentration in the upper airways where it plays a more important role (Vogelmeier *et al*., 1991) although immunohistochemistry staining showed that it is present also in contact with elastin fibres in lower airways which might suggest that it has physiological role in lung (Kramps *et al*., 1989; Korkmaz *et al*., 2010). It is produced by mucosal epithelial cells and bronchiolar goblet cells in human airways. Some of the chemical properties of SLPI enhance its anti-elastase activity, as it is acid resistant and resistant to oxidation and cleavage by most other proteinases ; all of which are important in the inflammatory setting (Wright *et al*., 1999). In addition it binds to

similar tissue sites to those of proteases and since it is a small molecule, it is able to penetrate the microenvironment around the neutrophil where other proteinases are unable to act (Owen *et al*., 1995). Of note, cells of mucosal surfaces produce SLPI, a barrier protein protecting cells from the destructive action of elastase and cathepsin G, but SLPI has minimal or no effect on proteinase 3 which was shown to degrade this inhibitor protein (Rao *et al*., 1993).

Table-1: Inhibitors of serine proteases

Protease inhibitors	Target Proteases	Source
I-Serpins		
AAT	Elastase, PR3, CatG	Plasma, neutrophils
Antichymotrypsin	Chymotrypsin	Plasma
Serpin B1	Cat G, Elastase, PR3	Neutrophils, Macrophages
II-Chelonianin		
SLPI	Elastase, Cat G	Epithelial cells, plasma , neutrophils
Elafin	Elastase, PR3	Bronchial secretion, skin
III- α Macroglobulins	Elastase, PR3, Cat G	Plasma
IV- TIMPs TIMP-1 TIMP2	Matrix metalloproteinases MMP-2 /MMP-9 MMP-1/ MMP-8	

TIMP: Tissue Inhibitors of Matrix Metalloproteases

1.2.4 NADPH oxidase system

This system is crucial for generation of reactive oxygen species (ROS) that mediate oxidative killing of pathogens. Electrons are transferred from cytoplasmic NADPH to oxygen outside

the cell or on the phagosomal side of the membrane generating superoxide along with several other ROS including hydrogen peroxide, hydroxyl radical, hypochlorous acid, nitric oxide, peroxide nitrite and chloramines (Hampton *et al*., 1998). The NADPH oxidase is a multicomponent enzyme which is inactive in resting cells with its multiple components kept separately in several membrane-bound and cytosolic subunits. The membrane-bound subunits form the cytochrome b_{588} which are incorporated into the membranes of secondary and secretory granules. The NADPH oxidase activity produces superoxide which is converted to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide reacts to form other ROS such as hydroxyl radical which can kill bacteria. Moreover, myeloperoxidase oxidizes halides in presence of hydrogen peroxide to form bactericidal acids, especially HOCl. Hydrogen ion influx into the phagosome increases initially to compensate for influx of negatively charged electrons.

1.2.5 Neutrophil adhesion and migration

Neutrophils reversibly adhere to the activated endothelial cells at site of infection or inflammation. A group of sugar binding cell surface glycoprotein known as selectins plays a significant role in this process. L-selectin is constitutively expressed on the surface of neutrophils and shed after cellular activation (Smith *et al.,* 1991). Endothelial, E- selectin is expressed on activated endothelial cells whereas platelet P-selectin is expressed on platelets as well as endothelium (Gahmberg *et al*., 1992; Carlos and Harlan 1994). The main leukocyte glycoprotein ligand is P-selectin glycoprotein which serves as a ligand for the other two selectins (L and E). Additionally, E-selectin recognizes a specific ligand on a leukocyte which is known as E-selectin ligand-1. The activation of neutrophils by these chemokines triggers surface upregulation of integrins. Thereafter, the integrin CD11b/CD18 mediates

firm adhesion to the endothelial cells and the density of these integrin complexes on the cell surface increases as a result of translocation from secretory vesicles, specific and gelatinase granules and also by de novo synthesis. CD11b/CD18 binds to its endothelial ligand, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 (Bailly *et al.,* 1995; Xie *et al.*, 1995; Henzen *et al*., 2000).

Neutrophil firm adhesion to the endothelium is followed by extension of neutrophil pseudopods between endothelial cells and penetration of the intercellular junctions, before moving into the interstitium (Carlos and Harlan 1994).

1.2.6 Chemotaxis

Leukocyte chemotaxis is regulated by a number of chemoattractants including bacterial by-product fMLP, complement proteolytic fragment C5a and chemokines. Neutrophils migrate along a chemokine concentration gradient across the wall of blood vessels and within the tissues. The migration is determined by chemokines like IL-8 and other related chemokines and also by expression of adhesion molecules (Roitt and Male 2001). Actin polymerization and de-polymerization within the neutrophils, upon interaction with chemokines, results in rearrangement of cell shape and upregulation of receptors before emigration (Springer, 1994).

1.2.7 Neutrophils phagocytosis and degranulation

Movement of granules and discharge of their contents is known as degranulation and different types of granules are released independently because their release is controlled by different control mechanisms. Granules fuse with phagocytic vesicles, phagosomes where they discharge their content to kill the ingested microbes, as a result of the high concentration of proteins in the granules; protein concentration in the phagolysosome is raised to 30-40%.

During phagocytosis, the plasma membrane of neutrophil tightly surrounds the microbe to limit the amount of extracellular fluid inside the phagosome vacuole. Eventually, the osmotic pressure of the protein inside the phagosome will withdraw water and the resulting influx will decrease the protein concentration. NADPH oxidase complex also assembles at the phagosomal membrane and produces oxygen radical antibacterial mediators: HOCl is probably the dominant mediator of oxidative bacterial killing inside the phagosomes (Hampton *et al*., 1998). Neutrophils rolling along the endothelial cells are exposed to chemokines released from the endothelium including platelet activating factor (PAF), complement fragments, leukotriene B4 and IL-8 and this will lead to upregulation of integrins on the surface of these leukocytes.

Subsequently, neutrophils are firmly adhered to the endothelium mainly by the integrin CD11b/CD18 which are expressed at relatively low levels on the surface of resting neutrophils. Neutrophil activation also results in the altered conformation (activation) of CD11b/CD18 as characterized by formation of new epitopes, which correspond to increased affinity for CD11b/CD18 to bind to ligands. It is also characterized by increased density of CD11b/CD18 on neutrophils and mobilization of these receptor from specific and gelatinase granules, as well as secretory vesicles, to cell surface (Borregaard and Cowland 1997).

1.2.8 Neutrophil Extracellular Traps (NET)

This is an additional pathogen combat strategy used by neutrophils in the extracellular killing of bacteria, fungi and parasites. Recent work revealed that uncondensed chromatin DNA and histone proteins are released from neutrophils to an extracellular structure or a lattice which is supplemented by proteins released from azurophilic, specific and tertiary granules and bound

to this structure. The resulting lattice is known as a neutrophil extracellular trap (NET). Immunoflourescence studies revealed that NET contains elastase, Cat G and MPO from azurophilic as well as lactoferrin from specific and gelatinase from tertiary granules. Histone proteins from the nucleus are an important component of NETs which also exert antimicrobial action at surprisingly low concentration (Brinkmann *et al*., 2004).

NETs also represent a physical barrier capable of trapping Gram positive and Gram negative bacteria. Moreover, high concentrations of several antimicrobial proteins and enzymes attained in the NET ensure efficient killing of the sequestered microbes. Neutrophil elastase present in NETs was shown to target specifically bacterial virulence factors (Belaaouaj *et al*., 2000; Weinrauch *et al*., 2002). In addition, containment of microbicidal granule contents minimizes their harmful effect on the surrounding tissues (Xu *et al*., 2009; Brinkmann *et al*., *2010*).

Some bacteria can resist bactericidal action of NETs. DNase enzyme from group A Streptococcus, a known virulence factor, is thought to mediate increased virulence through the ability to destroy NETs (Buchanan *et al*., 2006).

Reactive oxygen species (ROS) are of pivotal importance in NET formation. The evidence for this conclusion comes from the demonstration of defective NET formation in patients with chronic granulomatous disease, who have a defect NADPH oxidase pathway leading to impaired ROS generation (Nishinaka *et al*., 2011)

Recalcitrant Aspergillus infection of chronic granulomatous disease patients was cleared and NET formation ability was restored in another study as a result of gene therapy, supporting this hypothesis (Bianchi *et al*., 2009). The role of ROS in NET formation is believed to act as a component of the signaling pathway (Papayannopoulos and Zychlinsky 2009).

More recently, neutrophil elastase was demonstrated to play an essential role in NET formation with synergestic activity of myeloperoxidase. ROS production triggers selective intracellular release of neutrophil elastase followed by myeloperoxidase from primary granules. Once translocated to nucleus, elastase digests nucleosomal histones and promotes extensive chromatin decondensation and subsequently myeloperoxidase binds chromatin and enhances further decondensation. Elastase knock-out mice failed to form NET which could contribute to their immune deficiency (Papayannopoulos *et al*., 2010). Moreover, histone hypercitrullination catalyzed by peptidylarginine deiminase 4 (PAD4) is involved in neutrophil chromatin decondensation hence in NET formation. Evidence came from inability of PAD4 knock- out mice to form NET leading to increased susceptibility to bacterial infection (Li *et al*., 2010).

The factors that direct the microbicidal strategy selection by a particular subset of neutrophils remain largely unknown. The individual neutrophils may respond differently within the population. In contrast, the length of time required for various neutrophil strategies may determine the order of putting them into operation: phagocytosis followed by degranulation and eventually NET formation before neutrophil death (Papayannopoulos and Zychlinsky 2009).

1.2.9 Neutrophils in neonates

Infection is a major cause of morbidity and mortality in newborn infants, particularly preterm neonates, and the innate immune system is of prime importance in this age group. Limitation in cellular and humoral components of this system is believed to contribute to newborn

susceptibility to microbial invasion. Interest in this concept is obviously reflected in the number of studies which compare aspects of innate immunity in adults and newborns seeking answers that could be utilized to provide novel therapeutic strategies against neonatal infections.

Neutrophil chemotaxis has been shown to be impaired in neonates (Abughali *et al*., 1994; Tan and Davidson 1995). Reduced responsiveness to chemoattractant in newborn neutrophils is due to impaired chemoattractant induced signaling (Weinberger *et al*., 2001).

Similarly, the leukocyte aggregation pattern in response to chemotactic peptide, N-formyl-L-methinoyl L-leucyl L-phenylalanine (fMLP) was different in newborns compared to adult neutrophils. The newborn neutrophil aggregation curve showed slow aggregation with no de-aggregation whereas adult neutrophils had faster initial aggregation and was followed by slow de-aggregation. Examination under an electron microscope revealed that adult neutrophils were loosely bound with no cell membrane projections in contrast to newborn neutrophil which were irreversibly aggregated due to the close proximity of the cytoplasmic membrane projections (Olson *et al*., 1983).

Neutrophil CD11b expression and transmigration ability (spontaneous and IL-8 induced) were higher in neutrophils from neonates whose birth was assisted than in neutrophil taken from neonates who were born by normal vaginal delivery, suggesting differential activation between these types of delivery. Transmigration and CD11b expression were least in neutrophils taken from neonates born by elective Caesarean section before the onset of labour, which likely reflect the minimum activation signaling for neutrophils (Yekataei-Karrin *et al*., 2007).

In addition, levels of several antimicrobial peptides were found to be low in newborn infants. Plasma bactericidal permeability increasing (BPI) protein level increased in newborn infants after infection. In spite of higher plasma BPI levels in septicaemic newborn than in healthy neonates and adults, the ability of newborn neutrophils to release BPI after stimulation with PMA was less in term newborn than in adults, and less in preterm than in term newborns (Nupponen *et al* ., 2002). Levy and coworkers (1999) in keeping with this study found that newborn neutrophils are relatively deficient in BPI. In another study, they demonstrated that adding a supplement of recombinant BPI to cord blood neutrophils enhanced their bactericidal ability and inhibited TNF release in response to different Gram negative pathogens (Levy *et al*., 2000). This report is promising in the management of Gram negative infection in newborns, but further studies are needed before its potential application in clinical neonatology. Moreover, plasma levels of hCAP 18 and NGAL in neonates and adults do not show significant differences, but their levels in newborn neutrophils were significantly lower than in adults. LL-37 has been found in skin of healthy neonates and detected along with lysozyme in proteins derived from Vernix caseosa, which is a cream like substance present on the skin of newly born infants (Marchine *et al*., 2002).

Bactericidal activity against 11 strains of Enterococcus was identical in adult and neonatal neutrophils isolated from term newborn infants. Neutrophil killing of Enterococcus was mediated mainly by complement and supplemented by immunoglobulin which played a potentially important, yet not indispensable role in elimination of this bacteria (Harvey *et al*., 1992)

Neutrophil functions were studied in 13 preterm (PT) neonates (mean birth weight 1,506 g) in comparison with adult and term newborn neutrophils as controls. Adherence, chemotaxis and

phagocytosis of Candida albicans were reduced in neutrophils from PT infants in comparison to adult neutrophils. Chemiluminesence and degranulation of elastase and lactoferrin in response to zymosan were also impaired in PT neutrophils (Bektas *et al*., 1999).

While some studies reported that O2 is produced equally in adult and PT neutrophils in response to PMA stimulation (Bektas *et al*., 1990), other studies found that generation of oxygen radicals is less effective in PT and full term infants than in adults particularly in stressed newborns (Driscoll *et al*., 1990; Usmani *et al*., 1991).

Comparative studies of adult and cord neutrophils have also addressed the recently described NET formation and the results were in agreement with the view of functional immaturity in newborn innate immune system. Initially, newborn neutrophils could not form NETs when observed for one hour after activation compared with adult neutrophils, which were able to form NETs within 15 to 30 minutes (Yost *et al*., 2009 a). Further research revealed that newborn neutrophil could eventually produce NET formation after 2-3 hours (Marcos *et al*., 2009). The delayed NET formation in newborn neutrophils was demonstrated when they were stimulated with live bacteria or with natural or synthetic agonists of TLR2 or TLR4 but no delay was found upon stimulation of TLR5, TLR8 or TLR 9 stimulation (Marcos *et al*., 2009). However, delayed NET formation for 2-3 hours could provide adequate time for invading bacteria to multiply to sufficient degree to escape containment and elimination by neutrophils (Yost *et al*., 2009 b). Delayed NET formation is probably due to delay in maturation of regulatory mechanisms and it was more marked in preterm newborn as neutrophils from preterm newborns never formed NETs (Yost *et al*., 2009 b).

1.3 Chronic Lung Disease of Prematurity (CLD)

Chronic lung disease (CLD) of prematurity is the most important complication seen in premature newborns who required mechanical ventilator support for neonatal respiratory distress syndrome (RDS). The incidence of CLD has been rising over the past decade despite improvement in management of RDS including antenatal steroid administration and surfactant treatment (Fraser *et al*., 2004). However, the rising trend seen recently seems to represent a different pathological entity since it is observed in extremely premature neonates (less than 28weeks) and believed to be mediated by inflammation and altered lung development (Manktelow *et al*., 2001; Viscardi, 2012).

The definition of CLD has varied in recent years. Initially it was defined as being the need for supplemental oxygen beyond 28 days of age with associated radiographic changes. Recently CLD is applied to infants who need supplemental oxygen at 36 weeks corrected gestational age and who have characteristic chest x-ray changes (Kotecha, 1999; Bancalari *et al*., 2003) (Table 2).

The pathogenesis of CLD is linked to a number of risk factors involved in initiation or continuation of inflammatory process in preterm lung. Historically, the neutrophil appears to be the key player in this unresolved inflammatory process.

Merritt *et al* 1983 examined the tracheal aspirate fluid (TAF) of 26 neonates and found that significantly higher granulocyte counts were present in preterm infants with respiratory distress syndrome (RDS) than in fluid aspirated from term infants ventilated for non-respiratory reasons. Granulocyte counts were particularly high in those preterm infants who later went on to develop CLD. By the third day of life, infants who later developed CLD had

neutrophil counts a hundred times higher than that of preterm infants who had resolved RDS. They also reported that the total cell count started to fall after three days in those infants in whom RDS resolved, whilst the cell count stayed high much longer in those preterm infants who went on to develop CLD (Merritt *et al*., 1983).

Table-2 Diagnostic criteria for CLD

Age at birth, weeks GA	Mild CLD	Moderate CLD	Severe CLD
<32 weeks	Breathing room air at 36 weeks cGA or discharge, whichever comes first	Need for < 30% oxygen at 36 weeks cGA or discharge. whichever comes first	Need for ≥ 30% oxygen and/or positive pressure, (PPV or NCPAP) at 36 weeks cGA or discharge, whichever comes first
≥32weeks	Breathing room air by 56 day postnatal age or discharge, whichever comes first	Need for < 30% oxygen at 56 day postnatal age or discharge, whichever comes first	Need for ≥ 30% oxygen and/or positive pressure (PPV or NCPAP) at 56 day postnatal age or discharge, whichever comes first

Table 1.2 Diagnostic criteria for CLD as set out by National Institute of Child Health and Human Development NICHD/ National Heart, Lung and Blood Institute NHLBI workshop (Jobe, Bancalari 2001). Definition of abbreviation: CLD chronic lung disease of prematurity; NCPAP = nasal continuous positive airway pressure; cGA corrected gestational age; PPV = positive-pressure ventilation.

1.3.1 Risk factors for CLD

The pathogenesis of CLD is complex and multiple risk factors have been identified. CLD is more common in extremely premature infants as well as in male, non- black infants. Hyperoxia and high pressure and volume ventilation are linked with the development of CLD. Other risk factors include the presence of a patent ductus arteriosus (PDA), neonatal sepsis and the presence of maternal chorioamnionitis, figure 1.4.

1.3.1.1 Oxygen

Hyperoxia has a harmful effect on developing lung. Animal models demonstrated that exposure of newborns to high concentrations of oxygen can lead to tissue injury similar to that seen in classical CLD with thickening of interstitium , parenchymal and peripheral fibrosis associated with a significant influx of inflammatory cells (Pappas, 1983).

ROS interfere with surfactant production and impair lung growth and repair. Preterm lungs are particularly vulnerable to oxygen related lung damage, partly due to immaturity of the lungs antioxidant defenses (Saugstad, 2003). Hyperoxia enhances the proteolytic action of elastase whereas it decreases the inhibition by AAT which is shown to be cleaved or oxidized when exposed to a fraction of oxygen in inspired air (FiO_2) more than 0.6 (Merritt *et al*., 1983; Bruce *et al*., 1992).

1.3.1.2 Barotrauma/ Volutrauma

Mechanical ventilation causes barotrauma and volutrauma particularly with high peak inspiratory pressures (> 35cm water) (Taghizadeh and Reynolds1979). Despite advances in ventilatory techniques, including the development of high frequency oscillatory ventilation, barotrauma and volutrauma continue to contribute to lung injury.

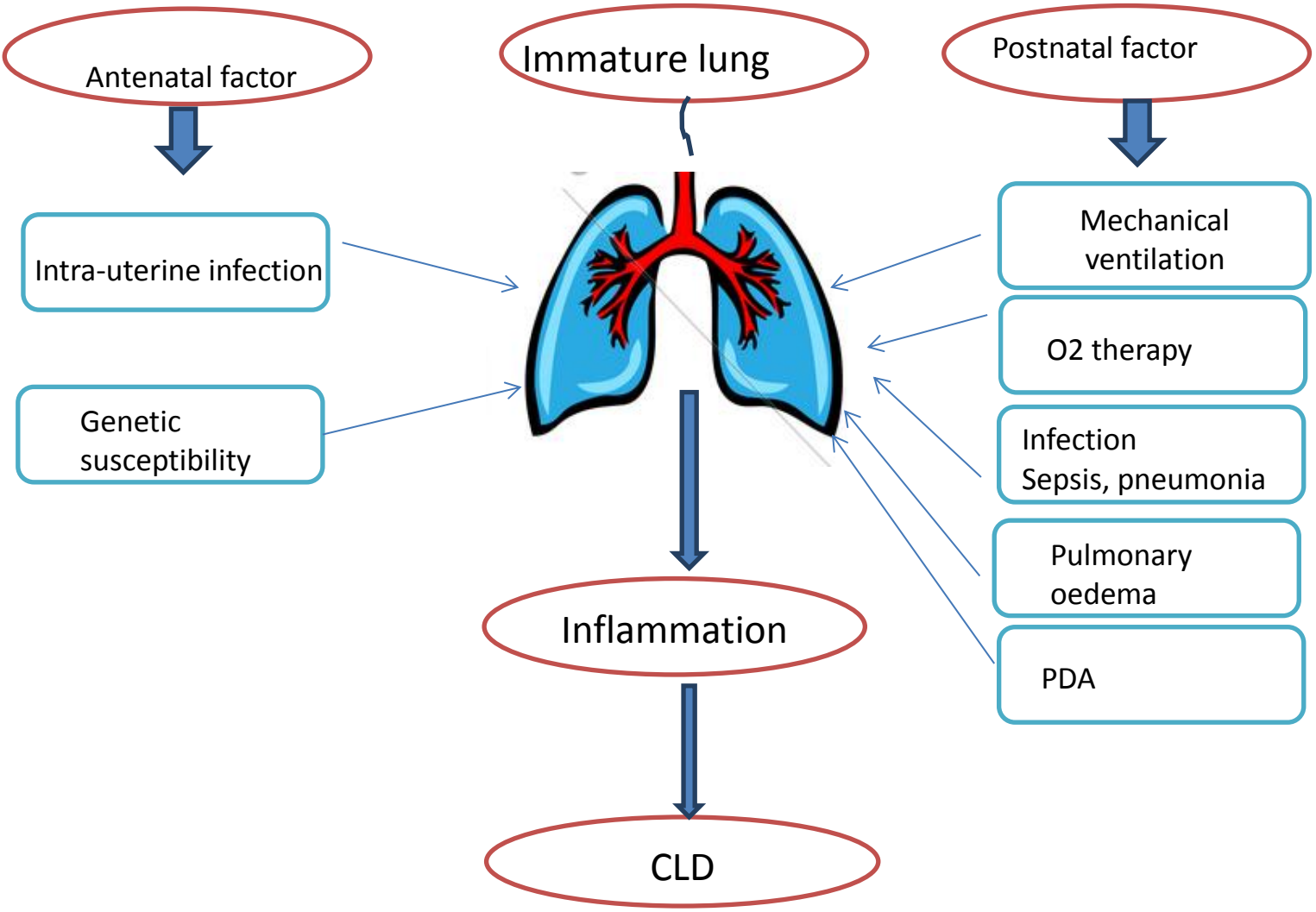


Figure1.4. Diagram shows the mechanism by which multiple risk factors lead to development of CLD.

Ventilation-induced lung injury results from excessive tidal volume (volutrauma) rather than the barotrauma of high inspiratory pressure (Speer, 2009). Positive airway pressure results in over distension of the airways and alteration of the alveolar capillary membrane permeability causing pulmonary oedema. Neutrophil recruitment and release of inflammatory mediators such as IL-6, IL-8 and other cytokines may also increase lung permeability contributing to lung injury (Vlahakis *et al*., 1999; Yoder *et al*., 2000). 'Priming' of the foetal lung by LPS triggers inflammation and mechanical ventilation may represent a 'second strike' that augments the inflammatory response (Speer, 2009). Moreover, the combined action of barotrauma and oxygen toxicity interfere with normal mechanisms for clearance of airways and the low humidity and temperature of inspired gas reduce the mucociliary surface which can predispose to nosocomial infection (Goodwin *et al*., 1985; Sherman *et al*., 1988).

1.3.1.3 Infection

Intrauterine and postnatal infections have been implicated in the development of CLD.

Antenatal infection is a known risk factor for preterm labour with 60 -80 % of preterm labour showing evidence of infection, with pathogens being isolated from placentas or amniotic fluids (Goldenberg and Rouse 1998). Preterm delivery is a risk factor for development of CLD. The most common organisms involved are *Ureaplasma urealyticum, Mycoplasma hominis, Gardnerella vaginalis* and *Bacteroides,* all of which are vaginal commensals (Goldenberg *et al*., 2000). Bacterial sepsis and viral infections (adenovirus and cytomegalovirus (CMV) are examples of post natal infection implicated as independent risk factors for the development of CLD (Sawyer *et al*., 1987; Cordero *et al*., 1997; Couroucli *et al*., 2000; Liljedahl, 2004).

Attention is focused on the role of *Ureaplasma* in the development of CLD since it is present in the lower genital tract of 40-80% of pregnant women (Van waarde, 1997). Moreover, 22% of women with preterm labour or premature rupture of membranes (PROM) were shown to have evidence of *Ureaplasma* in their amnioitic fluid (Kirchner *et al*., 2007). In addition, intraamniotic and maternal inflammatory responses were shown to be more intense in intraamniotic infection with genital mycoplasma than in infection with other microorganisms (Oh *et al*., 2010).

Animal models in which *Ureaplasma urealyticum* is injected into the amniotic fluid of pregnant baboons have demonstrated the ability of organism to provoke an inflammatory response in the lungs of the preterm animal once delivered (Yoder *et al*., 2003) which may go on to develop CLD. Similarly, intraamniotic inoculation of clinical isolates of genital *Mycoplasma* and *U Parvum* SV1 in rhesus Macaque monkeys resulted in release of proinflammatory cytokines and induced uterine contraction. The proinflammatory response in rhesus monkeys contributed to preterm labour and foetal lung injury (Novy *et al*., 2009). Thus many of the risk factors implicated in the development of CLD have inflammation of the lung as the mechanism through which injury occurs.

In the Alabama preterm study, *Ureaplasma* and *Mycoplasma* were detected in cord blood of 23% of extremely premature infants and these neonates were more likely to have systemic inflammatory response and CLD (Goldenberg *et al*., 2008). A meta-analysis of 17 clinical studies included 1479 neonates and supported a significant association between *Ureaplasma* respiratory tract colonization and CLD development (Wang *et al*., 1995). A more recent meta-analysis analysed 36 cohort studies until 2004 involving approximately 3000 preterm infants showed a significant association between *Ureaplasma* respiratory colonisation and

CLD development (P<0.001). However, the greatest effect was seen in small studies and this increased effect could be due to reporting bias towards positive association (Schelonka *et al*., 2005).

Despite the accumulating evidence from epidemiological studies and the experimental studies using animal models to study *Ureaplasma* and development of CLD, no consensus was reached in this matter and the role of *Ureaplasma* remains controversial (Speer, 2009).

1.3.2 The changing pattern of CLD

Classical CLD affects neonates born at 32-36 weeks of gestation who need mechanical ventilation and oxygen supplement for treatment of respiratory distress syndrome (RDS). Characteristic histopathology includes variable areas of atelectasis and hyperinflation, smooth muscle hyperplasia and extensive fibroproliferation (Northway *et al*., 1967). In preterm infants with RDS, CLD appears to proceed as a continuous process from acute lung injury in the first few days through proliferative and reparative phases lasting for approximately 2 weeks and characterised by regeneration of alveolar epithelium by alveolar type II pneumocytes, granulocytic infiltration in the alveoli and increasing numbers of myofibroblasts in the interstitial tissue before development of the chronic phase of CLD (Cherukupalli *et al*. 1996; Toti *et al*., 1997).

Interstitial and alveolar protein-rich oedema in early stages progressed to persistent airway inflammation with significant alveolar septal and peribronchial fibrosis and bronchiolar and pulmonary vascular smooth muscle hypertrophy, which can lead to right heart failure (Stocker, 1986; Hislop and Haworth 1990). Chest radiographs showed severe over-inflation with a mixture of cystic emphysema and areas of fibrosis with volume loss and extrapulmonary air leak (Jobe and Ikegami 1998; Bancalari *et al*., 2003).

The more recent pattern that is seen in extremely preterm infants (23-26 weeks) is quite different, with a decreased number of large, over simplified alveoli and significantly less fibrosis than seen in classical CLD (Figure 1.5).

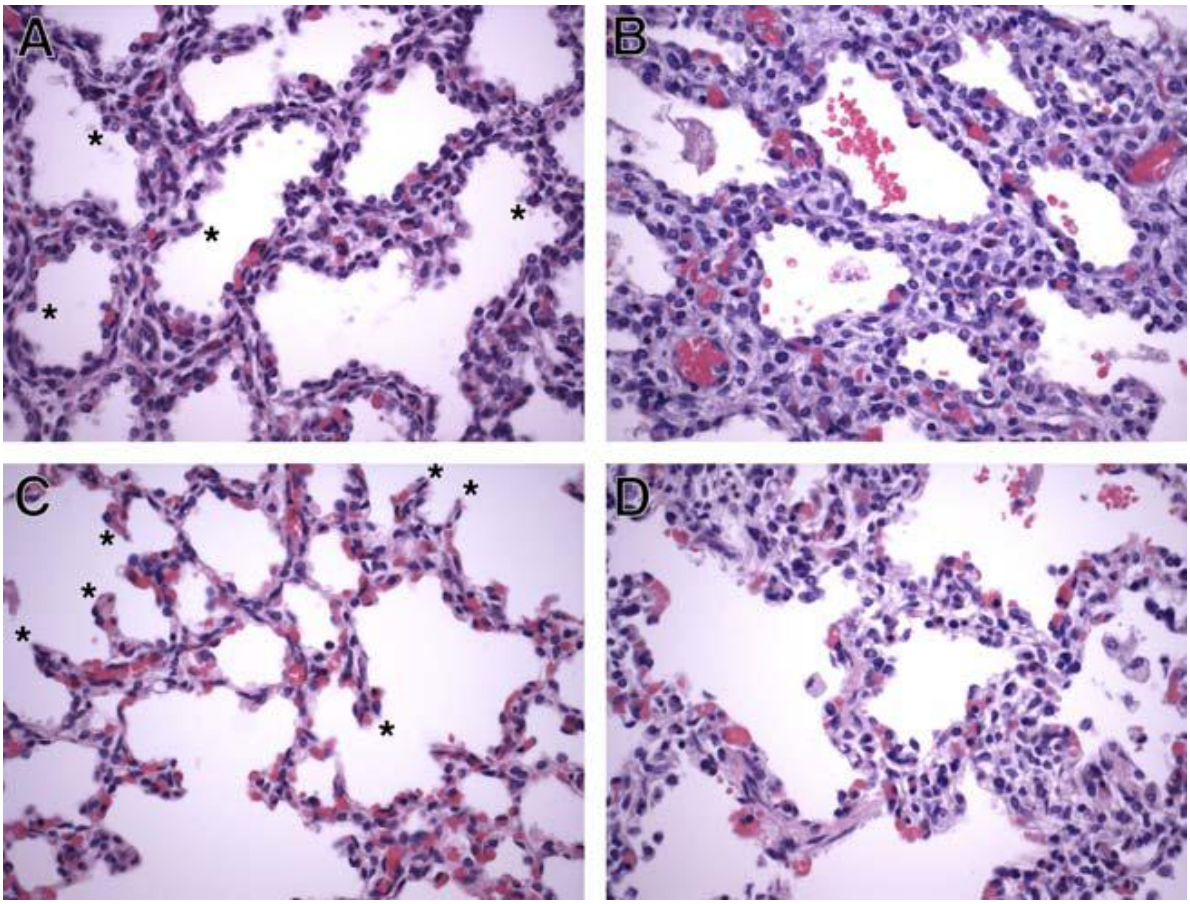


Figure1.5 Histology of developing lung: (A) Late canalicular/early saccular stage of lung development (GA 24 weeks, died 2 hours after birth) showing large simple air spaces with early secondary crest formation. (B) Ventilated premature lung (GA 23 weeks, lived for 7 days), shows cellular infiltration of the widened septa with focal haemorrhages within airspaces. (C) Late saccular/early alveolar lung (full term) showing well developed gas exchange parenchyma; compare it with (A), with thin septa and abundant secondary crest formation. (D) CLD in ventilated preterm infant (GA 27 weeks, lived for 12 weeks) showing simple, large-sized air spaces with hypercellular and thickened septa. (De Paepe et al., 2006). Reprinted with permission of the American Thoracic Society. Copyright © 2013 American Thoracic Society.

Therefore, it is felt that there has been a change in CLD that we see now from the consequence of a destructive process to represent an aberration of normal lung development, so called new CLD. New CLD has less fibrosis which is more diffuse, more uniform lung inflation; fewer and larger alveoli increased elastic tissue and decreased pulmonary microvascular development.

Clinical observation showed that extremely premature neonates (GA 23-27 weeks), which coincide with saccular stage of lung development, are at highest risk for CLD whereas the risk of CLD development for infants born at GA >32 weeks (late saccular-alveolar stage) is much lower (Jobe, 2011). New CLD described in this era of surfactant therapy and prenatal corticosteroid has different pathophysiology characterized by growth arrest of lung tissue and pulmonary vessels rather than by fibrosis and less fulminant pulmonary inflammation (Jobe, 2011).

Imbalance in proinflammatory and anti-inflammatory mediators leads to activation of the cellular death pathways in the lung, which is followed by resolution of normal lung architecture (healing) or disorganised repair (Bhandari and Bhandari., 2003) characterized by impaired alveolarization and dysregulated angiogenesis, which lead to fewer, larger simplified alveoli and a dysmorphic pulmonary vasculature: the pathologic hallmarks of CLD (Baraldi and Filippone 2007).

Inflammation-mediated injury during a critical window of vulnerability alters the development program of the foetal or newborn lung leading to abnormal alveolar development (Jobe, 1999; Viscardi, 2012). A similar pattern of abnormal alveolar formation has been observed in animal models of CLD, with preterm lambs treated with surfactant and

minimal oxygen supplementation and ventilator support developing fewer and larger alveoli than control animals (Albertin *et al*., 1999).

1.3.3 Proteases in CLD

A potential role has been attributed to proteases in the pathogenesis of CLD in preterm infants. An imbalance between proteases and anti-protease inhibitors has been implicated in the pathogeneses of CLD. In the pre-surfactant era increased elastase and decreased elastase inhibitory activity was detected in the majority of lung lavage samples from infants who developed CLD (Merritt *et al*., 1983; Watterberg *et al*., 1994). However, more recently, a number of studies have found elastase in only a minority of samples from the lung of premature infants and questioned the relationship of elastase to the development of CLD (Speer *et al*., 1993; Sveger *et al*., 2002). On the other hand, elastase is capable of digesting elastin. Elastin seems to have a key role in alveolar-genesis, the process by which septae form across the terminal air sacs to form alveoli. The process requires alveolar fibroblasts to form at the tips of the developing septae and synthesise elastin. Studies using elastin knockout mice (Wendel *et al*., 2000) demonstrate that absence of elastin leads to dilated distal air sacs with abnormally large cavities, consistent with an arrest in terminal airway development which is similar to that observed in new CLD. Thus loss of elastin through disruption of elastase regulation in the lung may impact on lung development.

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

1.4 Complement system overview

The complement system in humans was discovered in the 1890's as the heat-labile anti-bacterial component of serum and its name is derived from the capacity to enhance antibacterial activity of antibodies. Antibody activation of the complement cascade is known as the classical pathway (CP). Over fifty years later, the alternative pathway (AP), used to describe antibody-independent activation of complement was first postulated in the 1950's by Louis Pillemer (although he referred to it as the properdin pathway). The AP was not accepted by the scientific community until substantiated by Mayer (Mayer, 1984) almost 30 years later and the dispute of its existence is thought to have contributed to Pillemer's suicide in the late 1950's. The lectin pathway, as characterised by activation through carbohydrate recognition by MBL, was first described in the 1990's however complement activation through carbohydrate recognition by ficolins is still not fully understood. The complement system consists of approximately 35 proteins in plasma and an array of receptors and regulators on cell surfaces (Volankkis and Frank 1998; Walport 2001a; Walport 2001b).

Liver is the principal site of production of most complement components (Hobart *et al*., 1977). One of the notable exceptions is C7, where less than 50% is of hepatic origin (Würzner *et al*., 1994). While baseline constitutive synthesis provides a normal range of circulating protein levels, almost all components are acute phase reactants and synthesis can be induced locally in fibroblasts, endothelia, astrocytes and other cell types by the primary acute cytokines (e.g. IL-6, IL-1β and TNFα). For example, pregnancy upregulates all complement components in the maternal circulation except for C8. Hepatic synthesis is thought to account for 90% of complement production.

Well established non-hepatic complement synthesis includes secretion of C3 from activated neutrophils (Botto *et al*., 1992). C6 and C7 are produced by neutrophils precursors in bone marrow as well, but not in mature neutrophils. However, large stores of pre-synthesised C6 and C7 are found in mature cells that can be released on stimulation (Høgåsen *et al*., 1995). In addition, early components of the classical pathway C1, C2, C3 and C4 are also synthesised by macrophages (Johnson and Hetland, 1988; Morgan and Gasque, 1997).

Prevalence of total or partial deficiency of individual complement components have been described and certain deficiencies are reported to have a high incidence in certain ethnic groups (Halle *et al*., 2001; Barroso *et al.*, 2004; Kang *et al*., 2005). Deficiency of components are associated with increased frequency of pyogenic infection (C3, C1, C4 and C2), while deficiencies of the terminal pathway (C5, C6, C7, C8 and C9), properdin and MBL are associated particularly with fulminant or recurrent Neisseria infection (Loirat *et al*., 1980; Sjöholm *et al*., 2006; Garcia-Laorden *et al.*, 2008). Autoimmunity has also been found to be linked to deficiency of classical components C1 or C1q (90%), C4 (75%) and C2 (25%) including dermatomyositis and SLE-like disease (Figueroa and Densen 1991; Pickering *et al*., 2000; Winkelstein *et al.*, 2003).

1.4.1 Pathways of Complement activation

The complement system is activated through three essential pathways: classical, alternative and lectin pathways (Volanakis and Frank 1998; Walport 2001a; Walport 2001b), figure 1.6.

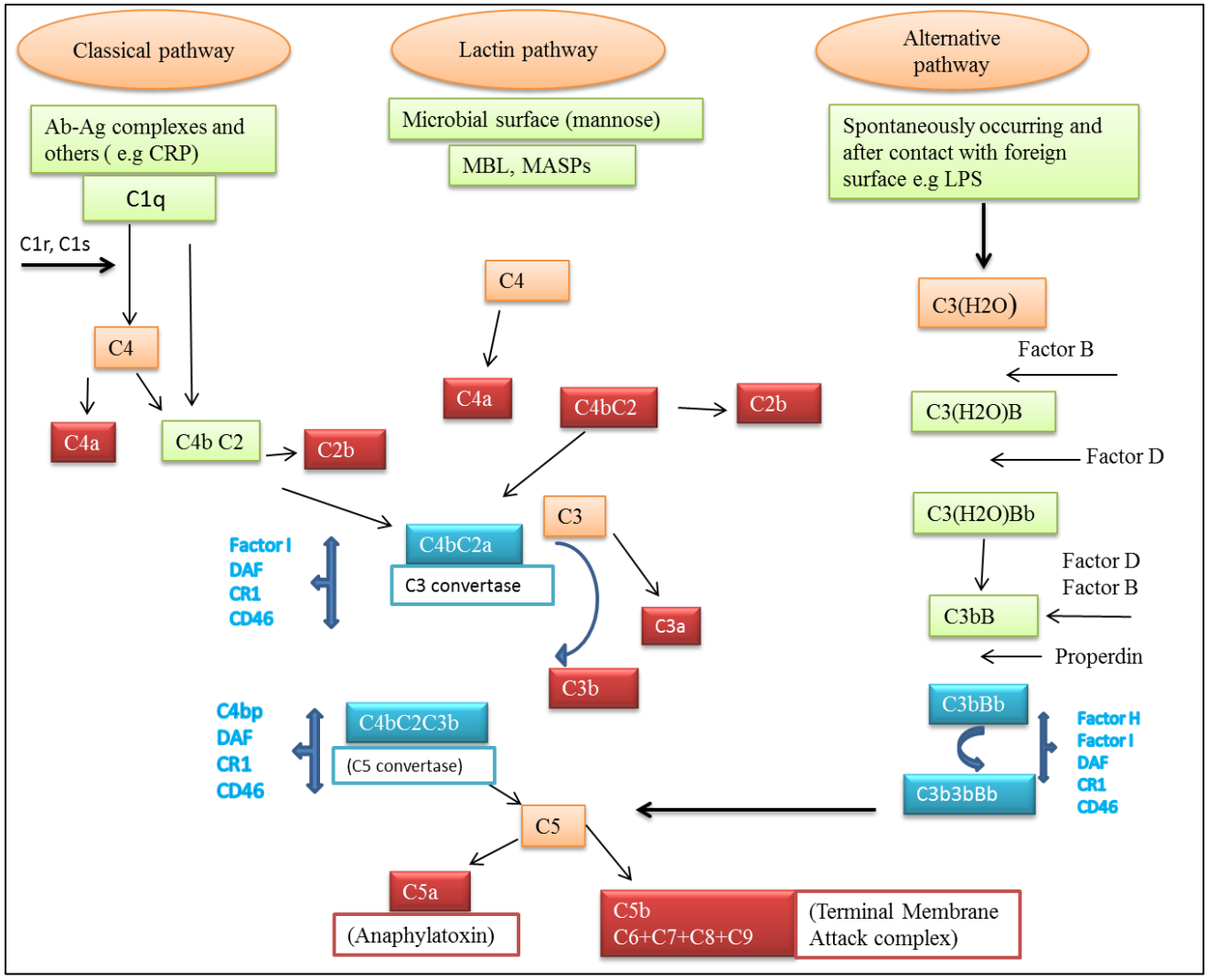


Figure 1.6 Summary of the three pathways of complement activation showing initiation via either the classical mode which is activated by an antigen/ antibody complex, or by the lectin or alternative mode, which are antibody-independent. All pathways converge at the conversion of C3 into C3a and it is active form C3b which participates in the formation of C5 convertase (C5b). All the three pathways converge to form the membrane attack complex. The complement system is tightly regulated by a number of regulators (blue lettering).

Complement has also been reported to be activated through incidental cleavage by non-complement system proteases, also known as extrinisic activation (Markiwski and Lambris 2007). Notably, the activity of cleavage of C3 into active C3a and C3b has also been described for human elastase released following neutrophil activation (Vogt *et al*., 1986).

1.4.1.1 The Classical pathways

The classical pathway is initiated by the interaction of C1 with Fc region of IgG or IgM antibodies bound to an activating surface (Walport, 2001a). C1 in serum is macromolecular complex consisting of 6 molecules of C1q and two molecules each of C1r and C1s held together in a complex $C1q_6r_2s_2$ stabilized by Ca^{+2} ions . Each C1q molecule is composed of 3 polypeptide chains, which combine to form six collagen-like triple helical arms with globular heads. The C1q globular head is the binding site for C1 complex, which binds to Fc region of aggregated or immune complex bound IgG (IgG1 or IgG3) or IgM antibodies found on a target surface. C1q can bind to the antibodies only when at least two IgG molecules are attached close enough to each other on the target to engage 2 separate C1q molecules (achieved by approximately 10^6 molecules per cell); therefore relatively high surface density of IgG is needed for the classical pathway activation. In contrast, only one target bound IgM molecule can activate the complement cascade because it has 5 C1q binding sites per molecule. In the absence of antibodies, C1q can bind to bacterial lipopolysaccharides (LPS) nucleic acids, immune complexes and some viruses (Loos *et al*., 1978; Loss *et al*., 1986; Spiller and Morgan, 1998).

Binding of C1q to Fc binding sites induces a conformational change in C1r that stimulates autocatalysis of C1r to an active serine protease enzyme which in turn cleaves C1s to an active serine protease enzyme. C1s has two substrates: C4 and C2. C4 is activated when C1s hydrolyzes a small fragment C4a, which acts as a weak anaphylatoxin (Gorski *et al*., 1979) from the amino terminus of the α chain, inducing a conformational change that exposes the thioester binding site on the larger fragment C4b. The C4b fragment covalently attaches to the target surface in the vicinity of C1, and the C2 proenzyme then associates with the conformationally altered C4b in the presence of Mg^{+2} ions, where the C2 is then cleaved by the C1s into C2a, an enzymatically active 70kDa carboxy-terminal fragment and a small fragment C2b, a 30kDa amino-terminal fragment which diffuses away (Nagasawa and Stroud 1977). The resulting C4b2a complex is called the classical C3 convertase (Xu *et al*., 2001), although it is identical to the convertase created by the lectin pathway.

C3 is an essential component of all complement pathways and the most abundant complement component in plasma. It is a large protein cleaved by C2a leading to hydrolysis of a small chemotactic fragment of C3a from its amino-terminus, and exposing an internal thioester group in the large fragment, C3b (Kindt *et al*., 2007). A single C3 convertase molecule can generate over 200 molecules of C3b, resulting in tremendous amplification at this step of the sequences, some of C3b binds to C4b2a to form a trimolecular complex, C4b2a3b (the classical/lectin C5 convertase), which in turn binds to C5 and causes conformational changes that enables C4b2a component to cleave C5 into C5a (a potent anaphylotoxin and chemotaxin) which is liberated leaving C5b associated to C3b on the activation surface. C5b has a greatly increased affinity for C6 binding and initiates formation of membrane attack complex (MAC) (Pettigrew *et al*., 2009).

1.4.1.2 The alternative pathway

The initiation of this pathway differs significantly from the classical and lectin pathways. It does not rely on antigen-antibody complexes. The alternative pathway activation involves four serum proteins C3, factor B, factor D and properdin. It is triggered by native C3, which contains an unstable internal thioester bond prone to slow spontaneous hydrolysis (called "tick over") to a C3b-like molecule, $C3_{H2O}$. The $C3_{H2O}$ binds to factor B, a single chain plasma glycoprotein structurally related to C2, in the presence of Mg^{+2}. Binding to the C3b-like molecule exposes a site on factor B that serves as the substrate for factor D which subsequently cleaves factor B into a small fragment (Ba) that diffuses away and a larger fragment, Bb that remains complexed with and forms $C3_{H2O}$ Bb, a soluble short lived C3 convertase which cleaves native C3 into C3b that covalently binds to hydroxyl or amino groups on bystander surfaces and remains active for longer time (Friec and Kemper 2009). Target bound C3b also binds factor B in the presence of Mg^{2+}, which when cleaved by factor D forms the alternative C3 convertase (C3bBb). C3 convertase activity of C3bBb has a short half-life but its binding to properdin greatly extends the half- life and stabilises the complex. Properdin can recognize and bind to the surface of certain pathogens such as *E. Coli*, *Neisseria* and *yeast* and facilitates complement activation. The complex properdin/C3b thereafter, binds factor B and cleaves it into Bb and Ba, and forms C3bBbP which acts as C3 convertase (Hourcade, 2006; Spitzer *et al.*, 2007; Kemper *et al.*, 2008; Friec and Kemper 2009).

Active surface-bound C3b which is required to initiate the alternative pathway cascade, can also originate from classical pathway activation, providing an amplification feed-back loop mechanism linking the two pathways. Alternatively, the "tick over" phenomenon occurs

spontaneously at rate of ≈1% of total C3 per hour. It is important to note that surface bound C3b on host cells is rapidly enzymatically inactivated by factor I to iC3b, which no longer binds factor B. Serum factor I cleavage is accelerated through C3b binding to cofactors CD46 (membrane co-factor protein), which is expressed on all human cells except erythrocytes, or serum factor H which is recruited to host surfaces by sialic acid (commonly added to surface glycoproteins on eukaryotic, but not prokaryotic, cells). Therefore, the alternative pathway on its own is only really effective for surfaces that lack the ability to regulate C3b. The C3 convertase activity of C3bBb generates the C3bBb3b complex, or the alternative C5 convertase (equivalent to the C4b2a3b complex in classical pathway), which then cleaves C5 and initiates the common terminal lytic pathway.

1.4.1.3 Lectin pathway

The lectin pathway is activated by soluble pattern recognition molecules (PRM), most notably mannan binding lectin (MBL) and the ficolins M, L and H. Both MBL and ficolins rely on the C1r/C1s homologue MBL-associated serine protease -2 (MASP-2) to activate complement system (Thiel , 2007): upon binding of MBL/MASP-2 complex to microbial surface through recognition of prokaryotic carbohydrate motifs, MASP cleaves C4 and C2 generating the C4bC2a, C3 convertase which finally leads to opsonization (deposition of C3b) and enhancement of phagocytosis, cell recruitment through chemotaxin release (C3a and C5a) and lysis of pathogens (Turner, 1996; Sorensen *et al*., 2005).

1.4.1.4 Terminal pathway

The common outcome of all three complement activation pathways is initiation of the terminal pathway. The terminal pathway differs from the activation pathways in that only C5 is cleaved by the C5 convertases for activation and the resulting complex has increased

affinity for the binding of the sequential components: C6, C7, C8 and C9. Complement component C6, C7, C8 and C9 are structurally homologous to each other. The C5b component is extremely labile and is rapidly inactivated unless stabilized by binding to C6. All complement reactions take place on the amphipathic surfaces of membranes or on immune complexes in fluid phase. The complex C5b6 binds to C7 and undergoes conformational change forming a C5b67-complex. If the reaction occurs on a target cell membrane, the hydrophobic binding sites enable C5b67 complex to insert into the phospholipid bilayer (Kindt *et al*., 2007; Murphy *et al*., 2007). There is some evidence that potential cytolytic effect is caused by deposition of C5b67 complexes on the membrane of nearby host cells (Podack *et al*., 1979). The C5b678 complex has also been referred to as a "leaky patch" and in sufficient numbers can induce lysis of red blood cells (but not nucleated cells) in the absence of C9 (Kindt *el al*., 2007). The final step in the formation of the MAC is the binding of multiple units of C9. As many as 10-18 molecules of C9 can be bound and polymerized by a single C5b678complex. The completed MAC consists of a C5b678 complex surrounded by a poly-C9 complex, although only 1 or 2 C9 molecules are sufficient for effective cytolysis of nucleated cells. The MAC, once inserted, kills the cells by osmotic lysis; therefore, many pores are required to overcome the active ion pumps on eukaryotic cells which will attempt to maintain osmotic balance.

1.4.2 Complement regulation

Complement system activation is kept under tight control to avoid the potential harmful effect on host cells. The system can attack bystander host cells as well as foreign cells, infected cells and invading microorganisms (Kindt., *et al* 2007). Inappropriate complement activation has been linked to pathogenesis of some diseases and ischemia-reperfusion injury.

Complement regulation occurs mainly at two steps within the cascades, at the level of convertases, both in their assembly and in their enzymatic activity, and during assembly of MAC. The regulatory proteins are soluble (C4bBP, factor H, factor I and C1 inhibitor) or membrane bound (DAF, MCP, CR1 and CD59).

1.4.2.1 Membrane bound regulators

The majority of regulatory proteins have similar structure and are encoded within the Regulators of Complement Activation (RCA) gene cluster on the long arm of chromosome 1 (Hourcade *et al*., 1989). Decay accelerating factor (DAF, CD55), complement receptor 1 (CR1, CD35), CD59 and membrane cofactor protein (MCP, CD46) are membrane bound regulatory proteins that belong to the RCA (Rey-Campos *et al*., 1988).

1.4.2.1.1 Decay Accelerating Factor (DAF) is a glycol-phosphoinositol (GPI) lipid-anchored protein widely expressed on various tissues and cells and a soluble form is found in different body fluids (Nicholson-Weller *et al*., 1983; Medof *et al*., 1987; Rooney *et al* 1992). It accelerates the dissociation of both alternative (C3bBb) and classical (C4b2a) pathway C3 and C5 convertases. It displaces C2a from C4b and Bb from C3b, which abrogates further C3 cleavage and as a result limits their ability to participate in further complement activation (Fujita *et al*., 1987). Many pathogens use DAF as a receptor such as some viruses (e.g Coxsackie virus and echovirus) and uropathogenic *E Coli* (Lindahl *et al.,* 2000).

1.4.2.1.2 Membrane Cofactor Protein (MCP) is classical type I transmembrane protein with two alternate cytoplasmic domains. It is present in a vast variety of tissues and circulating cells (Seya and Atkinson 1989; Johnstone *et al.,* 1993). It serves as a cofactor for factor I mediated cleavage of C3b and C4b (with lesser affinity) deposited on host cell surfaces leading to the formation of iC3b, C4c and C4d cleavage products (Seya *et al* 1986; Seya and Atkinson 1989). Some microbes like measles virus, HHV-6, Neisseria gonorrhoeae

and N meningitides use MCP as a receptor to facilitate entry to nucleated cells and adherence to epithelial cells (Lindahl *et al*., 2000). Of importance, CD46 has also emerged as a key signalling molecule on the surface of T-cells that modulate adaptive immune responses (Kemper *et al*., 2001).

1.4.2.1.3 Complement Receptor 1(CR1; CD35) is expressed on the surface of erythrocytes, neutrophils, monocytes and podocytes in glomeruli (Fearon, 1980). CR1 mediates phagocytosis of particles opsonized with C3b and act as cofactor for factor I mediated breakdown of C3b and C4b and also cleavage of iC3b to C3c and C3d. CR1 on erythrocytes play an interesting role in the transport and clearance of immunocomplexes from the circulation to the reticuloendothelial system (Medof *et al*., 1982), which is hypothesised to underpin development of autoimmunity when CR1 is absent or present as certain isoforms. Moreover, CR1 acts as a receptor for C1q and their interaction further augment clearance of immunocomplexes (Klickstein *et al*., 1997).

1.4.2.1.4 CD59, initially called membrane inhibitor of reactive lysis (MIRL) is the most abundant cell surface regulator of complement, expressed by all blood cells, endothelial and epithelial cells (Davies *et al*., 1989; Meri *et al*., 1991). It blocks formation of MAC by binding to C5b-8 and preventing binding of C9 to the complex (Meri *et al*., 1990; Rollins and Sims 1990).

1.4.2.2 Fluid phase regulators

1.4.2.2.1 Factor I -is a serine protease which prevents the formation of active convertases through catabolism of C3b and C4b into inactive fragments, such as C4d, iC3b, C3c and C3dg (Sim *et al* 1993; Sim and Laich 2000). Factor I cleaves C3b in presence of cofactors MCP, CR1 and factor H. The inactivation of C4b by factor I take place in presence of C4 binding protein (C4BP), CR1 or MCP as cofactors (Nagasawa and Stroud 1977).

1.4.2.2.2 Factor H (FH) is soluble protein similar to C4BP; it is a fluid phase inhibitor which plays an important role in self-recognition and protection from the alternative pathway complement. It acts as a dominant cofactor for factor I in cleavage of C3b preventing formation of functional C3 convertases (Weiler *et al*., 1976; Whaley and Ruddy 1976; Pangburn *et al.,* 1977). It also binds to C3b preventing initial factor B binding or it accelerates the dissociation of Bb from the convertase. The importance of factor H is underpinned by factor H deficiency association to diseases like atypical haemolytic uremic syndrome (aHUS), membranoproliferative glomerulonephrits and age-related macular degeneration (Atkinson and Goodship, 2007).

1.4.2.2.3 C4BP is able to bind C4b in the classical and lectin pathway convertases C (4b2a) can compete with C2 for binding to C4b, but its dominant mode of action is the cleavage of C4b to inactive fragments by acting as a factor I cofactor (Seya *et al*., 1995). This not only prevents formation of the convertase, but also accelerates its decay and blocks the convertase reformation (Gigli *et al.,* 1979).

1.4.2.2.4 C1 inhibitor (C1INH) is a soluble regulatory protein that inhibits C1 by binding to the activated C1r and C1s, similar to the mechanism for AAT control of elastase (suicide inhibitor) and removing them from C1 complex (Ziccardi and Cooper, 1979). It also prevents autoactivation of C1 that could occur in the absence of activating antibodies (Ziccardi, 1982). Deficiency of C1INH causes hereditary angioedema (HAE) (Carugati *et al*., 2001).

1.4.3 Complement system in neonates

Complement activation is of prime importance in neonatal defence against infections, particularly in presence of low antibody levels and poorly developed specific immunity.

Moreover, newborn infants, especially those born prematurely, are at higher risk of developing infection. All complement components appear in foetal serum by 18 weeks of gestation and increase gradually afterwards (Adinolfi and Beck 1975). Previous studies have shown that neonates have a low concentration of most complement factors as well as low haemolytic activity compared to adults (Fireman *et al*., 1969; Ferriani *et al.,* 1990; Wolach *et al.,* 1997). The exceptions to this trend are factor D and C7 which are reportedly identical or higher in newborns than in adults (Johson *et al.,* 1983; Wolach *et al.,* 1994; Stonntag *et al.,* 1998 b). Johnson *et al* found factor D concentration is higher in cord than in maternal blood.

The level of complement components is lower in preterm infants than in term neonates (Drew and Arroyave 1980). Similar or lower levels are noted when classical and alternative pathway components from preterm were compared to term newborns (Kohler, 1973; Stunk *et al.,* 1979; Johnston *et al.,* 1979). A significant correlation has been reported between complement components level and gestational age (Fireman *et al.,* 1969; Drew and Arroyave 1980; Shapiro *et al.,* 1981; Notarangelo *et al.,* 1984). Gestational age probably had a greater influence than birth weight on complement development. Furthermore, intra-uterine growth retardation (IUGR) does not seem to affect the development of complement (Drew and Arroyave 1980; Shapiro *et al.,* 1981; Kitajima *et al.,* 1990).

Complement hypoactivity in neonates is believed to be a predisposing factor to microorganism invasion (Sonntag *et al.,* 1998 a). On the other hand, activation of the complement system has been linked to the pathogenesis of meconium aspiration (Mollnes *et al.,* 2008) ischemia-reperfusion injury associated with pre- or perinatal asphyxia as well as in preterm infants that underwent cardiopulmonary bypass to aid in oxygenation when their lungs were very underdeveloped (Enskog *et al.,* 1996; Sonntag *et al.,*1998a). Regardless, the

mechanisms of activation in general appear to be intact in term infants, even if they are diminished (Zilow *et al.,* 1993; Wagner *et al.,* 1996) and in some cases it may be difficult to distinguish between low complement levels due to underproduction relative to those where complement is being consumed due to some underlying pathology associated with the precipitation of premature birth. On the other hand, serum concentrations of the soluble regulator proteins also appear to be low in neonates and preterm neonates, which may allow lower effector component concentrations to function prior to inhibition.

1.4.3.1 Complement Component of Classical and Lectin Pathways in Neonates

Many studies found that the functional activity and the components levels of classical and lectin pathways are low in cord blood compared to adult levels (Fireman *et al*., 1969; Adinolfi and Beck, 1975; Feinstein and Kaplan 1975; Strunk *et al.,* 1979).

Fireman and his colleagues found that the level of C1, C2 and C4 were only about 50% of maternal level (Fireman *et al*., 1969). C1 level was found in term and preterm to be between 53-79% and 27-65% respectively. In term newborns, C2 and C4 level ranged between 62-85% and 42-73% (Miyano *et al*., 1996; Sonntag *et al*., 1998b). The level of C2 and C4 were found to reach adult values at 1 to 6 months respectively whereas C1q level was lower than adult levels even by 6 months of age (Davis *et al*., 1979).

Newborn infants are able to synthesise MBL and maternal MBL is not transferred to the foetus (Kilpatrick *et al*., 1996; Van der zwet *et al*., 2007). In addition, Aittoniemi *et al* 1996 demonstrated a correlation between gestational age and MBL levels in newborns. Their results were in keeping with other studies which demonstrated that MBL is detectable in cord blood and the level of MBL increased with the duration of gestation (Molhortra *et al*., 1994;

Lau *et al*., 1995; Maruyama *et al*., 2003). In contrast, other studies found no correlation between MBL level in cord blood and gestational age (Kilpatrick *et al*., 1996; Swierzko *et al*., 2009). Moreover, mothers with high MBL level during pregnancy were more likely to have premature infants (Van de Gijn *et al*., 2008). MBL can modulate cytokine production *in vitro* and *in vivo* and this may influence the response of neonates to even minor infections (Jack *et al*., 2001). Synthesis of circulating MBL concentration is determined by variations in the structural and promoter region of MBL-2 gene (Turner *et al*., 1993). On the other hand, variants of MBL gene resulting in low level of MBL in neonatal blood were also associated with CLD complicating RDS in preterm infant (Hilgendorff *et al*., 2007). This was in agreement with the findings of Frakking *et al*., 2007 who reported a 15-fold higher risk of early onset sepsis in neonates with variant MBL-2 genotype that gives low MBL levels compared with neonates with wild type MBL-2 genotype.

Low MBL levels in premature neonates reflect insufficient production due to physiological immaturity of the liver as noted for other complement and liver proteins (Polberger *et al*., 1990; Kanakoudi *et al*., 1995). De Benedetti *et al* 2007 also found that low MBL levels were associated with sepsis in newborns. MBL's protective role against neonatal sepsis was demonstrated and the low MBL levels at birth were associated with an increased risk of hospital-acquired sepsis in infants admitted to the neonatal intensive care unit (NICU). Similar results were reported by Frakking *et al*., 2007 who demonstrated high susceptibility to infection in premature and term NICU patient with low MBL level.

MBL serum concentrations in cord blood samples were significantly lower in preterm infants compared to term newborn. The lowest levels of MBL as well as SP-D were seen in infants less than 28 weeks of gestational age especially in those with lower Apgar scores. This may be a predisposing factor to pulmonary and systemic infections (Hilgendorff *et al*., 2005).

MBL levels in term neonates increased in the first five days of life (Terari and Kobayashi, 1993) and tripled in the first 3 month of life (Thiel *et al*., 1995; Van dez zwet *et al*., 2007).

1.4.3.2 Alternative pathway

The complement components and the activity of this pathway exhibit similar trends to those of the other pathways with significantly lower levels in infants compared to adults. Factor B level was shown to be less in neonates compared to adult level ($P<0.001$) with level between 27-64% in term (wolach *et al*., 1997; Sonntag *et al*.,1998b) and 31-53% in preterm compared to adult values (Feinstein and Kaplan 1975; Miyano *et al*., 1996; Wolach *et al*., 1997).

Moreover, properdin ranges in term and preterm infants compared to adults were 33-72 and 13-41% respectively (Strunk *et al*., 1979; Drew and Arroyave 1980; Johnson *et al*., 1983; Wolach *et al*., 1994). Properdin levels correlated with birth weight (Adamkin *et al*., 1978). Davis *et al* reported that the level of factor B reached adult level by 6 months of age while properdin levels were still lower than adult levels even at 6 months and only reached adult levels at 16-24 months after birth (Davis *et al*., 1979).

1.4.3.3 Complement functional activity

The functional activity of alternative and classical pathways in term infants was decreased by 50% relative to the adult level (Adinolfi and Beck 1975; Johnston *et al*., 1979; Anderson *et al*., 1983; Notrangelo *et al.*, 1984; Wolach *et al*., 1994). Total haemolytic complement (CH50) was found to be between 52-73% and 32-78% in term and preterm infants, respectively. However, several studies indicated that alternative pathway function (AP50) in term or close to term infants is around 60-70% of adult levels (Wagner *et al*., 1996; Wolach *et al*., 1997; Sonntag *et al*., 1998 b). Low haemolytic activity of the neonatal complement system was attributed to the inability of these sera either to generate adequate number of

target sites or to stabilize sufficiently the enzymatic sites on the target cell membrane (Edwards, 1986). Furthermore, Drew and Arroyave (1980) reported that nearly absent levels of C3 and factor B split products in the sera of a majority of their normal study infants suggest that the low CH50 activity and low concentrations were developmental and not due to consumption of components.

CH50 was also studied in preterm newborns where adult/cord CH50 ratio was 32-78% (Adamkin *et al*., 1978; Strunk *et al*., 1979; Drew and Arroyave 1980; Miyano *et al*., 1987; Wolach *et al*., 1997; Høgåsen *et al*., 2000). Wolach *et al* studied cord/ adult CH50 ratio in term neonates and in 2 groups of preterm neonates with a gestational age of 34-36 weeks and 28-34 weeks respectively. They found the highest ratio (78%) in 28-34 weeks preterm followed by 34-36 weeks preterm newborns (75%) and the lowest level was in term group (69%) but the difference was not statistically significant (Wolach *et al*., 1997). In a similar study, Petrova *et al*., 2003 compared CH50 of cord blood and CH50 at 3rd or 4th postnatal day with maternal CH50 in 2 groups of preterm infants. Group I were gestational age (GA) of 28-32 weeks, whereas group II GA was 33-36 weeks. Group I showed significantly lower cord/maternal ratio (34.4%) than neonatal/maternal ratio (54.5%) whereas group II percentages were 62.9% and 56.4% respectively and the difference between these 2 ratios was statistically insignificant. Significant correlation between CH50 and gestational age was found in many studies (Ueda *et al*., 1980; Notarangelo *et al*., 1984; Miyano *et al*., 1987; Kitajema *et al*., 1990; Petrova *et al*., 2003).

Ferriani *et al*. (1990) studied CH50 in three groups of neonates; healthy infants aged 1 to 24 months and healthy adults. They found that neonates have the lowest values among groups studied. Surprisingly, they demonstrated that serum lytic activity among children aged 7-24 months was higher than normal adults. This finding disagreed with data reported by Norman *et al*. (1975) who did not find a difference in CH50 results of newborns and children up to 14

years old (Norman *et al*., 1975). Another study reported CH50 was proportional to gestational age and reached adult levels at 3-6 months of age (Fireman *et al*., 1969). Similarly, Pedraz *et al*. (1980) demonstrated that CH50 levels reached maternal level at six months of age.

Strunk *et al*., 1979 found that CH50 and AP50 were significantly correlated with birth weight. Moreover, they found that alternative and classical pathway functional activities were equally deficient in newborns as suggested by unchanged CH50/ AP50 ratio in cord and adult serum. In contrast, Drew and Arroyave 1980 did not find any correlation between concentrations of alternative pathway components (factor B and properdin) and gestation age and they believed that components of alternative pathway have steady rate of production during foetal life unlike classical pathway. Stossel *et al* have suggested that low levels of alternative pathway components may be more important in predisposing infants to infection than defects of classical pathway (Stossel *et al*., 1973)

On the other hand, Ferriani and co-workers (1990) reported that classical pathway activity matures at earlier age than the alternate pathway (1-3 months and 13-18 months respectively). Inter individual variation was demonstrated by Johnston *et al* when they measured the rate of haemolysis in 27 babies. Only 14 had CH50 lower than 2SD below the mean compared with 21 normal adult sera and so not every term baby had abnormal complement activity, at least of the classical pathway (Johnston *et al*., 1979).

Drew and Arroyave, 1980 studied effect of gestational age and birth weight on CH50. They demonstrated that preterms have lower CH50 than full term neonates and found statistically significant correlation between gestational age and serum concentrations of complement and CH50 activity. They suggested that intrauterine growth retardation has no effect on haemolytic activity and Shapiro *et al*. in agreement with them had shown equal CH50 levels

in small for gestational age and newborn with appropriate weight for age (Shapiro *et al*., 1981). Drew and Arroyave also compared haemolytic activity and components levels in normal and infected newborn and suggested that relative absence of C3 and factor B split products in the sera of the majority of normal infants is indicative of developmental aetiology (low production) for the low complement component levels and CH50 activity rather than due to consumption, secondary to activation of the complement system by either the classical or alternative pathways.

1.4.3.4 Complement component C3

C3 is central complement component which has a major role in bacterial opsonisation by all pathways. The synthesis of this component starts from 9th week of foetal life and increases with foetal development (Adinolfi, 1970). The level of C3 has been found to be lower in term and preterm infants compared to adults and also in term compared to preterm. Mean C3 concentration in small for gestational age group was significantly lower than in the appropriate weight for gestational age group $P<0.02$ (Shapiro *et al*., 1981). Johnson *et al.* found the level of C3 in newborn serum 88% of adult level using electroimmunoassay (Johnson *et al*., 1983). Lower values were reported by Miyano and co-workers using a different method. They found that level of C3 was only 45-47% of adults using radial immunodiffusion. Measurement of C3 in 32-42 gestational week neonates by ELISA was shown to be 56% of adults. The median level in full term neonates was 0.67g/l compared to 0.8-1.2 g/l in adults, rising to adult levels by 3-6 months of age (Fireman *et al*., 1969; Norman *et al*., 1975). Quantitative maturation of complement components C2, C4, C5, C6 and factor B occurred in an age related fashion, so their levels were not significantly different from adult level by six month age (Davis *et al*., 1979).

There are several reports of increased formation of complement activation products such as Ba, C3a, C3d and SC5b-9 in infants with neonatal infections compared with healthy newborns, indicating that activation fragments are valid indexes of infection and underscore normal complement activation in neonates (Drew Arroyave 1980; Peakman *et al*., 1992; Zilow *et al*., 1993; Zilow *et al*., 1997).

1.4.3.5 Terminal pathway in neonates

Activation of C5-C9 in the terminal pathway occurs as a result of C3 convertase activation irrespective of which pathway cleaves C3 and ultimately results in formation of MAC responsible for pathogen killing. Levels of terminal complement components were lower in term and preterm infants compared to adults except for C7 which was equal to or higher than adult level (Johnson *et al.,* 1983; Wolach *et al.,* 1994; Wolach *et al.,* 1997; Sonntag *et al.,* 1998b). The exception to these studies is Adinolfi and Beck who showed that C7 concentration in neonates was only about 70% of adult levels (Adinolfi and Beck, 1975). Fireman and his colleagues found that the level of C6 to be about 50% of maternal level (Fireman *et al*., 1969) but comparison with maternal level may underestimate complement level in newborns because complement components (except C8) are acute phase reactants and pregnancy induces higher levels than in non-pregnant females.

Wolach and co-workers studied complement levels in two groups of preterm and a group of term infants. They found a significant correlation between levels of complement and gestational age and the most significant surge in levels occurred later in pregnancy (Wolach *et al*., 1997).

C9 concentration varies considerably, possibly due to different methodology. Ballow *et al* in 1974 found that the level of C8 and C9 are in range of 10-25% of adult level whereas Zilow *et al* in 1994 reported that the level of C9 was as low as 1% of adult value. Lassiter used

radial immunodiffusion to measure C9 concentration and found that C9 level was 17% of the mean serum concentration from their mothers. He also found that reconstituting C9 levels corrected the limited *E coli* bactericidal capacity of neonatal sera with low C9 concentration (Lassiter *et al*., 1992). Høgåsen *et al* studied C9 using ELISA which is more quantitative and found that 15% of neonates in their study had C9 levels less than 2% of adult values (Høgåsen *et al*., 2000). Levels of C9 were also found to correlate with the gestational age.

1.4.3.6 Anaphylatoxins

The anaphylatoxin C3a, C5a, C5a-desArg are pro-inflammatory polypeptides generated after proteolytic cleavage of C3 and C5 in response to complement activation. C3a and C5a regulate vasodilation, increase the permeability of small blood vessels and induce the contraction of smooth muscles and may impair cardiac function (Ember *et al*., 1998; Klos *et al*., 2009). The anaphylatoxins in humans are rapidly inactivated by carboxypeptidase B which cleaves off the C-terminal arginine (hence the designation des-Arg) residue from the anaphylatoxins. Degradation products C3a desArg and C5a desArg molecules have much reduced anaphylatoxin activities. They increase vascular permeability and trigger smooth muscle contraction and release histamine from mast cells and basophils (Hugli, 1984). C3a and C3adesArg are eliminated by the kidney, while C5a is internalized and degraded by neutrophils. They only appear in plasma when the binding capacity of the neutrophils is exceeded. There is a correlation between C3a and MAC, while no correlation was found between C3a and C5a. C5a and C5adesArg activate neutrophils and macrophages, while both C3a and C5a induce the release of proteases and cytokines from leukocytes (Enskog *et al.*, 1996). C5a has potent chemotactic activity and plays an active role in pulmonary inflammation (Struck *et al*., 1988) Sonntag *et al* and Enskog *et al* found that anaphylatoxin

concentrations were higher in term neonates than in healthy adults. The reason for the higher plasma concentration in newborns may be due to the limited capacity of leukocytes to bind anaphylatoxins (Enskog *et al*., 1996; Sonntag *et al.,* 1998b). In contrast, Zilow *et al* used a capture sandwich ELISA to measure C3a-desArg and found that the level was similar in healthy term and adult.

It was found that premature babies who had birth complications tend to have high plasma anaphylatoxin concentrations and C5a was high in fluid aspirated from tracheobronchial fluid in preterm infants who were at risk of lung disease (Groneck *et al*., 1993; Enskog *et al.,* 1996). The levels of C3a were found to be increased in asphyxiated neonates as well as those with septic shock (Hack *et al*., 1989; Schrod *et al*., 1992).

Miyano *et al* reported that the level of C3, C4 and C3d as well as CH50 and factor B were lower in infants with RDS compared to infants with other lung diseases such as pneumonia or normal lung function (Miyano *et al*., 1991), while C1q and other serum component concentrations were similar between infants with and without RDS.

1.4.3.7 Regulatory proteins

The lack of some regulatory proteins, such as factor H, factor I and properdin are associated with greater susceptibility to infections and may represent a life-threatening condition. There are several studies investigating the concentration of regulatory proteins in neonates compared to adults. De Paula *et al* (2003) found significantly lower levels of factor H, factor I, C4BP, properdin and vitronectin in healthy neonates when compared to adults. Schwartz and co-workers have reported that free protein S levels in cord blood (gestational age 29-34 weeks) was 74% of that of adult plasma, and total protein S (which is usually bound as a component of C4BP) only 23% of adult mean. This is because of higher levels of C4BP in the term infants relative to adults (Schwartz *et al*., 1988). Other studies on preterm and term

infants have also showed low levels of C4BP had a marked correlation with gestational age; (Malm *et al*., 1988 Melissari *et al*., 1988), while the levels of protein S were found to reach adult levels by 4 months of age (Sthoeger *et al*., 1989).

Melissari *et al* (1988) found that C4BP was lower in term (18%) and preterm (6%) cord blood when compared to adult plasma (P<0.001) while neonates were reported to have 35-78% factor H and 25-75% of adult factor I concentrations (Davis *et al*., 1979). Reduced levels and function of C1INH was reported in neonates compared to adults: term levels of C1-INH were about 62% of adult and 77% of maternal level (Cat *et al*., 1993).

1.4.3.8 Complement activation and pathogenesis of some newborn diseases

Uncontrolled complement activation was implicated in pathogenesis of organ damage induced by premature rupture of membrane (PROM). Complements levels were normal in maternal and cord blood of preterm neonates born after premature rupture of membranes of more than 24 hours, but C5a levels increased significantly in the third postnatal day (Petrova and Mehta 2007). Amnionitis stimulated production and triggered activation of complement component in preterm infants (Kitajima *et al*., 1990). The level of C3, C4, C3d, CH50 and factor B were demonstrated to be high in infants born with associated chorioamnionitis whereas C5, C1q, C2 and C9 levels showed insignificant change compared to control (Kitajima *et al*., 1990; Miyano *et al*., 1996)

The level of C9 in CSF obtained from 16 infants with hypoxic injury encephalopathy (HIE) has been reported to be reduced compared to CSF from 7 control infants due to the activation of complement to form MAC and consumption of C9 during complement activation in the CNS following resuscitation (Schultz *et al*., 2005). C9 was deposited on cerebral neurons, and they also found the concentration of MAC in cerebrospinal fluid (CSF) of newborns with HIE to be greater than in infants without hypoxia.

Evidence that the classical pathway contributes to the hypoxic-ischemic brain injury has come from animal studies. A study on neonatal rats showed that complement activation contributes to hypoxic–ischaemic injury in the brain; however, depletion of complement activity by cobra venom factor (CVF) did not eliminate complement deposition within injured brain. This may indicate a role for local complement component synthesis in the CNS contributing to pathology (Cowell *et al*., 2003).

Meconium aspiration syndrome (MAS) is infrequent and severe disease of newborns characterised by respiratory distress, pulmonary inflammation, hypoxia and persistent pulmonary hypertension. The pathophysiology of MAS is complex with activation of different immune pathways and pro-inflammatory mediators (Van Ierland and de Beaufort 2009). Complement activation probably contributes to the pathogenesis of MAS (Castelheim *et al*., 2004; Mollnes *et al*., 2008). It is also responsible for neutrophil inflammatory responses mediated by C5a (Castellheim *et al*., 2005). These findings are substantiated by a neonatal pig model. Intrapulmonary instillation of meconium in piglets resulted in massive elevation of membrane attack complex followed by similarly substantial increase in cytokine levels. MAC and cytokine levels correlated with the degree of lung dysfunction and were higher in animals that died than in surviving animals (Lindenskov *et al*., 2004). Additionally, a recent study had demonstrated that meconium can activate the lectin pathway as well as alternative pathway in human serum (Salvesen *et al*., 2009).

1.5 *UREAPLASMA*

Ureaplasma spp is a member of the Mollicutes class together with mycoplasma. They were initially named *T- Mycoplasma* due to their tiny colonies compared to other *Mycoplasma* colonies but later renamed as *Ureaplasma* to reflect their ability to utilize urea as energy source. The species *Ureaplasma urealyticum* was later sub-divided into 2 biovars. Biovar 1 included serovars (SV) 1, 3, 6 and 14 whereas biovar 2 included SV2, 4, 5, 7, 8, 9, 10, 11, 12 and 13. The classification was reviewed in 2002 and the biovars 1 and 2 were reclassified in to 2 species (based on 16S rRNA homology): *Ureaplasma parvum* SV1, 3, 6 & 14) and *Ureaplasma urealyticum* (remaining SV), respectively (Robertson *et al*., 2002). Both of these species have been detected in clinical samples particularly *U. parvum* (Abele-Horn *et al*., 1997; Kong *et al*., 1999). The common features of these species are lack of cell wall, small genome size, and utilization of urea as primary energy source. Despite being recognized as a commensal in the female genital tract of low virulence, it is increasingly linked with various obstetric and gynaecological problems including infertility, foetal loss, chorionamnionitis and preterm labour (Waites *et al*., 2005).

1.5.1 *Ureaplasma* in neonates

Ureaplasma urealyticum is known to cause non-gonococcal urethritis (Horner *et al*., 2001). It was also reported as a causative agent for meninigitis in an immunocompromised adult after renal transplant (Geissdörfe *et al*., 2008). *Ureaplasma* is believed to be a major cause of chorioamnionitis and thereby linked to preterm labour, spontaneous abortion and chronic lung disease in neonates (Cassell *et al*., 1986; Yoon *et al*., 1998). It is also known to cause meningitis in term and premature neonates (Waites *et al*., 2005).

Neonatal *Ureaplasma* colonisation has been associated with development of CLD in preterm infants (Yada *et al*., 2010). Moreover, a cohort of 1,988 pregnant women had cervical culture

for *Ureaplasma* at the initial antenatal visit and followed up during pregnancy. Abnormal vaginal flora were detected in 22.7% of which 53.6% had *Ureaplasma spp*. In all intrauterine models, *Ureaplasma* infection was persistent which highlights the limited capacity of newborns to clear these bacteria (Viscardi, 2012). *Ureaplasma* colonisation in preterm newborn that subsequently developed CLD could be persistent or transient (Castro-Alcaraz *et al*., 2002). Using logistic regression to analyze the outcome with other risk factors of PT labour taken into consideration. PT labour was significantly correlated (p= 0.02) with positive *Ureaplasma* culture with an odds ratio (OR) of 1.64 and 95% confidence interval 1.08-2.48 (Breugelmans *et al*., 2010).

In contrast, a recent study investigated the prevalence of *Ureaplasma* species in amniotic fluid obtained trans-abdominally at 16-20 weeks of pregnancy from 121 asymptomatic pregnant women. *Ureaplasma* were not isolated by culture in any of the studied amniotic fluid samples but 4 cases were identified on multiplex PCR (2 *U. parvum* and 2 *U. urealyticum*). All the 4 cases had normal labour and gave birth to normal weight babies. Four other cases of PT delivery in the series were from *Ureaplasma* negative mothers. Their prematurity is not associated with *Ureaplasma* infection. (Rodríguez et *al*., 2011). This was in agreement with another study which did not demonstrate association between preterm with *Mycoplasma hominis*, U. *urealyticum* or U.*parvum* colonization and labour outcome (Govender *et al*., 2009).

One animal model study suggested a role for *Ureaplasma urealyticum* in the development of chronic lung injury of some immature infants, but the critical finding is the differential response to *U. urealyticum* among an apparently homogeneous group of antenatal exposed, immature, nonhuman primate infants (Yoder *et al*., 2003). The same study revealed that approximately 50% of immature baboon infants (GA 125 days; full term is 185 days) could

clear *Ureaplasma* from their pulmonary system within the first week after delivery and those who failed to clear the bacteria where at higher risk of pulmonary complications (Yoder *et al*., 2003).

Moreover, epidemiological human studies suggest that *Ureaplasma* infection is associated with pathogenesis of CLD. The Alabama preterm birth study was undertaken to evaluate the prevalence of *Ureaplasma* and *Mycoplasma spp* infection in neonates born at gestational age of 23-32 weeks and its association with adverse outcomes. The study included 351 mothers and their newborn babies and it demonstrated that *Ureaplasma spp* and *Mycoplasma hominis* infections were significantly more common in spontaneous PT labour. Positive cultures were found in 23% of the women and associated with systemic inflammatory response in newborns and probably with CLD (Goldenberg *et al*., 2008). Another prospective study on very low birth weight (<1501 gram birth weight; VLBW) preterm neonates agreed with Alabama study results and suggested that invasive *Ureaplasma* infection probably increases the risk of severe intraventricular haemorrhage in VLBW infants (Viscardi *et al*., 2008).

Another experimental study in Rhesus monkeys showed that intraamniotic inoculation of *M. hominis* or *U. parvum* as a sole pathogen elicits a remarkable pro-inflammatory response that contribute to PT labour and foetal lung injury. Chorioamnionitis and pneumonitis also worsen with duration of in *utero* infection (Novy *et al*., 2009).

In addition, VLBW newborns colonized with *Ureaplasma* were twice more likely to develop pneumonia than non-colonised VLBW neonates (Pacifico *et al*., 1997). All the past studies were reviewed by Viscardi and Hasday (2009) who concluded that the balance of reports showed that *Ureaplasma* infection definitely contributes to the pathogenesis of CLD and is augmented by the barotrauma of the ventilatory support and adverse effect of oxygen therapy. These factors collectively lead to dysregulated inflammatory response in the immature lung that impairs alveolarization and stimulates collagen and elastin deposition secondary to

fibroblast proliferation and activation (Viscardi and Hasday 2009). More convincing evidence of the significant association between *Ureaplasm*a infection and subsequent development of CLD comes from a meta-analysis of 23 cohort studies that recruited VLBW infants colonized or infected with *Ureaplasma.* More than 200 newborn were included in the analysed studies published between 1988 and 2004 (Schelonka et *al*., 2005).

A recent case report also presented autopsy results of a newborn that died, concluding the cause of death to be *U. parvum* congenital pneumonia which was confirmed by PCR (Morioka *et al*., 2010).

Recent interest in collectins, in particular surfactant protein A (SP-A) have resulted in a report where SP-A played an important role in clearance of *Ureaplasma* infection. Evidence came from *in vitro* study showing enhancement of macrophage cell line (RAW 264.7) ability to phagocytose and kill *Ureaplasma spp* when SP-A was added in a concentration of 10-50 µl/ ml (Okogbule-Wonodi *et al*., 2011). Moreover, SP-A deficient mice when infected with *Ureaplasma* were less efficient in clearing the infection and also exhibited an exaggerated inflammatory response compared to wild type mice. Simultaneous administration of SP-A and *Ureaplasma* to the deficient mice did not improve the rate of bacterial clearance but on the other hand, reduced the inflammation which denotes the importance of this collectin in regulation of pulmonary inflammatory response to infection (Famuyide *et al*., 2009). Expression of secretory leukoprotease inhibitor and anti-neutrophil elastase activity were also reduced in bronchoalveolar fluid from SP-A deficient mice, suggesting anti-inflammatory action of SP-A is in part due to its ability to inhibit cleavage of secretory leukoprotease inhibitor by matrix metalloproteases (Ramadas *et al*., 2009).

1.6 Hypothesis and Aims

Hypothesis: Neonatal neutrophils are deficient in proteases relative to adult levels and newborn cord serum is also relatively deficient in terminal complement components (C6-C9) which impairs complement function and capacity of cord serum to kill pathogens.

Aims

The objectives of this research were:

- To compare the total and releasable concentration of cathepsin G and proteinase 3 between normal adults and normal caesarean-delivered term neonates, including the expression and surface binding of proteinase 3 and its high affinity neutrophil surface receptor CD177.
- To examine surface proteinase 3 expression on the surface of neutrophils lacking CD177 (both in deficient individuals and normally occurring CD177-negative subsets) in adults and neonates.
- To measure proteinase 3 concentrations in bronchoalveolar lavage samples (BAL) from ventilated premature neonates, correlated to clinical outcome (resolved respiratory distress versus chronic lung disease) and to assess its relationship with other neutrophil proteases, elastase and MMP-9.
- To examine developmental insufficiency of complement in term neonates (relative to normal adults) by developing ELISAs for C6, C7, C8 and C9 and establishing accurate reference ranges for these components and relating the deficiencies to decreased complement function as measured by lysis of sensitized sheep erythrocytes (CH50 assay) and bacteriocidal activity against the important neonatal pathogen, *Ureaplasma parvum*.

Chapter Two

Materials and Methods

2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a biomedical technique used to detect antigens (Ag) in biological samples. It is based on the direct interaction of the Ag with an antibody (Ab) which is labeled with an enzyme. The antibody bound to the antigen is detected by the addition of the enzyme's colourless substrate (chromogen). The coloured reaction product indicates the presence of Ag. The coloured change can be measured and quantitated by reading the Absorbance in a spectrophotometer *(*Murphy *et al.,* 2008).

Optimised sandwich ELISAs were created for measuring C6, C7, C8 and C9, choosing the most sensitive monoclonal antibody from commercial and non-commercial sources. Where necessary, sensitivity and range of assay were enhanced by immobilising monoclonal antibodies with rat anti-mouse IgG1 monoclonal antibody. Amino acid hydrolysis (performed by the supplying company, Quidel Corporation), was used to accurately quantify affinity purified C6, C7, C8 and C9. Confidence intervals for complement concentration were determined in 20 normal adults and 20 cord sera from uncomplicated caesarean-delivered term newborns. Study was approved by Research Ethics Committee (REC) and written informed consent was obtained from mothers prior to section and from volunteers. Serum preparation is described in section 2.3.4.

2.1.1 Complement C9 ELISA

2.1.1.1 Antibodies

The suitability of different monoclonal antibodies (mAb) as capture Abs for purified C9. ELISA was tested in order to choose the most sensitive antibody. Monoclonal antibodies tested against human C9 were supplied by Quidel, anti-C9 (cat.A223), and from Hycult Biotechnology (clone WU13-15, X179 and aE11). Non-commercial monoclonal anti-C9

antibody (B7) was kindly provided by Professor Paul Morgan. In addition to testing this antibody's ability to capture purified Quidel C9, they were also tested for their ability to capture C9 in C5b-C9 complex as well as native C9 in diluted serum samples. Only Quidel goat anti-C9 polyclonal antiserum was tested as a detecting antibody, which was visualised by Jackson Laboratories' HRPO-conjugated minimum cross-reactivity Donkey anti-goat antibody and OPD (measured at 490 nm).

2.1.1.2 Varying the concentration of mAbs to coat C9 ELISA plate

In order to optimise C9 ELISA, The effect of different concentrations of B7, X179 and aE11 mAbs, were tested to detect the most suitable working concentration of these antibodies to act as capture mAb for C9 ELISA. Tested mAbs concentrations started from 1 µg/ml to 4 µg/ml. Where nessary, sensitivity and range of assay were enhanced by immobilising monoclonal antibodies on plates coated with rat anti-mouse IgG_1 monoclonal antibody at final concentration of 4 µg/ml (Stock concentration 1mg/ml, Lifespan Bioscience).

2.1.1.3 C9 ELISA antibody selection

The capture anti-C9 monoclonal antibodies (mAbs) were diluted in coating buffer ($NaHCO_3/Na_2CO_3$, pH 9.6) at a final concentration of 2-4 µg/ml and used to coat ELISA plates (96-well flat bottom, Nunc-Immuno Maxisorb, Thermo Fisher Scientific, Denmark) by adding 100 µl of diluted antibody into each well. The plates were then sealed with an adhesive strip and incubated at 37°C for one hour, followed by complete removal of liquid by inverting and tapping the plates against clean paper towel. Non-specific binding sites were then blocked by filling the wells with 115 µl blocking buffer [5% bovine serum albumin (BSA) in phosphate buffered saline (PBS), containing 0.05% Tween 20 (PBST)]. The plates were incubated for one hour at 37°C, before being washed once with PBST. The purified C9

standard (Quidel C9) serial dilutions ranging from 0.78 to 200 ng /ml of C9 were prepared in blocking diluent buffer and used for the preparation of the standard curve. Similar steps were performed to obtain C5b-C9 standard curves. Standard curves were run in duplicates for each ELISA, 100 µl of the standard was dispensed into designated wells and the plates were incubated for 1 hour at room temperature (RT).

The detecting Ab (polyclonal goat anti- human C9 Ab) was diluted to a final concentration of 1:1000 in blocking buffer and added to each individual well. The plates were sealed and incubated at room temperature for one hour. Plates were washed three times with PBST and incubated at room temperature with 1:1000 of Horseradish peroxidase(HRPO) anti- goat antibody (Jackson Laboratories), diluted in blocking buffer, for one hour. Plates were washed three times with PBST then once with PBS. The plates were developed with a substrate solution made up by adding two tablets of Ortho-Phenylenediamine, (OPD-EASY) (each contains 3.5 mg of 1,2-phenylendiamine dihydrochloride) (Fisher Scientific), to 6.4ml of sterilised water mixed with 2.5 µl of hydrogen peroxide (Aldrich, Steinheim, Germany). One hundred µl of substrate solution was added to each well and incubated for about 10-20 minutes, depending on the rate of colour change of the standard curve wells. The reaction was stopped by adding 100 µl of 2N sulphuric acid (H_2 SO_4) (Sigma-Aldrich) and read on a plate reader (Dynex technologies, Chantilly, VA) at 490nm.

2.1.1.4 C9 ELISA in cord and adult sera

The most sensitive capture antibody, locally available anti-C9 (B7) was coated onto 96-well Nunc-Maxisorb plates at a concentration of 2µg/ml. The standard curve ranged from 0.78 to 200 ng /ml, cord and adult serum samples were assayed at 1:1000 and 1:2000 dilution as detailed above in section 2.1.1.3.

Table 2.1 List of monoclonal antibodies used in ELISA

Antibody specificity	**Clone**	**Isotype**	**Concentration**	**Source**
Monoclonal antibody to human C9	A223	IgG2bk	>1 mg/ml	Quidel,san, Diego USA
Monoclonal antibody to human C9	WU 13-15	Mouse IgG1	100 µg/ml	Hycult-Biotechnology
Monoclonal antibody to human C9	X197	Mouse IgG1	100 µg/ml	Hycult-Biotechnology
Monoclonal antibody to human C9	aE11	Mouse IgG1	100 µg/ml	Hycult-Biotechnology
Monoclonal antibody to human C9	Non-commercial B7	Unknown	0.6mg/ml	Paul Morgan Lab-Cardiff university
Monclonal to human C6	WU6-4	MouseIgG1k	100 µg/ml	Hycult-Biotechnology
Monclonal to human C6	A219	Mouse IgG1	1 mg/ml	Quidel,san, Diego USA
Monclonal to human C7	WU 4-15	MouseIgG1k	100 µg/ml	Hycult-Biotechnology
Monclonal to human C7	A221	MouseIgG1K	1mg/ml	Quidel,sanDiego USA
Monoclonal to human C9	A249	Mouse IgG1	1mg/ml	Quidel, San Diego USA
Monoclonal to human C8	Clone αC8 E2	BPM	0.411 mg/ml	Non-commercial Morgan Lab

2.1.2 Complement C6 ELISA

2.1.2.1 Optimising C6 ELISA

Different monoclonal antibodies(mAbs) were tested for their ability to capture purified C6 listed in table 2.1 as well as human serum. In addition, working concentration of the most sensitive antibody was optimised by testing serial different concentration ranging from 1 to 4 µg/ml as illustrated above in C9 ELISA section 2.1.1.3. Quidel goat anti-C6 polyclonal antiserum was tested as a detecting antibody, which was visualised by Jackson Laboratories' HRPO-conjugated minimum cross-reactivity Donkey anti-goat antibody and OPD (measured at 490 nm).

2.1.2.2 Cord and adult C6 ELISA

C6 levels in cord and adult sera were measured using an ELISA assay. A 96-well plate which was coated with the selected Hycult anti-human C6 (clone WU 6-4), diluted in carbonate coating buffer at final concentration of 4 µg /ml stock concentration 0.88mg/ml, Hycult-biotech). Standard C6 (Quidel) along with cord and adult sera were diluted using blocking buffer as mentioned above. The standard C6 range was 500 to 1.23 ng /ml, and samples were assayed at 1:500 and 1:1000 dilution, all done in duplicate at 100 µl /well. After 1 hour incubation at RT, the plate was washed three times with PBST then 100 µl /well of polyclonal anti-C6 (Quidel), diluted to 1:1000 in blocking buffer were added. The detection goat antibody was detected as detailed above, section 2.1.1.3.

2.1.3 C7 ELISA

2.1.3.1 Optimising C7 ELISA

Different monoclonal antibodies were tested to capture purified C7 as listed in table 2.1 Titration of the most sensitive one was done to reach the best working concentration. In addition, rat anti-mouse IgG_1 antibody was used to enhance the binding of capture antibody and allow using it at lower concentrations. Only one detecting antibody (Quidel polyclonal anti-C7) was used and the reaction was visualised using (HRPO) anti-goat antibody.

2.1.3.2 C7 ELISA in cord and adult sera

Rat anti-mouse IgG_1 antibody was pre-coated into 96-well microtiter plate by incubation (100µl/well) of a 4µg/ml dilution in ELISA coating buffer at 37°C for one hour. Plates were blocked with (0.5% BSA in PBS containing 0.05% Tween 20), 115µl/well at 37°C for 45 minutes. Rat anti-mouse IgG1 coated plates were used to immobilise the best mAb, murine anti-human C7 (Ouidel, stock concentration 1.16mg/ml) at a concentration of 2µg/ml. The plate was washed once with PBST, before adding standard ranging from 1.23 ng/ml to 500 ng/ml (Quidel). Serum samples (100µl/well) were tested at 1:500 and 1:1000 dilutions in blocking buffer. After incubation for one hour at room temperature, the plate was washed three times with PBST, before addition of goat polyclonal anti –sera to human C7 (Quidel) at 1:1000 dilution in blocking buffer, 100 µl/well. The detection antibody was measured as detailed above in section 2.1.1.3.

2.1.4 C8 ELISA

2.1.4.1 Optimising C8 ELISA

96 well Maxisorb microtiter plates were coated with three different monoclonal antibodies specific for C8 (Ouidel, Hycult, and non- commercial BPM antibody) listed in table 2.1. Different concentrations of these antibodies were used to capture purified C8. The empirically-determined most sensitive antibody (BPM) was titrated for its optimal working concentration as described for C9 mAbs. Only Quidel goat anti-C8 polyclonal antiserum was tested as a detecting antibody, which was visualised by Jackson Laboratories' HRPO-conjugated minimum cross reactivity Donkey anti-goat antibody and OPD (measured at 490 nm).

2.1.4.2 C8 ELISA in cord and adult sera

96 well Maxisorb microtiter plates were coated with the selected best mAb, BPM anti-C8 (clone $\alpha C8E_2$, stock concentration 0.411 mg/ml) at 2µg/ml diluted in coating buffer, 100µl/well and incubated at 37°C for one hour. Plate was incubated for further one hour with blocking buffer (0.5% BSA in PBS with 0.05% Tween 20) and washed once with PBST. The standard was diluted in blocking buffer ranging from 1.953ng/ml to500 ng/ml. Cord and adult serum samples were diluted 1:500 and 1:1000 and aliquots of 100 µl were measured. The plates were incubated for one hour at room temperature before being washed three times with PBST. Polyclonal goat anti-sera to human C8 diluted 1:1000 in blocking buffer was used as detection antibody and was measured as above for other assays, section 2.1.1.3.

2.2 Complement Haemolytic activity (CH50) assay

The CH50 assay was used to assess the ability of cord sera to haemolyse sensitised sheep RBCs (complement-mediated haemolysis). Sheep erythrocytes (SRBCs) were gently resuspended in the stock bottle by tapping the side of the bottle and by gentle inversion and pipetting (TCS serological products, UK). Six mls of sterile SRBCs were aseptically transferred to a 15 ml conical tube using a biological safety cabinet. Tube was topped up with fresh PBS and mixed by gentle inversion, then centrifuged at 1000xg for 4 minutes. Supernatant was removed and the pellet was gently re-suspended twice in 10 ml PBS. The tube was centrifuged at 1200xg for 4 minutes and the supernatant was removed. Five ml of PBS was added to two lots of universal containers, one of which received 20 µl of amboceptor antibody (rabbit α-sheep erythrocyte antibody) while the other received 400 µl of washed sheep erythrocyte pellet. These two lots were pooled together. The resulting suspension of erythrocytes was incubated in water bath, 37° C for 30 minutes and mixed gently every 10 minutes. Following sensitisation the tube was gently mixed and centrifuged at 1500 xg for 4 minutes. The pellet was washed with 20 ml PBS and centrifuged at 1500 xg for 4 minutes and then subsequently washed in 10 ml of veronal buffer saline (VBS) followed by centrifugation for 4 minutes before being resuspended in 20 ml of VBS as the working stock (sensitised erythrocytes diluted to 1% in VBS). Cord and adult serum samples were then diluted to 1:20 in VBS in preparation for the assay. A flat bottom 96-well plate was prepared for each assay: Row (A) contained 50 µl of extran (1 ml of H_2O in 50 µl of Tween 20) was added to all wells in row A. 50 µl of the 1:20 serum (internal control) was added to row B wells. C-H received 50 µl VBS. Wells in row C received an additional 100 µl of the 1:20 serum dilution (final dilution 1:30) from both cord and adult sera.

Where indicated serum samples were supplemented with 50 micrograms/ml C9 and compared to matched unsupplemented samples in parallel. All samples were run in triplicate. Serial 2:3 dilutions (100 µl) were made down the plate from well C to well G after which the remaining 100 µl was discarded. Row H wells were left as a serum-free VBS negative lysis control. The 4% erythrocyte solution was diluted 1:1 to give a working 2% solution of which 50 µl was added to each well. The plate was then incubated at 37° C for 30 minutes with absorbance reading being taken every 3 minutes in a plate reader at wavelength of 690 nm.

2.3 *Ureaplasma* killing assay

2.3.1 *Ureaplasma* Strains used

All experiments were conducted using prototypic *Ureaplasma parvum* strains of serovar (SV1) (DKF-1; obtained from health protection agency (HPA); Colindale, London, UK), SV3 (HPA5), SV6 (HPA2) and SV14 (HPA32). All strains were maintained in *Ureaplasma* specific medium (USM; purchased from *Mycoplasma* Experience, Surrey, UK).

2.3.2 *Ureaplasma* selective culture medium and culture conditions

Ureaplasma serovars were grown in *Ureaplasma* selective medium (USM) purchased from Mycoplasma Experience Ltd. (Surry, UK). USM consisted of a simple broth base medium supplemented with yeast extract, 7 mM urea, 10% heat-inactivated porcine serum, with phenol red as the pH indicator (starting pH, 6.65) and with 2.5 µg/ml amphotericin B and 0.25 mg/ml ampicillin. Cultures were performed in flat-bottom 96-well plates covered with

adhesive strip (Elkay, Basingstoke) or in 5 ml bijoux containers (Elkay, Basingstoke) and incubated in a humidified tissue culture incubator set at ambient CO_2 concentration at 37 °C.

2.3.3 Quantification of *Ureaplasma parvum* growth

Ureaplasma growth was expressed as colour changing units (CCU). To determine the number of CCU within a sample or culture a series of 1:10 dilutions are made as follows. 180 µl of USM was added to a single column (8 wells) of a 96 well flat plate. 20 µl of sample was incubated into the first well to give an initial 1:10 dilution. From this well the process is repeated down the plate to 7^{th} well give a dilution gradient to 10^{-7}. The final well remained un-inoculated to serve as a negative colour, to rule out false positives. Plates were then incubated for 48 hours at which time colour change had ceased. The final well in which colour change occurred was denoted as 1 CCU. By knowing the dilution of the well giving 1 CCU, the number of CCU in the initial sample could be calculated.

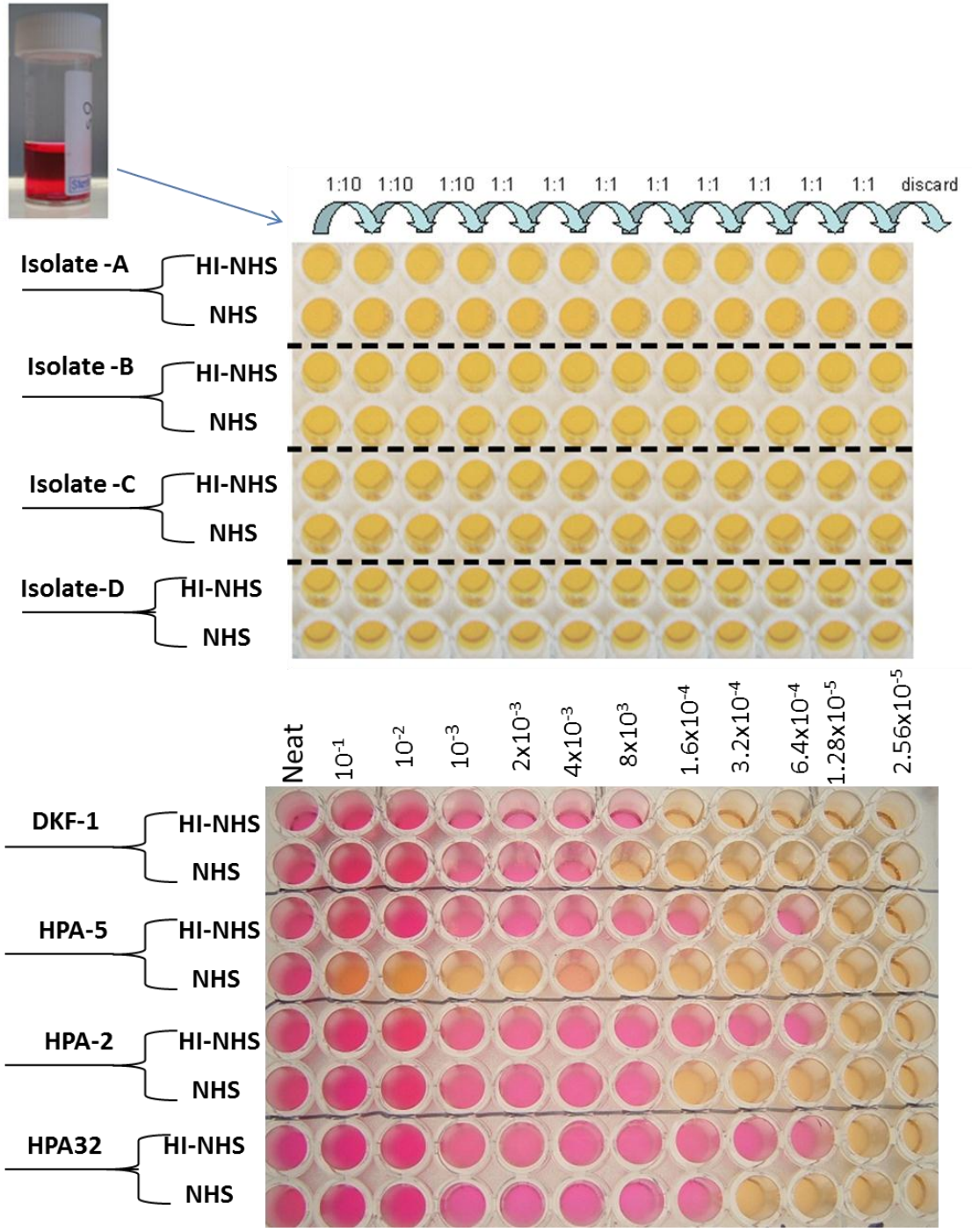


Figure 2.1 Diagram of complement killing assay, a 96 well plate setup for four Ureaplasma Parvum isolates (A-D) under two conditions. 50% heat inactivated normal human serum (HI-NHS) and 50% normal human serum (NHS). Following complement attack Ureaplasma were titrated out as set out in the plate above, initially 1:10 dilutions followed by 1:1 dilutions, to determine the fold reduction of colour changing units between HI-NHS and NHS. (Yellow wells indicate no growth while pink colour indicates positive growth).

2.3.4 Preparation of human serum

Stored serum was collected from 20 healthy adult volunteers and 20 full term umbilical cord samples of unknown *Ureaplasma* colonisation status. Blood samples were originally obtained by our laboratory from the adult volunteers and from placentas of consented Caesarean section mothers. Blood was collected in 20 ml glass universal bottles and allowed to clot at room temperature for one hour. The clot was dislodged from the wall of the bottle and incubated for an additional hour on ice to allow the clot to contract further. Samples were centrifuged at 3600 xg for 10 minutes. Supernatant (serum) was then dispensed into 1.5 ml tubes and centrifuged again at 16000 xg for 1 minute to remove remaining coagulated debris. Aliquots of 250 µl of normal human serum (NHS) were dispensed into 0.5 ml (Eppendorf) tubes and stored at -80° C until required. Heat inactivation of serum was done by incubation at 56°C for 30 minutes to eliminate heat labile complement components. Heat inactivated normal human serum (HI-NHS) lacks functional C2 (required for the classical and lectin pathways) and factor B (required for the alternative pathway). A mixture of 50% NHS and 50% HI-NHS was prepared by mixing equal volumes of them with veronal buffered saline (VBS or Complement Fixation Buffer; Oxoid, Basingstoke).

2.3.5 Complement killing assay in cord and adult sera

A volume of 200 µl of each *Ureaplasma parvum* serovar culture was dispensed in duplicate in a point-bottomed 96 well plate. Plates were sealed and centrifuged at 3600 xg for 10 minutes at room temperature followed by careful aspiration of the supernatant to avoid disrupting the pellet. Pellets were re-suspended in 200 µl of 50% NHS diluted in VBS in parallel with a matched 50% HI-NHS control to compare the degree of killing. Plates were sealed and incubated at 37° C for 1 hour then centrifuged at 3600 xg for 10 minutes.

Supernatant was discarded and pellets were re-suspended in 200 µl USM and transferred to the first column in another 96 well flat bottom plate. Matched serum and heat-inactivated control were titrated in adjacent wells to ensure accurate calculation of killing in the assay. In each row, serum exposed *Ureaplasma* were diluted 1:10 for three consecutive wells followed by serial dilution 1:1 in the remaining 8 wells in the same row giving a dilution gradient to 2.56 $x10^{-5}$. Plates were sealed and incubated for 48 hours until colour change had ceased. Degree of killing was determined by calculating the fold decrease in CCU from HI-NHS relative to the NHS for each serovar and each serum (figure 2.1).

2.3.6 Killing assay in cord sera supplemented with terminal complement complex (C6-C9).

Complement killing assay in cord sera was performed as described in section 2.3.5. Killing assays were also performed in parallel where purified C6, C8 and C9 (Quidel Corporation, San Diego, CA) were added to give an excess of 50 µg/ml concentration for each (to correct the ELISA determined deficiency of these components) in each cord serum for comparison of ureaplasmacidal activity. Degree of killing was determined by comparing fold decrease of *Ureaplasma* survival following incubation with heat inactivated versus normal sera.

2.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.4.1 Western Blot for anti-*Ureaplasma* antibodies in cord sera

Western blotting, also known as immunoblotting, is a well-established technique to detect a specific protein within a sample (Burnette, 1981). Proteins were separated by mass using gel electrophoresis and then probed using specific antibodies to a given protein of interest. It is a technique that has been used previously on bacteria and human serum.

Prior to the experiments, *Ureaplasma parvum* samples were prepared for Western blot by culturing 5ml from each *Ureaplasma parvum* serovar overnight in a bijoux tube. An aliquot of 1.25 ml from each culture were transferred to a universal container (1.5ml Eppendorf tube) and centrifuged at 13000 x g for 10 minutes at RT. After discarding the supernatant, the pellet was resuspended in another 1.25 ml of the same culture to accrue a cumulative pellet for the entire 5 ml in a single eppendorf tube. Pellets were then washed three times by suspension in 1 ml PBS followed by re-centrifugation. Pellets were then re-suspended in 25 µl PBS along with 25 µl LDS gel loading buffer (Invitrogen, Paisley). Samples were boiled for 2 minutes then cooled prior to loading for separation. SDS-PAGE gel was set up as follows: Gel casting apparatus was set up as directed by manufacture (BIORAD, Hertfordshire, UK) and resolving gel were made up by using the volumes indicated in the table 2.2 and the mixture was poured between gel plates and allowed to set leaving 1.5-2 cm space at the top, 400 µl of butanol was immediately overlaid to remove unwanted air bubbles and to level out the top of the resolving gel which was washed off with de-ionised water after the gel had polymerised. The stacking gel was prepared as shown in table 2.2 and then layered on top of the set resolving gel between the gel plates, with the addition of a gel comb

which was removed after the gel had polymerised. All gels were poured at 1.5 mm thickness with a 15-well comb. Plates containing set gels were set up in the running tank with the central reservoir filled with running buffer composed of 25 mM Tris, 192mM glycine and 0.1% SDS at pH 8.3 (Biorad, Munich, Germany). Five µl of molecular mass standard containing a mixture of equal volumes of EZ Run TM pre-stained recombinant protein ladder (Fisher Scientific, UK, Ltd) and Magic Mark^{TM} XP Western protein standard (Invitrogen, Paisley) was loaded a long side with 7 µl of each solubilised *Ureaplasma serovar* into adjacent wells of the gel with elongated pipette tips. Gels were electrophoresed at 150 Volt (V) and approximately 120mA for 1 hour (until the dye front reached the bottom of the gel).

2.4.2 Western blotting

The proteins were electrophoretically transferred from gels to 0.22 µm nitrocellulose membranes (Anachem) using a BioRad Mini-Protean 3 system as indicated by manufacturer instructions. Transfer was carried out in a transfer tank with the transfer cassette, using transfer buffer (14.4 g/l Glycine, 3.03 g/l Tris base, 20% methanol), an ice pack to keep the transfer buffer cool and a magnetic stir bar to circulate buffer. Transfers were run at 100 V for 1 hour. Nitrocellulose membranes containing transferred proteins were placed within a 50 ml falcon tube (Elkay, Basingstoke) and blocked with 10 ml of blocking buffer using 5% w/v skimmed milk (Marvel, Premier International Foods, Spalding, UK), in 50 ml PBS with 0.05% Tween 20 detergent (Fisher Scientific) (PBST) on a roller for one hour. Membranes were probed with 1:200 of cord serum, diluted in blocking buffer and incubated overnight in cold room (4°C). Probing was followed by washing three times with 15ml of PBST each for 10 minutes on a roller. Five microliters of a goat anti-human IgG (FC specific) peroxidase-conjugated secondary antibody (Sigma, UK), diluted 1:1000 in blocking

buffer, was incubated on a roller for one hour. Membranes were washed three times in PBST as described above followed by one wash in PBS. Blots were developed by using a mixture of 1.5 ml of peroxide solution and 1.5 ml of luminol enhancer solution (Pierce® ECL (Thermo scientific, Loughborough) poured on the nitrocellulose membrane. In a photographic dark room the nitrocellulose membrane was exposed to x-ray film in a light-proof cassette for varying lengths of time before being developed. The length of exposure time was determined empirically by the intensity of signal once developed, but was usually about two to five minutes.

Table 2.2 Composition of Western blot stacking and resolving gel

Stacking gel		**Resolving gel**	
Reagent	**4%**	**Reagent**	**7.5%**
40% Bisacrylamide 37.5:1	1.1012 ml	40% Bisacrylamide 37.5:1	1.93 ml
dH_2O	6.4 ml	dH_2O	5.47 ml
Upper buffer pH6.8	2.4 ml	Lower buffer pH8.8	2.5 ml
10% APS w/v	100 µl	10% APS w/v	100 µl
TEMED	40 µl	TEMED	10 µl

APS= Ammonium persulphate. TEMED= Tetramethylethylenediamine.

2.5 Adult and Cord neutrophil study

2.5.1 Blood samples collection

Blood was taken by venepuncture from consented healthy adult volunteers. Cord blood was collected from the umbilical veins of term infants delivered by elective Caesarean section after normal pregnancy with no history of premature rupture of membrane or known risk of infection. Ten pairs of cord and adult samples were used for these experiments; study was approved by REC and written informed consent was obtained from mothers prior to section and from volunteers.

2.5.2 Isolation of peripheral blood neutrophils

Percoll method was used to isolate neutrophils and blood was obtained by venepuncture from the placental end of umbilical cord by midwife immediately after delivery. Umbilical cord blood was used as it is normally discarded following delivery of the infant and the volumes of up to 40 ml can be obtained relatively easily in most samples. After collection of each cord sample, a similar volume of blood from a healthy adult volunteer was obtained and simultaneously prepared in identical steps to cord blood sample. All samples were collected in 50 ml syringe which contains citrate 1/10 volume 3.8 % sodium citrate (Sigma, Life Science) to prevent coagulation. Citrated anti-coagulated blood was gently transferred to a 50 ml conical tube and mixed by gently inversion of the tube then centrifuged for 20 minutes at 450xg at room temperature. The supernatant (platelet rich plasma) was carefully removed and transferred to a clean tube then centrifuged at 1300 xg for 20 minutes at room temperature to remove any remaining red blood cells and platelets. This platelet poor plasma (PPP) was saved in clean 15 ml conical tubes and used to make up the plasma percoll gradient later.

The bottom layer (leukocytes and erythrocytes) were differentially sedimented by adding 3 ml of 6% warm sterile Dextran (Sigma Life science) per 20 ml blood and then made up to the original blood volume with warm 0.9% sterile saline. This mixture was left to settle for 45 minutes in 37° C water bath loosely covered with the lid on until a clear line of demarcation could be observed between the lower layer containing red cells and the upper layer containing the white blood cells. Leucocyte rich supernatant was carefully removed from the tube and centrifuged at 200 xg for 6 minutes at room temperature. The supernatant was discarded and the cell pellet gently re-suspended in 2 ml of PPP.

Percoll (Sigma-Aldrich, Dorset, UK) is suitable for density gradient experiments due to its low viscosity and low osmolarity. Percoll consists of polyvinylpyarrolidone (PVP) - coated colloidal silica particles which are 15-30 nm in diameter (23% W/W in water). The PVP coating renders the percoll non-toxic to cells. In a bijoux tube, 1.02ml of 90% percoll and 0.98ml of PPP were mixed (51% gradient layer). In a separate bijoux tube, a 42% gradient layer was made consisting of 0.84 ml of 90% percoll and 1.16 ml of PPP. The 51% gradient mixture was put carefully in a 15 ml conical tube. The 42% mixture was very carefully layered over the 51% gradient layer in the 15ml tube to avoid mixing of the layers. The leukocyte fraction was then carefully layered on the top of the appropriate adult or cord plasma percoll gradient once again to avoid mixing of the layers.

The gradients were centrifuged at 350 xg for 13 minutes at room temperature without braking. This allows the formation of 3 layers of cells in each tube (figure 2.2). The upper layer consists of mononuclear cells whereas the middle layer contains neutrophils and the cell pellet in the bottom of the tube contains any remaining red blood cells.

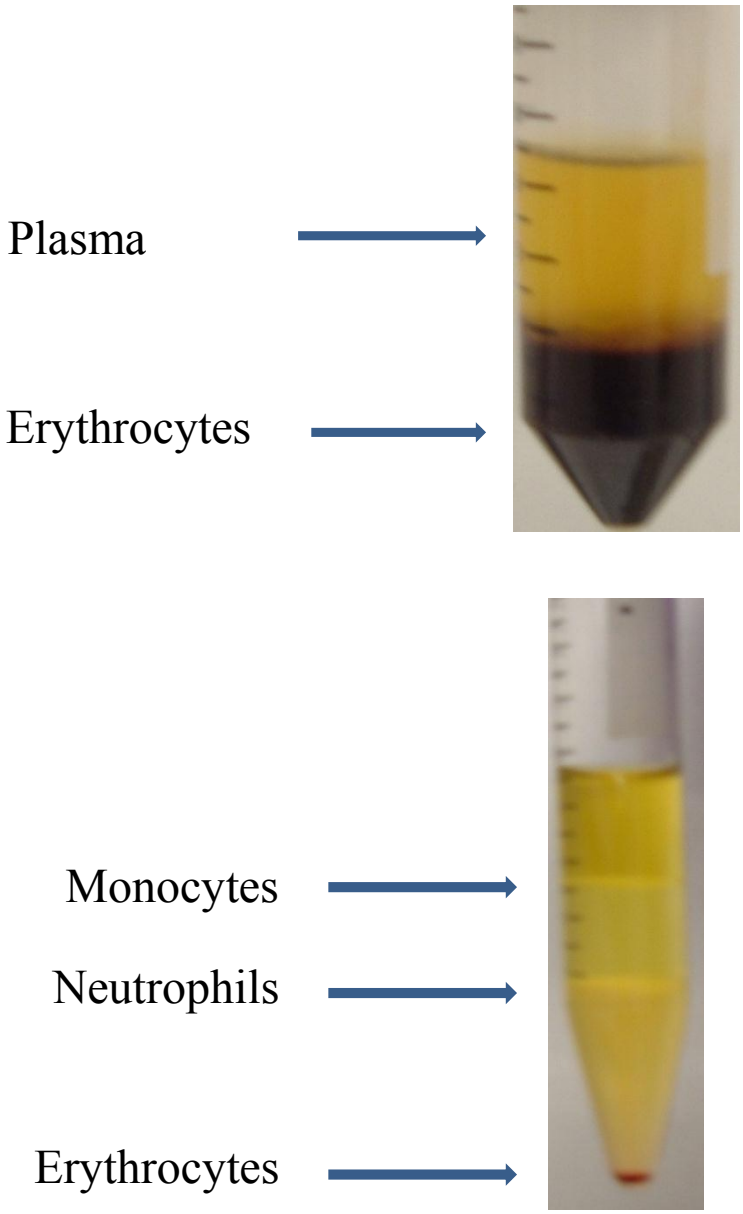


Figure 2.2 Separation of neutrophils following purification by Percoll density gradient centrifugation.

The neutrophil layer was carefully aspirated from the gradient and placed into a clean tube and re-suspended in flow cytometry medium (FACS buffer) (PBS containing 0.1% bovine serum albumin, 15 mM EDTA, 30 mM sodium azide, pH7.35) (Oxoid, Basingstoke, UK), before being centrifuged at 1000 xg for 2 minutes at 4°C. The neutrophils were then washed once in 10 ml of warmed Hanks buffered salt solution (HBSS) without calcium and magnesium and twice more in HBSS with calcium and magnesium to remove the trace of EDTA, before being counted on a haemocytometer and re-suspended at a density of ~10^6 cells/ml in HBSS buffer for FACS staining and analysis. Cell used for intracellular staining were not washed in HBSS.

2.5.3 Neutrophil stimulation

The neutrophil suspension was divided into seven aliquots of one million cells in one ml as below:

1) Unstimulated cells: no additional stimulant added
2) A final concentration of 1 μM formyl-met –leu-phe (fMLP) (Sigma-Aldrich).
3) A final concentration of 5 μg/ml cytochalasin B (Sigma –Aldrich).
4) A combination of 1 μM of fMLP and 5 μg of cytochalasin B (final concentrations).
5) A final concentration of 10 μg/ml of lipopolysaccharide (LPS).
6) A final concentration of 100 ng /ml IL-8.
7) A final concentration of 1μg /ml PMA.

LPS is a bacterial membrane polysaccharide, whilst fMLP is a synthetically derived peptide, representative of bacterial peptides that stimulate the fMLP receptor on neutrophils.

IL-8 is a chemotactic cytokine signalling through the CXCR2 receptor. All of these are capable of mildly stimulating neutrophils. Cytochalasin B is a cell permeable mycotoxin, isolated from fungus that acts by chelating actin filaments within the cell. When cytochalasin B is combined with fMLP their combined action is significantly augmented as a powerful stimulant of neutrophil degranulation (Saeki *et al*., 2001). All seven tubes were mixed using a vortex and incubated in a water bath at 37°C for 15 minutes. The tubes were vortexed again and part of the sample removed (400µl) and prepared for flow cytometry analysis. Of the remaining 600 µl of cell suspension, 500 µl were put into an Eppendorf tube and centrifuged at 1300 rpm for 3 minutes to separate released extracellular proteases from those retained within the cells. The supernatant, containing any released proteases was then removed and 100 µl of the supernatant was placed in a clean Eppendorf tube containing 10 µl of Phenylmethanesulphonyl fluoride PMSF (Sigma Aldrich) which is a serine protease inhibitor. The remaining supernatant was placed in a clean Eppendorf tube without any inhibitor. The cell pellet was dissolved by adding 250 µl of lysis buffer (distilled water containing 10 nM Tris and 0.5% triton X100, pH 7.4). All samples were stored at -20° C until used for ELISA and functional activity assay.

Table 2.3 List of antibodies used in neutrophil flow cytometry

Antibody specificity	**Clone**	**Isotype**	**Conjugated**	**Source**	**Volume used**
Proteinase 3	1B10	IgG2a	Phycoerythrin-(PE)	HyTest Ltd	0.5µl/100µl
CD177	MEM-166	IgG1	Allophycocyanin	Caltag Medsystems	0.5µl /100µl
CD66b	80H3	IgG1	Allophycocyanin	SeroTec.UK	0.5µl/100µl
CD63	MEM-259	Mouse IgG1	Phycoerythrin	BioLegend	5 µl/100µl
CD43	L-10	MouseIgG1	Phycoerythrin	Invitrogen	5 µl/100µl
CD35	7E9	Rat IgG2a	Allophycocyanin	Invitrogen	1µl/100µl
Control		Mouse-IgG_1	Phycoerythrin	Caltag Medsystems	2µl/100µl
Control		Mouse- IgM	Allophycocyanin	Caltag Medsystems	2µl/100µl
Control		Mouse-IgG_2b	FITC	Caltag Laboratories, CA, USA	2µl/100µl
serpinB1		IgG1	FITC	Prof. Remold-O'Donell, Harvard University	5µl/100µl

2.5.4 Flow cytometry analysis

Flow cytometry is an invaluable technology that allows for quantitative analysis of single cells present in a complex mixture. Cell antigens (surface or intracellular) can be detected using fluorescent conjugated Abs. The most common fluorescent molecules used include: fluorescein isothiocyanate (FITC), which emits at 530 nm, and R- phycoerythrin (RPE), which emits at 578 nm, after excitation with the 488 nm Argon laser. In contrast to RPE, FITC produces significant amount of orange and yellow light that overlaps into the RPE channel, and this spurious signal needs to be compensated during two colour flow cytometry (Brown and Wittwer, 2000).

In the present study, flow cytometry was used to analyse neutrophil surface and intracellular expression of the CD177, PR3, CD35, CD43, CD66b, CD63, C3aR, C5aR and SerpinB1.

2.5.4.1 Extracellular preparation for flow cytometry

Using a round bottomed 96 well plate, 100 μl /well of flow buffer was dispensed, followed by 100 μl /well of stimulated or unstimulated cell suspension (10^5 cells) added to each well. The plates for cord and adult samples were centrifuged for 2 minutes at 1000 xg at 4°C, before the supernatant was discarded. The antibodies listed in table 2.3 were prepared in flow buffer according to their concentration. The pellets were then re-suspended with the appropriate antibody and each antibody was tested for all six conditions in duplicate. The plates were incubated for 25 minutes at 4°C before adding 150 μl of FACS buffer to all wells. The plates were centrifuged again at 1000 xg for 2 minutes, followed by two washes with 200 μl/well of FACS buffer in repeated sequence of centrifugation and re-suspension. Thereafter,

cells were re-suspended in 200 μl/well of FACS buffer before being transferred to test tubes ready for immediate FACS analysis.

2.5.4.2 Intracellular Staining

The following flow cytometry preparation on the neutrophils was performed by Dr Brad Spiller using a fixation and permeability kit from An Der Grub Bio Research GmbH (Kaumberg, Austria). However I have assisted and analysed the data collected.

For intracellular staining, isolated neutrophils were suspended only in cold FACS buffer without exposure to HBSS. In a 96 well round bottomed plate, 100 μl of cell suspension were dispensed in each well mixed with 100 μl of cold FACS buffer. The plates were centrifuged at 1000 xg for 2 minutes at 4°C followed by cell pellet re-suspension in 100 μl of flow cytometry buffer and 100μl of fixation buffer and incubated for 15 minutes at RT. The wells were topped with 150 μl of flow buffer and the plate centrifuged. The cells were washed in 200 μl of flow buffer and centrifuged once more at 1000 xg for 2 minutes. Pellets were then re-suspended in 100 μl of saponin-containing permeability medium which contained 1:10 diluted antibodies listed in table 2.3. This was incubated at RT for 15 minutes. The plate was then centrifuged and the pellet re-suspended in 200 μl of flow cytometry buffer before centrifuged again at 1000 xg for 2 minutes. Finally, pellets were re-suspended in 200 μl of flow cytometry buffer and transferred to test tubes for analysis.

Flow cytometry analysis was performed using a Becton-Dickinson FACSCalibre. The data was analysed using the associated Cell Quest software. All experiments were performed in duplicate.

2.5.5 Cathepsin G (Cat G) activity assay

Cat G kinetic activity assay was performed on supernatants taken from cord and adult blood neutrophils as well as on solubilised cell pellets for all conditions including unstimulated cells as well as conditions described above in section 2.5.3. Integral Dynex Revelation software in the spectrophotometer calculated the rate of chromogenic conversion of the colourless SUC-Ala-Ala Pro-Phe-pNA substrate to yellow product by comparing the rate of conversion with a standard curve of purified Cat G (purchased from Athens Research and Technology, Athens, Georgia). Stocks of standard human neutrophil Cat G (Athens Research and Technology, Georgia, US) were prepared by reconstituting 100μg of lyophilized cathepsin G powder in a 200 μl solution of 50 mM sodium acetate, pH 5.5, 150 mM sodium chloride. The 16.9 μM enzyme solution was then divided into 20 μl aliquots and stored at -20°C. To make up a standard curve, 5 μl aliquot of Cat G stock was thawed and diluted using "activity buffer" (0.1M tris, Fisher Scientific, Loughborough, UK; 0.5M sodium chloride, pH 7.4, 0.05% triton X-100). Standard concentrations of Cat G ranged from 170 nM to 0.3 nM. These standard dilutions were then added in duplicate in a volume of 100μl per well in flat-bottomed 96 well plates. Supernatants and cell lysate samples were thawed and 100μl from each sample dilution was added to plate wells, again in duplicate. A stock solution of neutrophil Cat G specific chromogenic substrate was thawed and diluted to 1:100 concentration using activity buffer. Each well of the 96 well plate containing sample or standard concentrations of neutrophil Cat G was topped up with 100 μl of substrate solution. The kinetic assay was read on a plate reader (Dynex Magellan Industries, Chelmsford, UK) warmed to 37°C while shaking the plate with readings recorded over 1 hour at 1 min intervals and wave length of 405nm using Integral Dynex Revelation software to calculate

concentration of Cat G by comparing the rate of substrate conversion in the samples against the standard curve of purified Cat G.

2.5.6 Proteinase 3 ELISA

Microtiter 96 well plates (Nunc-maxisorb) were pre-coated with monoclonal rat anti-mouse IgG_1 antibody at 4μg/ml dilutions in carbonate binding buffer ($NaHCO_3/Na_2CO_3$, pH 9.6). Aliquots of 100 μl/well were dispensed and plate sealed and incubated at 37°C for one hour then fluid was discarded. The plate was blocked with 115 μl/well of blocking buffer (5% BSA in PBS containing 0.05% tween 20) for 45 minutes at 37°C to block additional protein binding sites. The plate was washed once with PBST (200 μl/well) followed by coating with monoclonal antibody (MoAb) Mouse anti-human PR3-G2 at a concentration of 2 μg/ml diluted in blocking buffer (Hycult, stock concentration 0. 94mg/ml). After that, the plate was incubated for 30 minutes at 37°C and washed three times with PBST. Stocks of standard human neutrophil PR3 (1mg/ml, Athens Research and Technology, Georgia, US) were prepared by adding 5 μl from stock solution to 45 μl PBS. Standard solutions were further diluted in blocking buffer which contains (PMSF) (Sigma, Aldrich; stock concentration-50 mg/ml) at 25 μl/ml (1/40) to a final concentrations ranging from 0.976 ng/ml to 250 ng/ml. A total of 100 μl /well were incubated along with samples described in section 2.5.3 at RT for 1 hour. Freshly thawed cell lysate diluted at 1:200 and supernatant samples, diluted at 1:10 in blocking buffer containing 1/40 PMSF, were incubated along with the standard as described. Plates were washed three times with PBST. To detect bound PR3, rabbit anti-human PR3 polyclonal antibody (Euro Diagnostic) was added at 1:1000 dilutions in blocking buffer and incubated for one hour at RT. After being washed three times with PBST, 100μl /well of

Horseradish peroxidase (HRPO) anti-rabbit antibody (Jackson Laboratories) diluted at 1:1000 in blocking buffer was incubated at RT for one hour. Plates were washed three times with PBST and once with PBS then developed with OPD-EASY (Sigma-Aldrich), and the reaction stopped with 2N H_2SO_4 before reading at 490nm.

2.6 PR3 in BAL samples

2.6.1 Sample information

Bronchoalveolar lavage samples were used from an ongoing study and collected by Dr Davies. All samples were collected after ethical approval and written informed consent from parents of patients located on the Neonatal intensive care or high dependency units at the University Hospital of Wales, Cardiff, U.K. The study included three groups of mechanically ventilated infants. Infants included in the first group were premature infants (<32 weeks gestation) who developed CLD whereas preterm infants who developed and recovered from neonatal respiratory distress syndrome (RDS) were in the second group. A control group including full term infants who were ventilated for non-respiratory reasons were also examined.

2.6.2 BAL PR3 ELISA

PR3 levels were measured using the method outlined above in section 2.5.6. One hundred microliter of standard or lavage supernatant samples diluted in (blocking buffer with stabilisers) at 1:10 and 1:50 was added to designated wells. This was then sealed and incubated at RT for one hour and bound PR3 detected as outlined in the previous section. Any samples with PR3 levels above the upper end of the standard curve were diluted further and re-assayed.

2.7 Statistical analysis

Statistical analysis for this thesis was performed using Microsoft Excel and Graphpad Prism software version 5.01 (Graphpad Software Inc). In general cord and adult data results were normally distributed and as such data was expressed as mean and standard error of the mean (SEM). Significance was taken as a *P* value <0.05. For lavage data was non-parametric in nature and comparisons between data sets were made using Mann Whitney U tests, while correlations between groups were performed using spearman correlation co-efficient. Data of lavages is expressed as median values.

Chapter Three

Proteases and other neutrophil markers in cord and adult blood

3.1 Introduction

Newborn infants are at higher risk of infection which is a major cause of morbidity and mortality. Neonates' susceptibility to infection is believed to result from quantitative and functional deficit in humoral and cellular arm of innate immune system (Wilson *et al*., 1986; Fleer *et al*., 1988). Cord blood neutrophils are relatively deficits in adherence, chemotaxis, phagocytosis, oxidative metabolism and bactericidal permeability-increasing (BPI) protein level (Levy *et al*., 1999; Kim *et al*., 2003). Neutrophil proteases are part of bactericidal arsenal. Another factor for susceptibility of neonates to infection could be that infant's neutrophils contain or release less proteolytic enzymes, required for bactericidal activity, than adults. Nevertheless, their untoward effects are believed to have an important role in the pathogenesis of inflammatory lung diseases. Studies of neonatal neutrophil elastase demonstrated that newborn neutrophil have lower elastase activity compared to adult (Davis *et al*., submitted). However, thus far no study on proteinase 3 and cathepsin G in neonate's neutrophils has been published.

- In this chapter, (A) I have examined neutrophil proteinase content and the ability of newborn neutrophils to release these enzymes compared to adult cells. In particular, I have examined cathepsin G functional activity and PR3 intra and cell-surface expression as well as measuring PR3 release following stimulation with a variety of pharmacological agonists.
- (B) I have sought to examine the levels of different neutrophil markers on the neutrophil surface and intracellular levels of theses markers, which are representative markers expressed by different granules (CD15, CD16, CD35, CD63, and CD66b).
- (C) I have examined the neutrophil intracellular and surface membrane expression of CD177 which is believed to be a surface receptor of PR3.

3.2 Results

3.2.1 Neutrophil surface expression of high affinity PR3 receptor, CD177

CD177 is a neutrophil specific protein reported to be found in the plasma membrane and secondary granules of neutrophils. It has been proposed as mPR3 receptor on the neutrophil surface (Goldschmeding *et al*., 1992; Stroncek *et al*., 1994; Bauer *et al*., 2007).

CD177 has a variable distribution on the surface of unstimulated neutrophils, ranging from completely absent to uniformly high, but usually is observed as a bimodal mixture of expression (Bauer *et al*., 2007). These will be referred to as the bright and dim sub-populations of cells in this chapter. Neutrophils taken from adult and cord blood were incubated with APC-conjugated (IgG1) isotype control and APC anti-CD177, then the samples were analysed by flow cytometry to determine the expression of CD177-bright and if the CD177-dim were different from the isotype control (i.e. to differentiate between CD177-low and CD177-negative)

The histogram for isolated adult and cord neutrophils showed a clear bimodal distribution of CD177 in the absence of stimulation (unstimulated cells) compared with the unimodal low expression of background (isotype control). The dim subset represents low or possibly negative CD177 expression, whereas the brighter subset expresses a substantial amount of CD177. Of note, negative expression of CD177 was detected in two cord sample as shown in figure 3.1.

Figure 3.2 shows that the mean cellular fluorescence (MCF) of $CD177^{bright}$ cells on unstimulated cord neutrophils was remarkably higher than that of adults (p = 0.002). This was a consistent observation for each paired set of neutrophils analysed, with the exception of the two CD177-negative cord samples. Moreover, the mean percentage of CD177- expressing unstimulated cells was also significantly higher in cord neutrophils (mean ± SEM, 79 % ±2.2) compared with adults (49 % ±5.9, p = 0.0005).

The mean fluorescence intensity (MFI) for $CD177^{dim}$ expression subset on unstimulated cord neutrophils was significantly greater than that of isotype matched control staining (24.8 units and 2.5 units respectively, p= 0.0006). Similarly, surface CD177 expression on the dim subpopulation of adult neutrophils was 14.26 units, which was significantly higher than background staining by the isotype control (p = 0.019), suggesting that the $CD177^{dim}$ subset are not completely deficient in CD177 expression. However, mean cellular fluorescence comparison of the $CD177^{dim}$ subpopulations between adult and cord neutrophils were not statistically different (figure 3.2). CD177 expression on the two negative cord samples could not be increased above background staining even after stimulation, confirming a lack of intracellular CD177 stores for these samples. Absence of CD177 from these two cord sample was also confirmed by intracellular FACS staining: background MFI =2.42 compared to negative CD177 cord MFI =3.15.

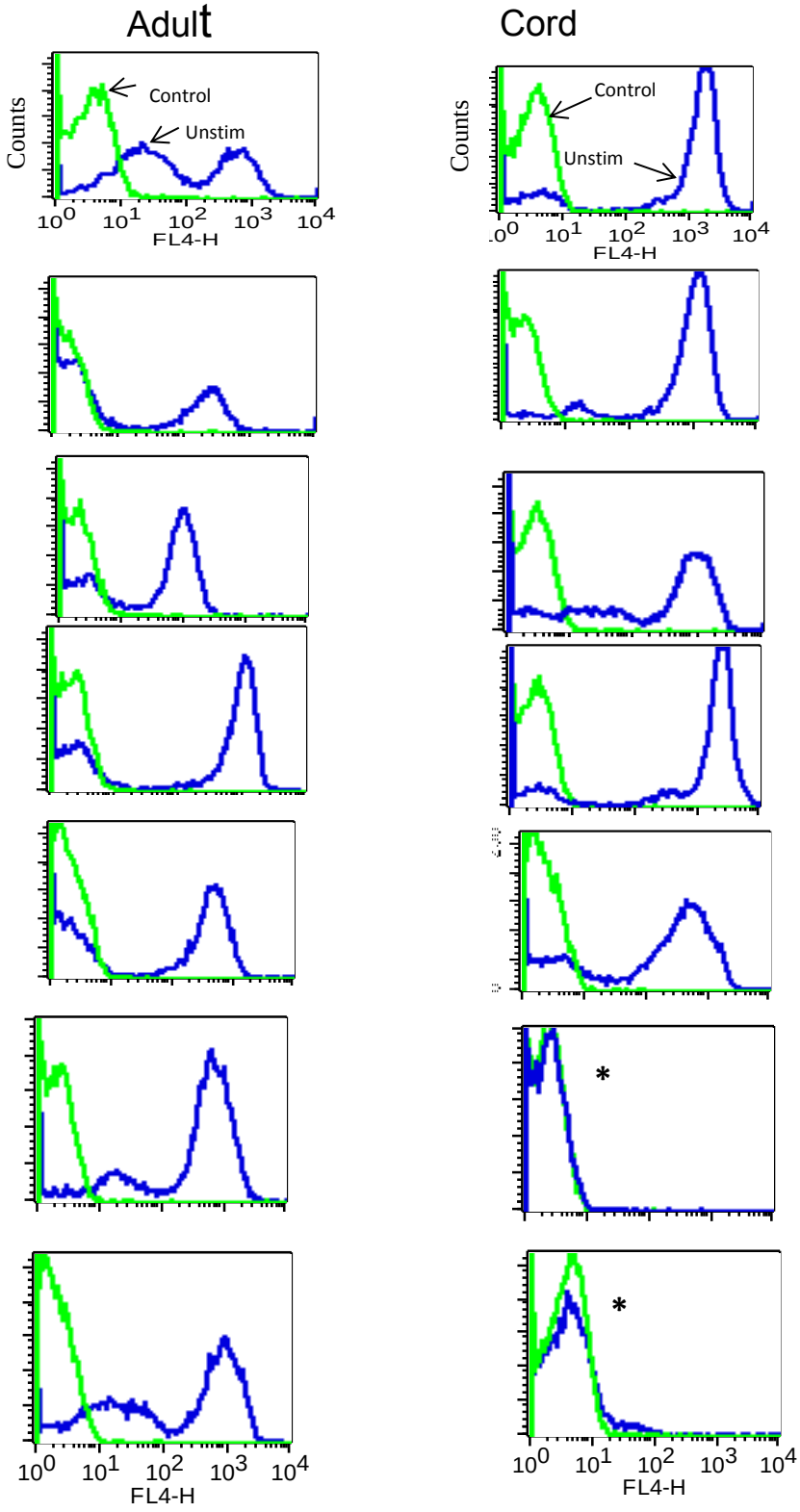


Figure3.1 Overlay histogram of mean cellular fluorescence showing the expression of CD177 on unstimulated cord and adult cells (blue) compared to the isotype control (green). Bimodal distribution of CD177 is observed in all cord and adult neutrophils except for the last two cord samples (*) which were CD177 negative. (10 pairs of cord and adult neutrophil samples were studied).

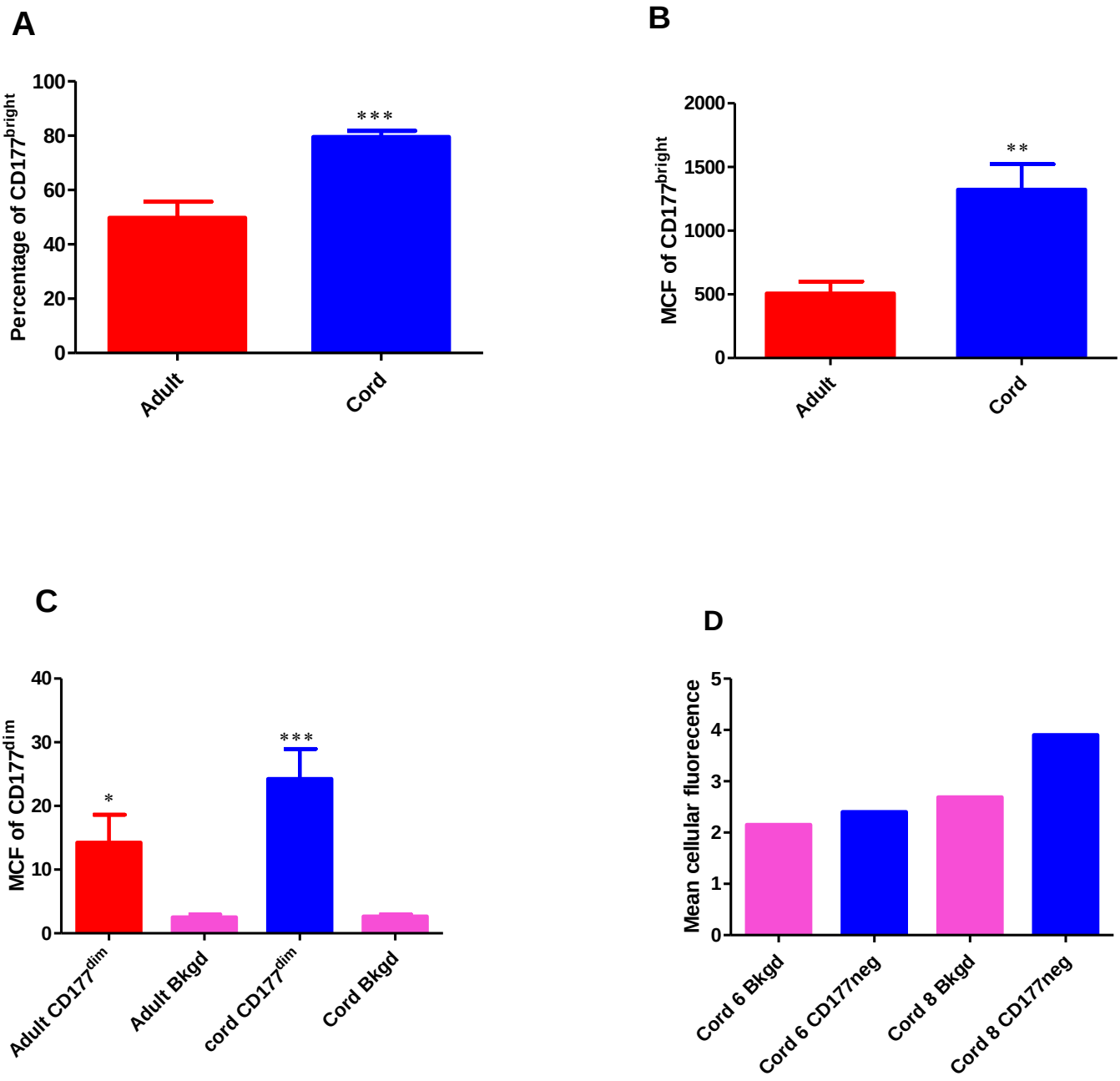


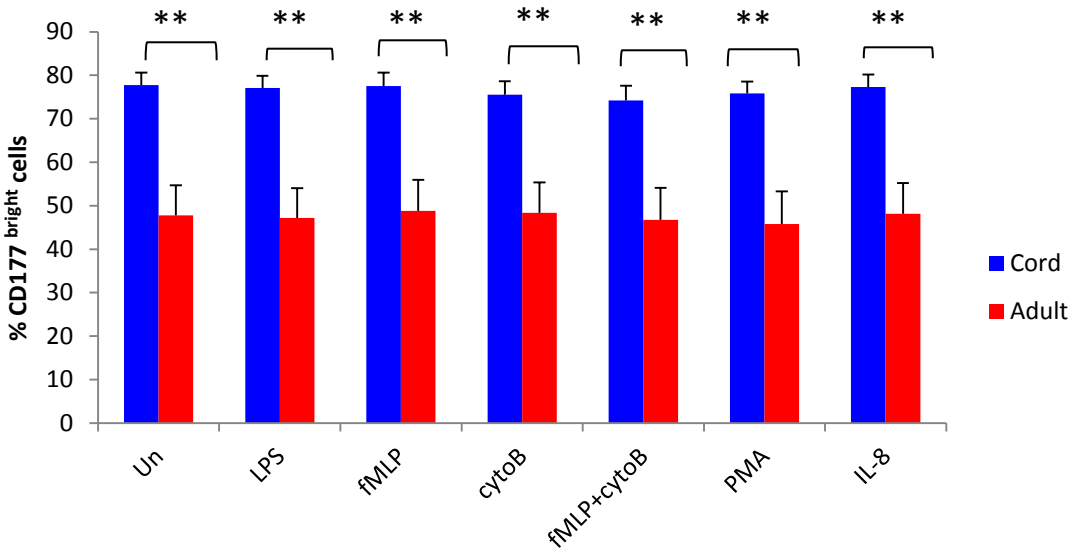
*Figure 3.2 Surface expression of CD177 on unstimulated cord and adult neutrophils. (A) Percentage of CD177^{bright} among adult and cord neutrophil samples. (B) Mean cellular fluorescence (MCF) of CD177^{bright} expression on unstimulated neutrophils. (C) MCF of CD177^{dim} cells for unstimulated cord and adult neutrophils compared to isotype control (background). (D) MCF of CD177 expression on the two CD177 negative cord samples in unstimulated cells compared to isotype control (background).Data expressed as mean, error bars demonstrate SEM, *= p< 0.05; **=p<0.01; ***=p<0.001 (n=10).*

3.2.1.1 **No change in the percentage of CD177^{bright} neutrophils following stimulation**

Alterations in neutrophils CD177 expression was analysed in response to LPS, fMLP, cytoB, PMA and IL-8. These pharmacological agonists are potent inducers of neutrophils degranulation and stimulation. Cells were treated with these stimulants for 15 min before staining with APC-conjugated anti-CD177. The percentage of CD177 expressing neutrophils was higher (77-79%) in unstimulated cord neutrophils compared to adult (45%-49 %, p = 0.001); and stimulation did not increase the percentage of CD177^{bright} cells. No alteration was seen in CD177 expression in the two negative cords following stimulation (leading edge of histogram only 1.6%-4.2% greater than the isotype control histogram), except when incubated with the phorbol ester (PMA). However, the isotype control histogram also increased following PMA treatment for the CD177-negative cord neutrophils (figure 3.3).

Alteration to the mean cellular fluorescence for the CD177^{bright} subpopulation following neutrophil stimulation was also measured. While the percentage of CD177^{bright} neutrophils did not change after stimulation, the amount of CD177 expressed increased in this subset. All agonists caused a significant right shift in the histogram of CD177 expression and increased mean cellular fluorescence compared with unstimulated cells ($p \leq 0.01$). Cord blood neutrophil showed the same response to all of the stimulants. However, cord neutrophils, both unstimulated and in response to all stimulants, expressed significantly more CD177 on the surface of CD177^{bright} cells compared to adult cells $p \geq 0.01$, figure 3.4.

A)



B)

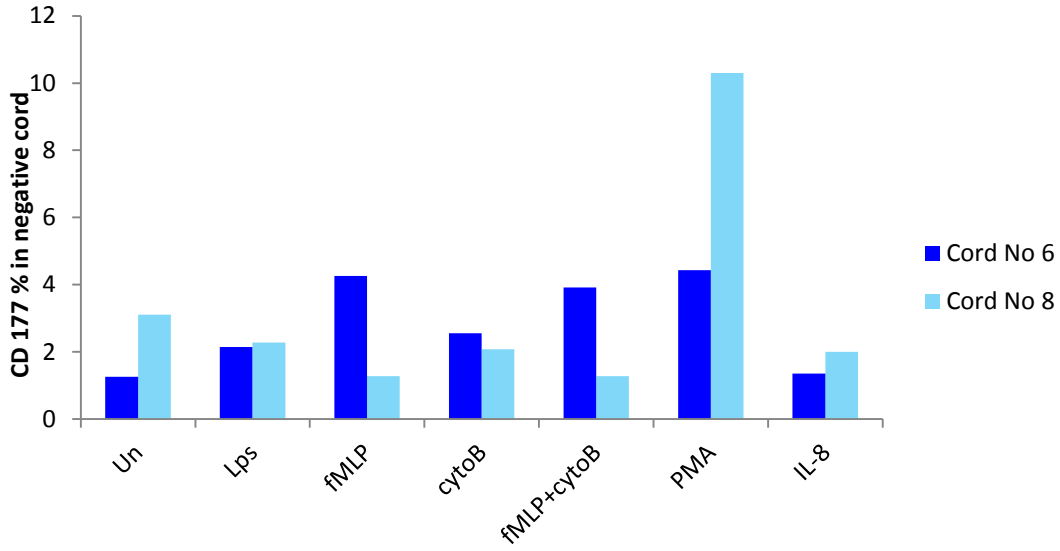


*Figure 3.3. (A) Percentage of CD177 expressing neutrophils (CD177^{bright} neutrophils) in cord and adult samples, data for unstimulated (un) as well as following stimulation with LPS, fMLP, cytoB, fMLP with cytoB, PMA and Il-8 are shown separately.The differences were highly significant under all stimulants ** = $p < 0.01$. (B) Precentage of CD177expressing neutrophil in two CD177 negative cord samples. All values are given as mean ±S EM.*

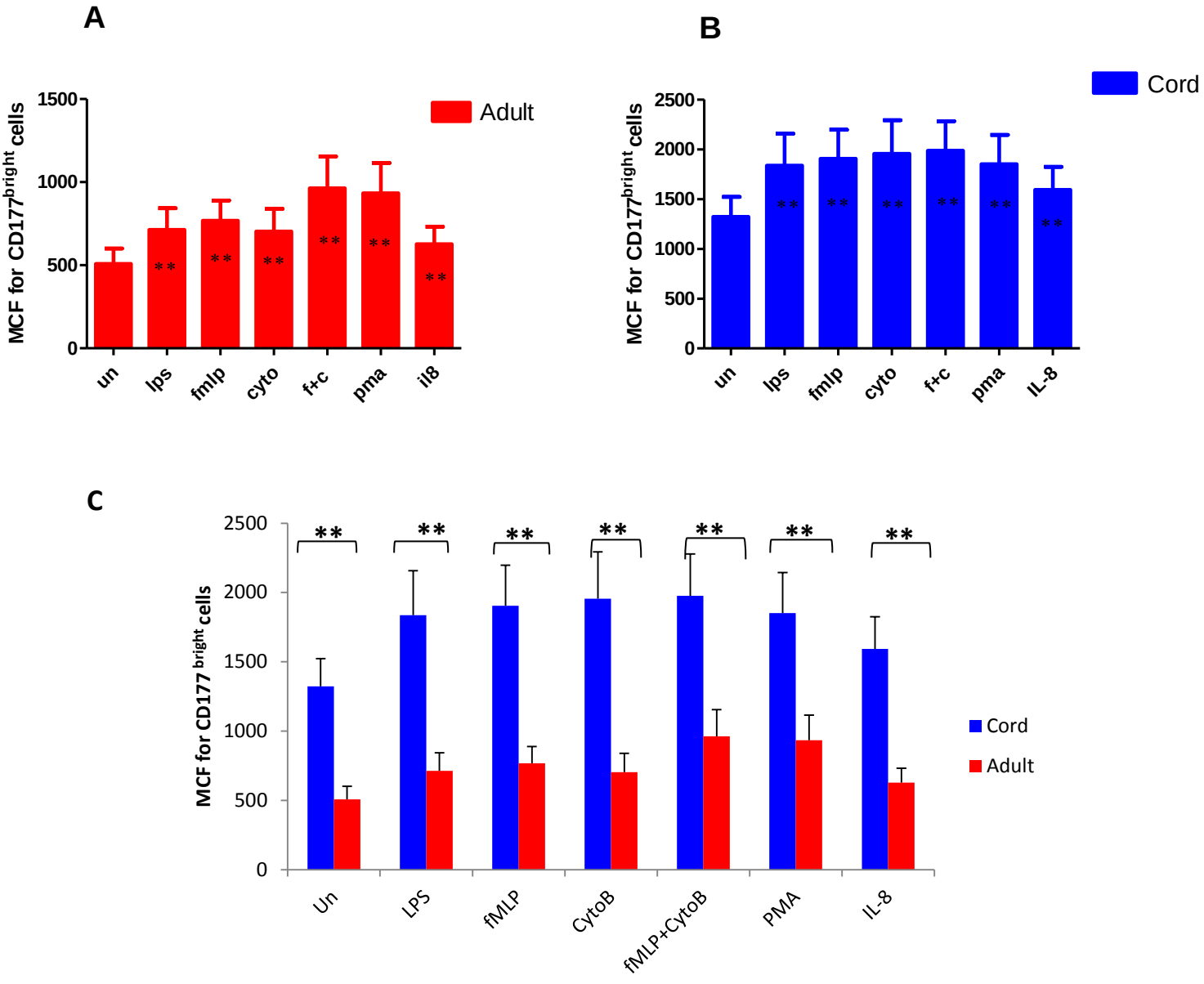


*Figure 3.4 Effect of pharmacological agonists on cell surface expression of CD177 by unstimulated and stimulated cells (A) Adult neutrophils; (B) Cord blood neutrophils. Note that all the stimulants induce significant increase in surface expression of CD177 when compared to unstimulated cells in both cord and adult neutrophils ** = $p<0.01$. (C) Comparison of cord and adult neutrophils on the same scale showing higher CD177 expression on cord neutrophils than adults, ** = $p<0.01$. The data are presented as mean cellular fluorescence ± SEM for ten adult and eight cord samples excluding the two CD177 negative cord samples.*

3.2.2 Surface expression of PR3 before and after stimulation

I analysed membrane PR3 (mPR3) on cord (n=10) and adult (n=10) neutrophil samples, before and after stimulation with LPS, fMLP, CytoB, fMLP combined with CytoB, PMA and IL-8. Figure 3.5 shows the pattern of mPR3 expression on unstimulated cord neutrophils and in response to stimulation with fMLP and fMLP plus CytoB. In 8 cord samples, bimodal mPR3 could be observed before and after stimulation. The last 2 rows in the figure showed monomodal pattern of PR3 expression in the two CD177 negative cord samples even after stimulation. Although CD177 negative cord neutrophils were able to express mPR3 on cell surface possibly via other mechanisms, CD177 seems to be necessary for bimodal PR3 expression pattern since this was not seen in the negative cord samples. Adult neutrophils express monomodal mPR3 on umstimulted cells but after stimulation especially with fMLP combined with CytoB, additional sub-population of neutrophil with low mPR3 expression was seen (figure 3.6; 3.7). On the other hand, CD177 expression was bimodal even without stimulation with the exception of the two CD177 negative cord samples (figure 3.8 and 3.9).

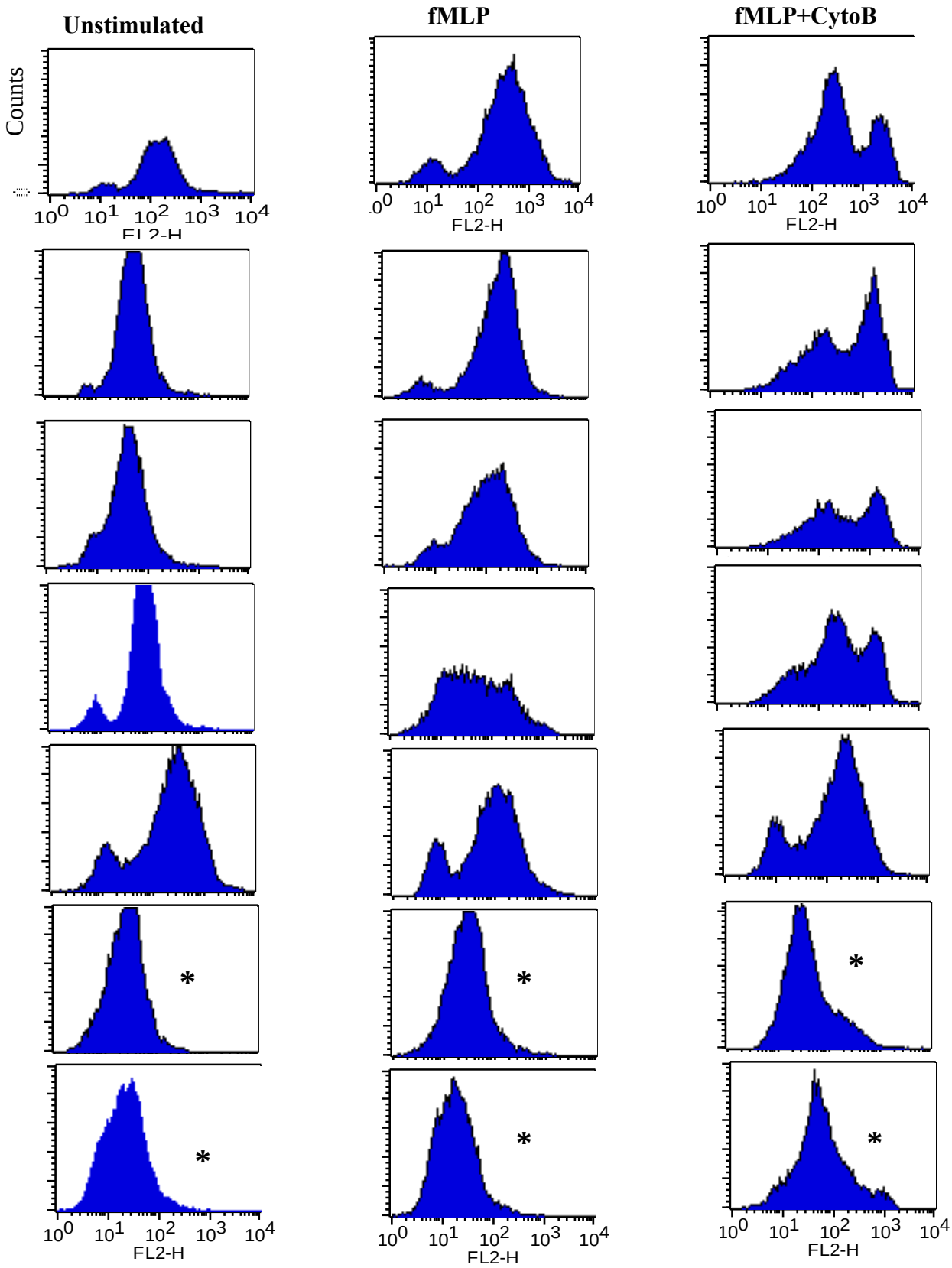


Figure 3.5 .Histogram shows bimodal distribution of mPR3 on cord blood neutrophils of unstimulated (control) and neutrophils stimulated with FMLP alone or fMLP combined with cytoB (f + C). denotes mPR3 expression of CD177 negative cord samples with monomodal expression of mPR3 observed before and after stimulation.*

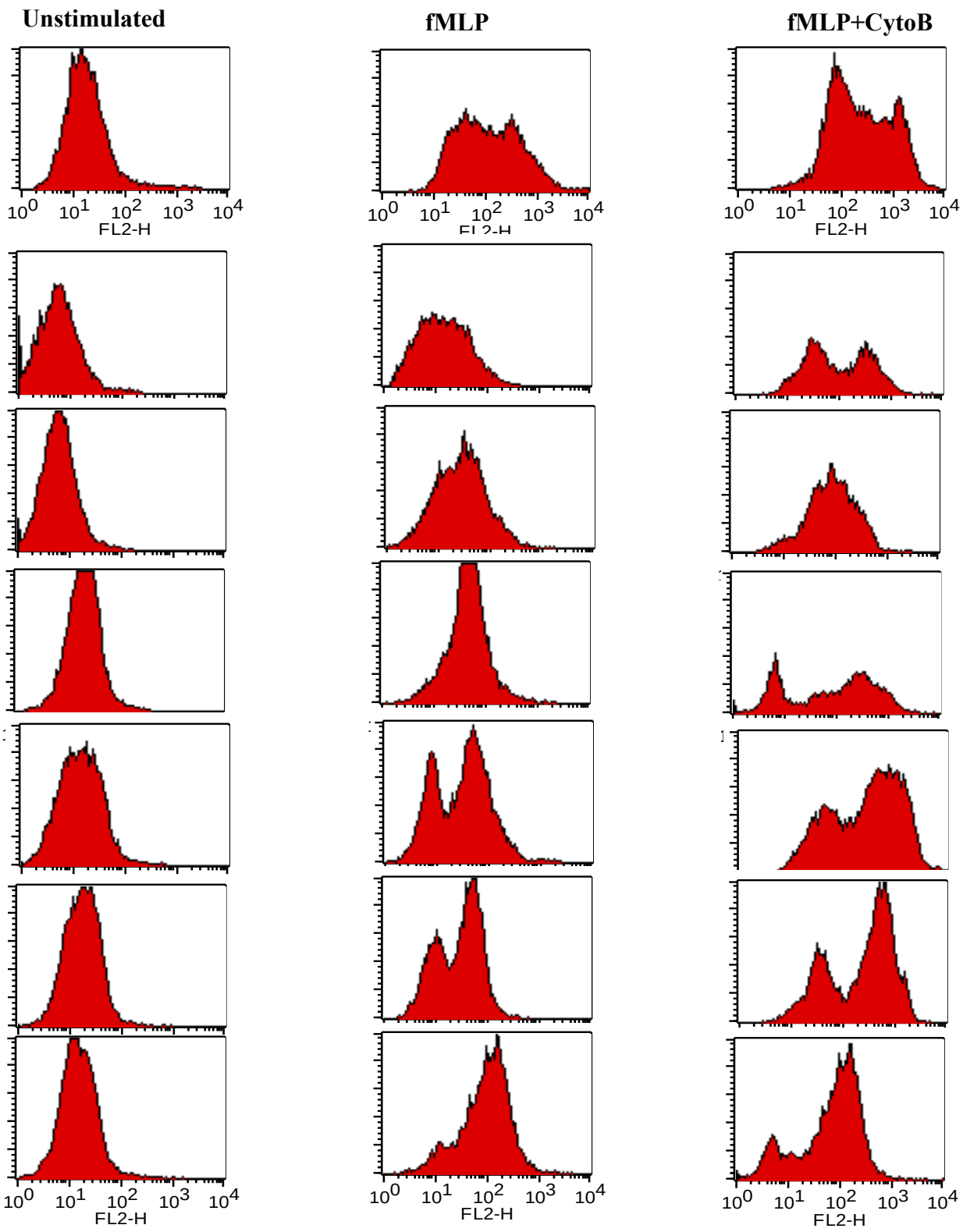


Figure 3.6 Effect of fMLP and fMLP with cytoB stimulation on expression of mPR3 in adult neutrophils. Neutrophils activated with fMLP alone or fMLP +cytoB induced bimodal pattern of mPR3 expression compared with monomodal expression pattern on unstimulated cells. Each histogram represents individual blood samples.

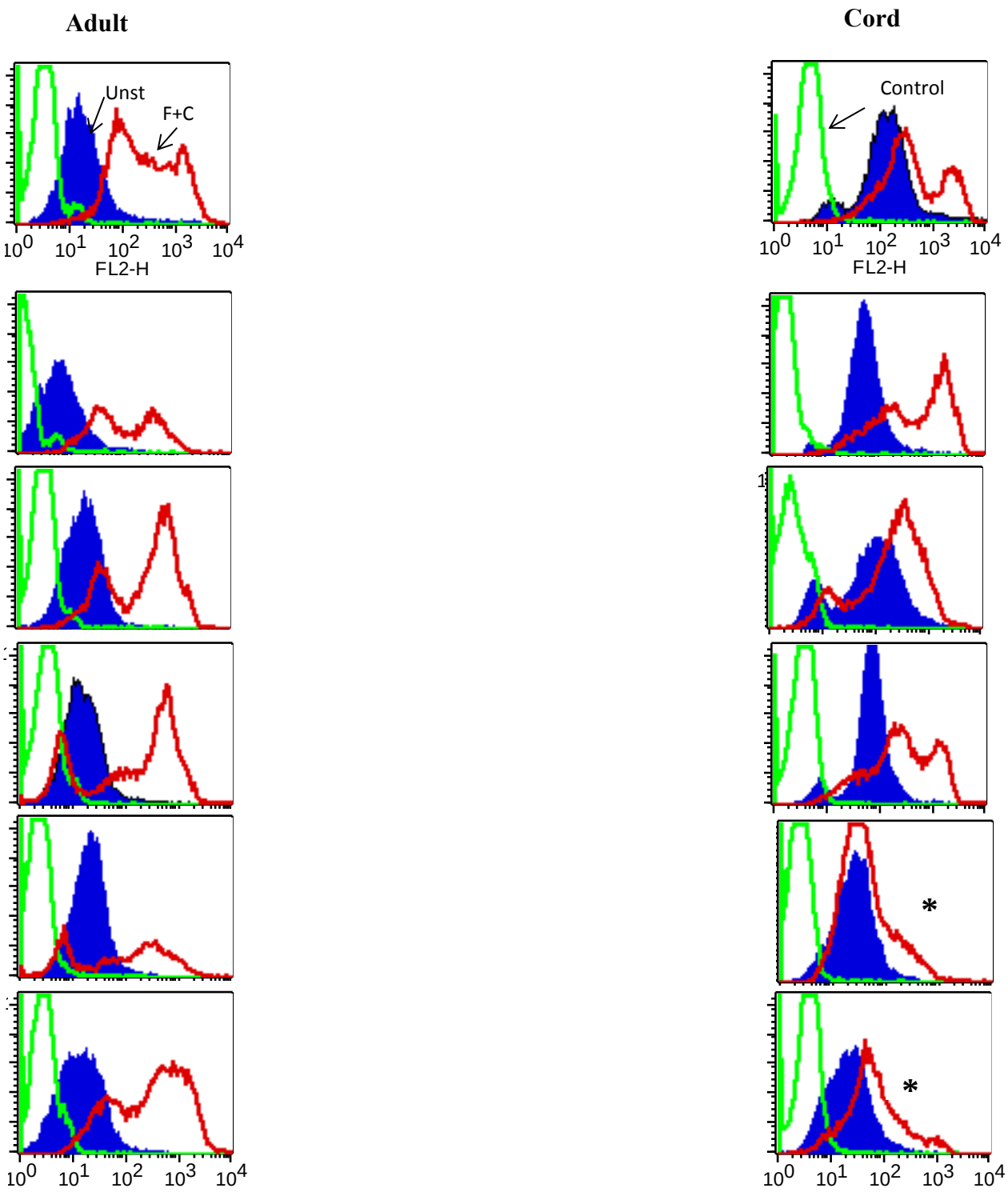


Figure 3.7 Overlay histogram showing the expression patterns of mPR3 on unstimulated cell (blue), and in the presence of fMLP plus cytoB (red) compared to the isotype control (green) on cord and adult neutrophils. denotes mPR3 expression on the CD177 negative cord samples.*

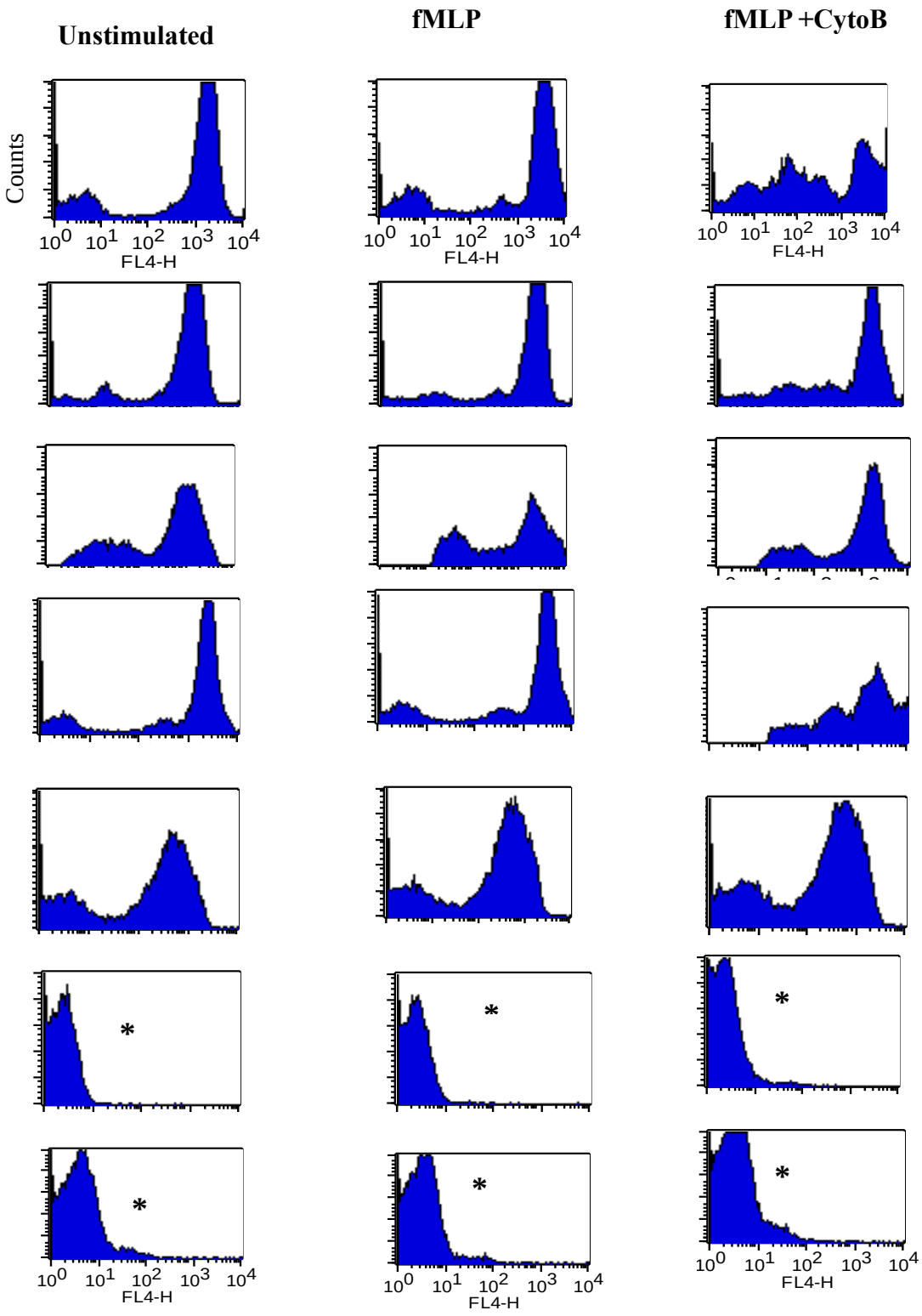


*Figure 3.8 .Histogram shows bimodal distribution of CD177 on cord neutrophils before and after stimulation with fMLP alone or combined with cytoB. * denotes CD177 pattern in the negative cord samples.*

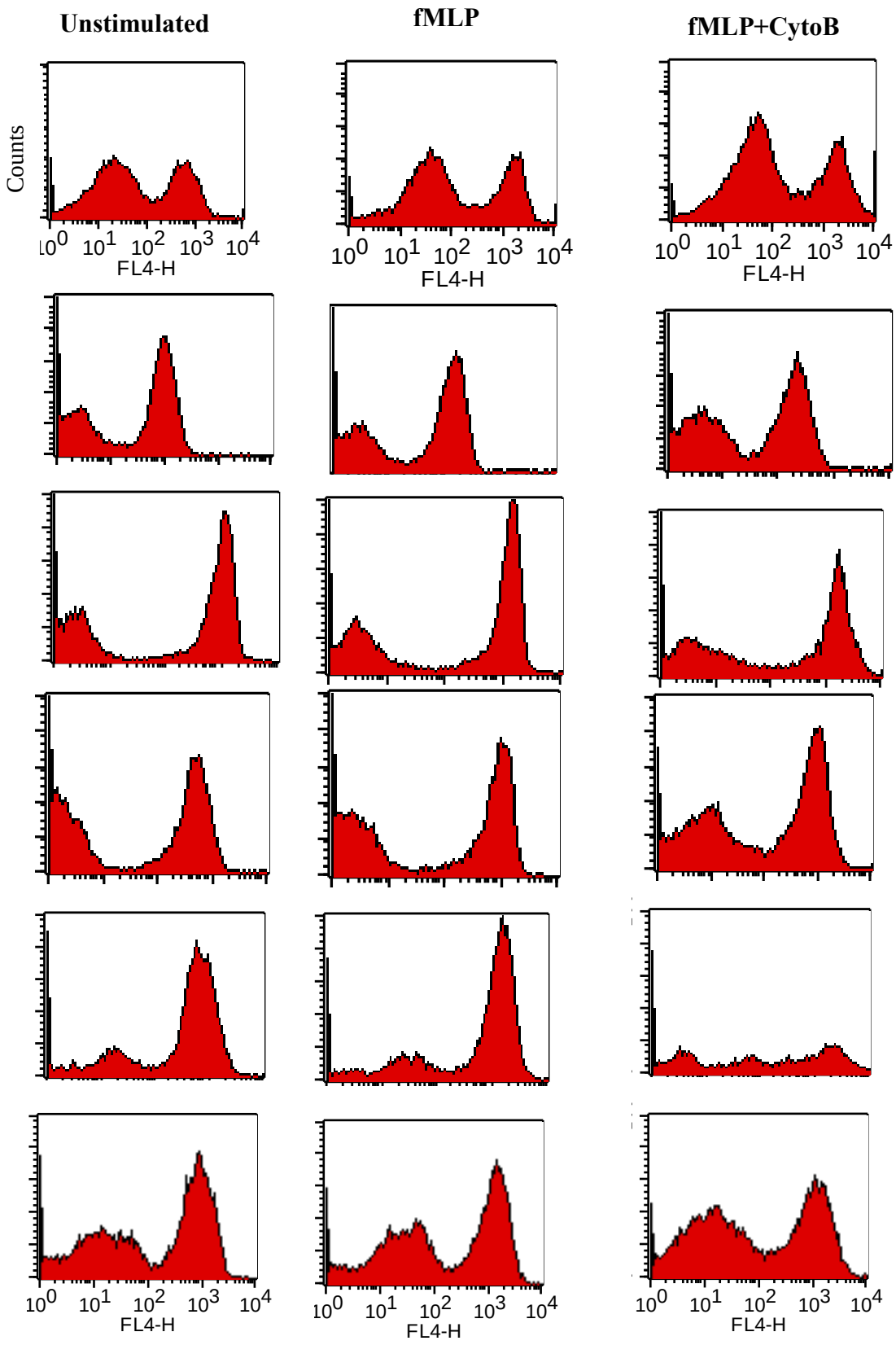


Figure 3.9 Histogram shows bimodal distribution of CD177 on adult neutrophils before and after stimulation with fMLP alone or with cytoB (f + C).

3.2.2.1 Expression of mPR3 on the neutrophils from CD177-negative cord samples

As demonstrated above, the expression of CD177 was not detected on the surface of neutrophils from two cord blood samples. Figure 3.10 shows the mPR3 expression on CD177 negative cord samples compared to the isotype control. The level of mPR3 was higher than the isotype control on unstimulated neutrophils in negative cord samples (mean 33.5 units) compared to the level of background (mean 3.2 units). Furthermore, after maximal stimulation with F+C, CD177-negative cord neutrophils expressed even greater levels of mPR3 (mean 53.4 units) compared to the isotype control level (5.2 units).

Therefore, neutrophils from these two cord samples, despite their negative CD177 status, were able to expresses membrane PR3 on their surface before as well as after activation, albeit at lower levels than on CD177-positive cells. These results suggest that expression of PR3 on the neutrophil surface was mediated by a CD177-independent mechanism or alternative low affinity receptor.

Activation of CD177- negative cord neutrophils with LPS, IL-8 and fMLP or cytoB alone did not change mPR3 expression compared to unstimulated cells. However, incubation with F+C or PMA consistently increased mPR3 on the cell surface of CD177-negative cord neutrophils. The expression of PR3 was increased by 3 fold with F+C and by 1.7 fold with PMA. However, as only two CD177 negative cord samples were available for analysis, statistical analysis of this observation was not possible.

Similar levels of mPR3 were observed on the surface of unstimulated CD177-negative cord cells and the unstimulated cells from matching adult samples processed in parallel on the same day. Activation of cells by fMLP combined with cytoB increased the mPR3 expression on adult neutrophils by 30 fold compared to unstimulated neutrophils (MCF 838.9 vs 27.1), but mPR3 expression only increased by 3-fold on the CD177-negative cord neutrophils after F+C stimulation. Whether this has functional implications (e.g. transmigration or bactericidal activity) for CD177 negative neutrophils is nevertheless unknown.

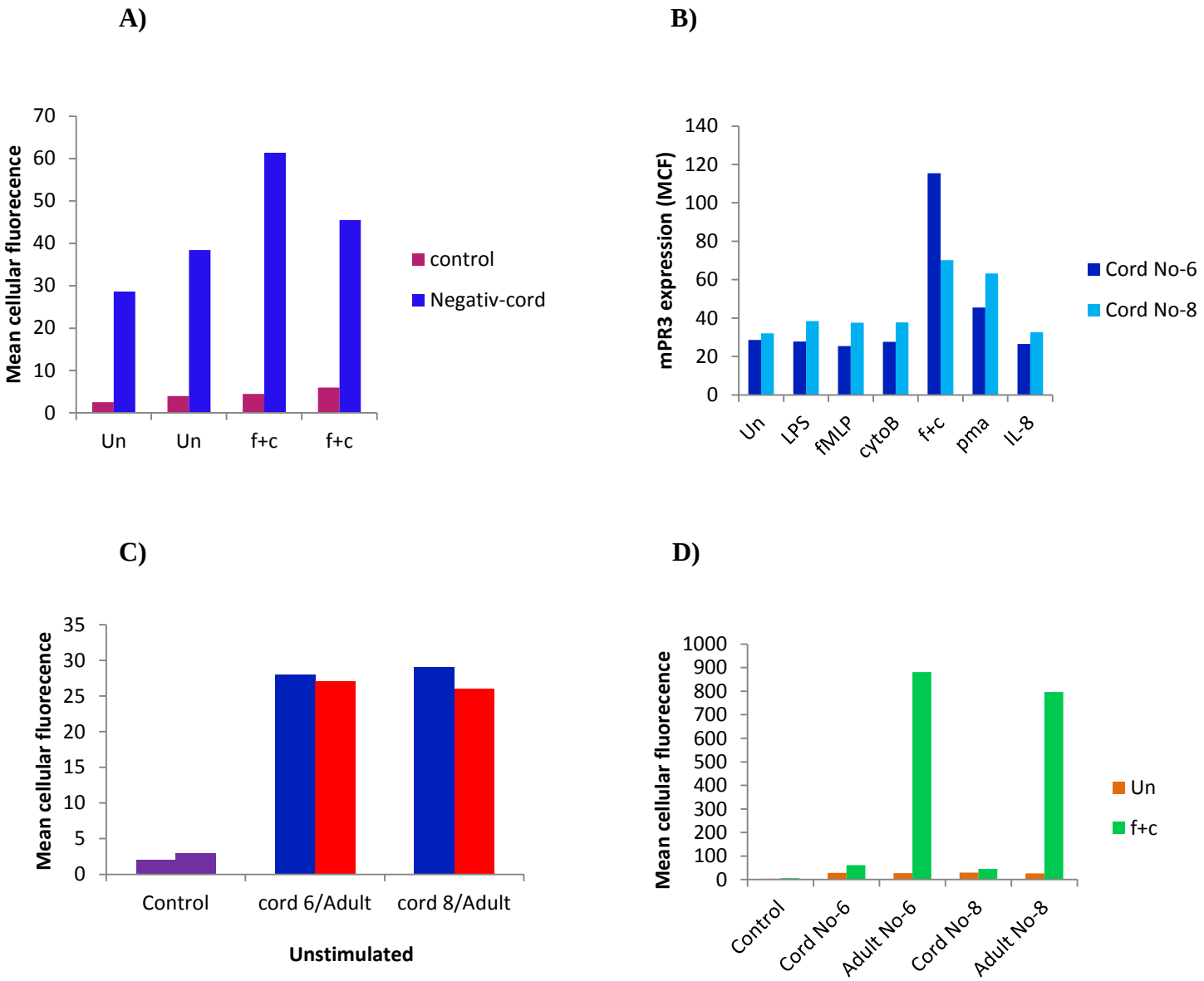


Figure 3.10 Expression of mPR3 on neutrophils from CD177-negative cord samples. (A) comparison of PR3 expression between isotype control and CD177 negative cord on unstimulated and in presence of fMLP plus cytoB (f+c), note that mean cellular fluorescence (MCF) of mPR3 expression increased compared with control (B) Variation of the mPR3 expression before and after stimulation in the two CD177 negative cord samples (C, D) Comparison of membrane PR3 expression on CD177-negative cord neutrophils and matched adult neutrophils.

3.2.3.2 Cell surface expression of PR3 in response to stimulation on CD177-positive cells

The mean fluorescence mPR3 expression for unstimulated cord blood neutrophils (46.6 ±SEM 6.6) was significantly greater than that of unstimulated adult neutrophils (19.4 ± 2.4; p = 0.001 by unpaired t-test), which may be related to higher cord CD177 expression. Following stimulation with LPS, PMA, IL-8, fMLP, or cytoB and F+C, the mean mPR3 expression in both adult and cord cells was higher than for unstimulated cells (figure 3.11). Cytochalasin B combined with fMLP were consistently the most potent inducer of increased mPR3 expression. Following stimulation with F+C, the mean cellular fluorescence for mPR3 on cord cells rose significantly to a mean of 972.7 units compared to a mean of 46.6 units on unstimulated cord neutrophils p = 0.009. Similarly, mPR3 expression was also significantly higher on adult neutrophils treated with F+C (MCF 446.7) than that of unstimulated cells which had mean cellular fluorescence of 19.4 units, (p = 0.002). On the other hand, stimulation with fMLP alone caused moderate increases in fluorescence intensity for mPR3 expression in adult and cord cells with p value <0.05 in both cord and adult.

Figure 3.13 demonstrates the ratio of MCF between stimulated and unstimulated cells. Stimulation of adult neutrophils with fMLP and cytoB resulted in a 25-fold rise in the mPR3 expression relative to unstimulated cells, while 14.4-fold increase in cord mPR3 was observed. For adult neutrophils, stimulation with PMA mPR3 increase of 18.4-fold, and LPS, fMLP alone, cytoB alone only increased mPR3 by 2.4-5.7-fold. PMA stimulation of cord neutrophils only increased mPR3 by 5.8-fold and other stimulants had a lower effect on cord cells. IL-8 consistently had the poorest mPR3 increase of 1.2-fold, which failed to reach statistical significance relative to unstimulated cells.

In agreement with CD177 expression results, the percentage of mPR3^{bright} cells measured for cord neutrophils ranged from 79%-94%, which was consistently higher than the 42%-86% observed for adult neutrophils (figure 3.12). However, unlike mPR3, percentages of CD177^{bright} cells were not altered by stimulation in each individual.

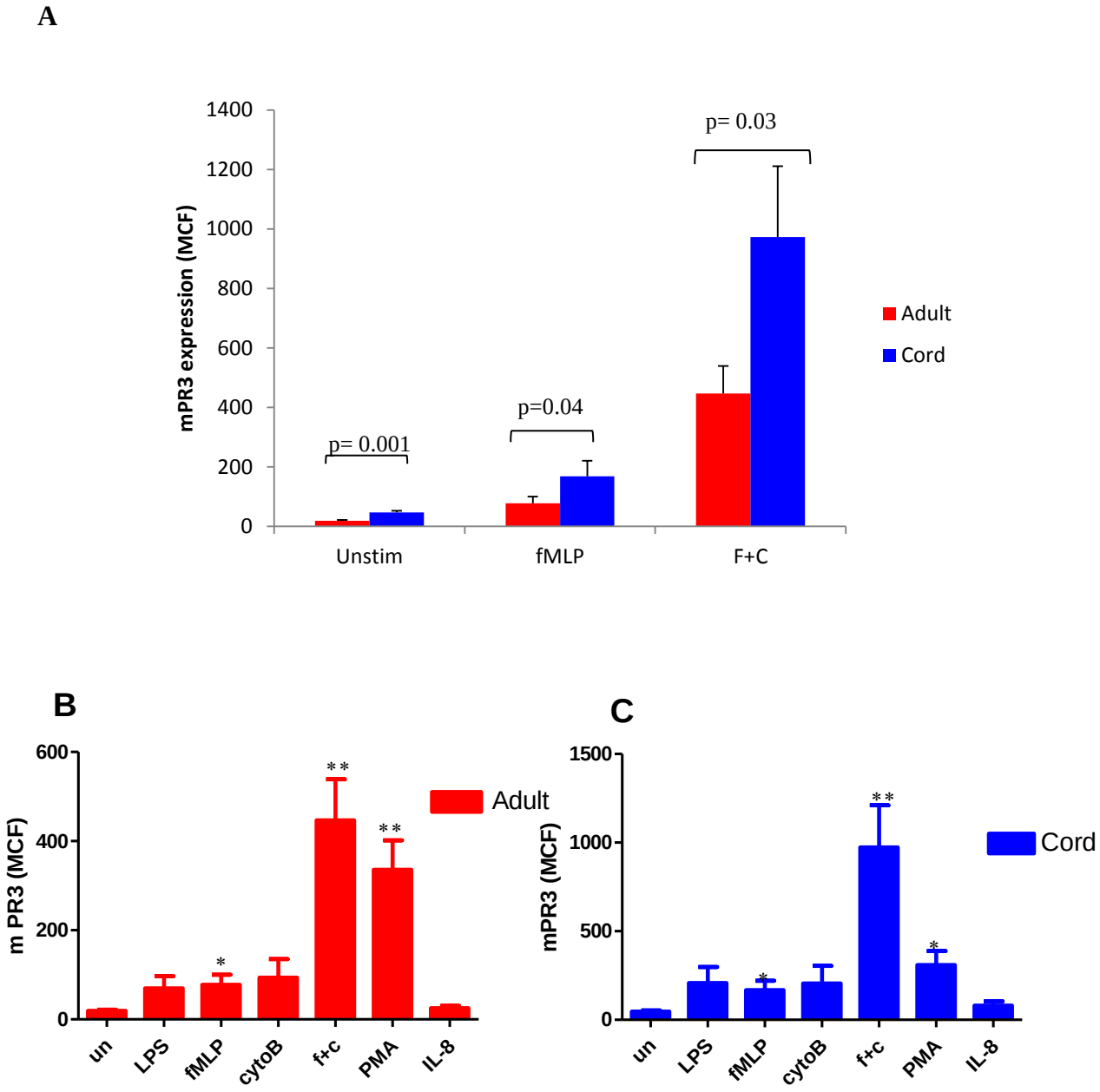


*Figure 3.11 Effect of neutrophil activation on membrane expression of PR3 as measured by flow cytometry. (A) Cell surface expression of PR3 on cord blood neutrophils compared to adult neutrophils. Neutrophils were either unstimulated (un) or incubated for 15 min at 37°C with fMLP or fMLP plus cytoB (f+c) significant differences are shown. B) Membrane PR3 expression on adult neutrophils, (C) membrane PR3 expression on cord neutrophils. Note that the fMLP, f+c and PMA induced significant increase in cell surface expression of mPR3 when compared with unstimulated cells in both cord and adult blood neutrophils. Data are shown as mean ± SEM. * $p<0.05$, ** $p<0.01$; $n=10$.*

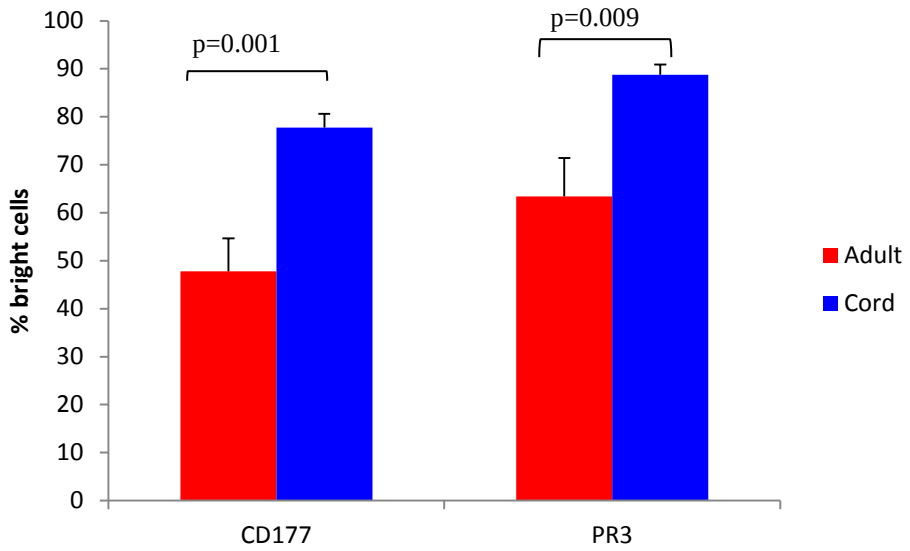


Figure 3.12 Percentage of mPR3 and CD177 expressing on unstimulated neutrophils in cord blood compared to unstimulated adult neutrophils, statistical significance is shown in the figure.

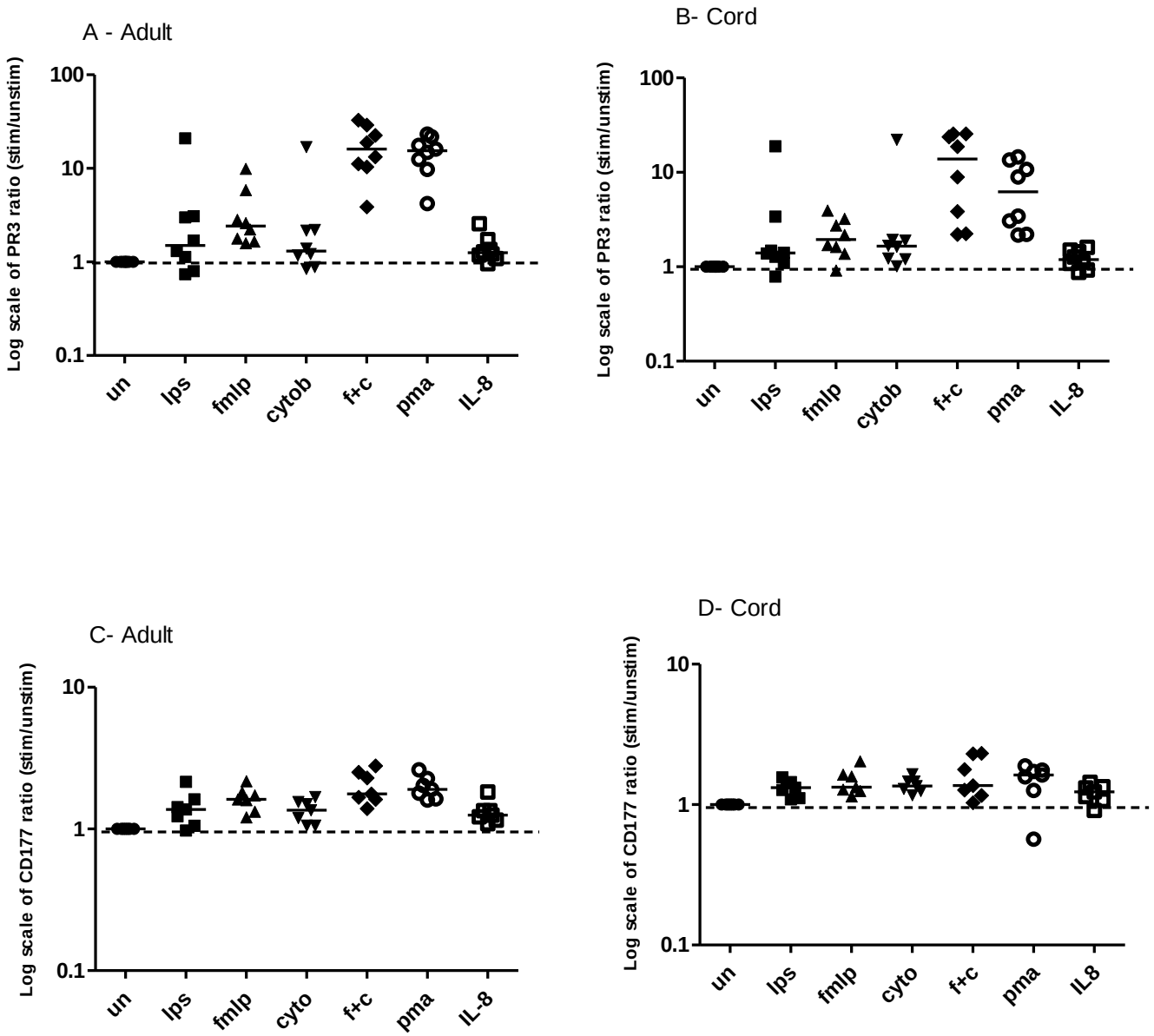


Figure 3.13 Scatter graph of CD177 and PR3 ratios of mean cellular fluorescence (MCF) following stimulations (stim) compared to unstimulated cells (un). (A and B) PR3 ratio in adult and cord neutrophils; (C and D) CD177 ratio in adult and cord neutrophils. Note all stimulation conditions with the exception of IL-8 resulted in statistically significant increase of CD177 and mPR3 expression on the cells surface compared to unstimulated cells in both adult and cord neutrophils $p<0.05$.

3.2.3 Neutrophil expression of CD63 and CD66b

Neutrophils primary granule marker CD63 expression was evaluated on the surface of neutrophils isolated from both adult and cord blood. CD63 expression on unstimulated cells appeared to be slightly higher in adult with mean fluorescence of (43.2± SEM 7.4) compared to (31.1± 4.2) in cord unstimulated cells, but the difference was not statistically significant, (p = 0.1). Significant surface expression of CD63 is only seen following maximal neutrophil stimulation resulting in overt degranulation (figure 3.14). The combination of fMLP and cytoB resulted in significantly elevated CD63 expression compared to unstimulated cells (mean 144.3; P =0.014) in adult and in cord neutrophils (mean 193; P = 0.009). However, no statistically significant difference was found in CD63 expression in response to maximal stimulation between adult and cord neutrophils. Stimulation of cord and adult neutrophils with LPS, fMLP, cytoB, PMA and IL-8 did not cause any significant increase in the surface expression of CD63 compared to unstimulated cells.

Effect of agonists on the expression of secondary granule marker CD66b was also studied on adult and cord blood neutrophils. Unstimulated cord and adult neutrophils had higher baseline level of surface CD66b expression (MCF, 246 and 203.5 units respectively), in contrast to the lower baseline expression of CD63 (31.1; 43.2 units), on both cord and adult samples (figure 3.15). In adult neutrophils much greater increase in CD66b expression was observed following stimulation with PMA or fMLP combined with cytoB p =0.0006 and p =0.0003 respectively compared to unstimulated condition. Activation of adult cells with LPS, cyto B and IL-8 induced statistically insignificant increase in CD66b expression compared to unstimulated cells; however, increase in CD66b expression after stimulation with fMLP achieved statistical significance (p =0.01). On the other hand, in cord blood neutrophils, all

stimulants caused significant increase in CD66b expression compared to unstimulated cells particularly fMLP plus cyto B (f+c) which was the most potent stimulant for CD66b (p = 0.006). Furthermore, the levels of CD66b on the surface of adult neutrophils stimulated with PMA and f+c appeared to be higher than on cord neutrophils, but failed to reach statistical significance. It is also interesting to note the difference between the stimulants as PMA showed evidence of degranulation of secondary granules marker (CD66b), but not primary granules marker (CD63), while fMLP and cytoB in combination could degranulate both sets of granules.

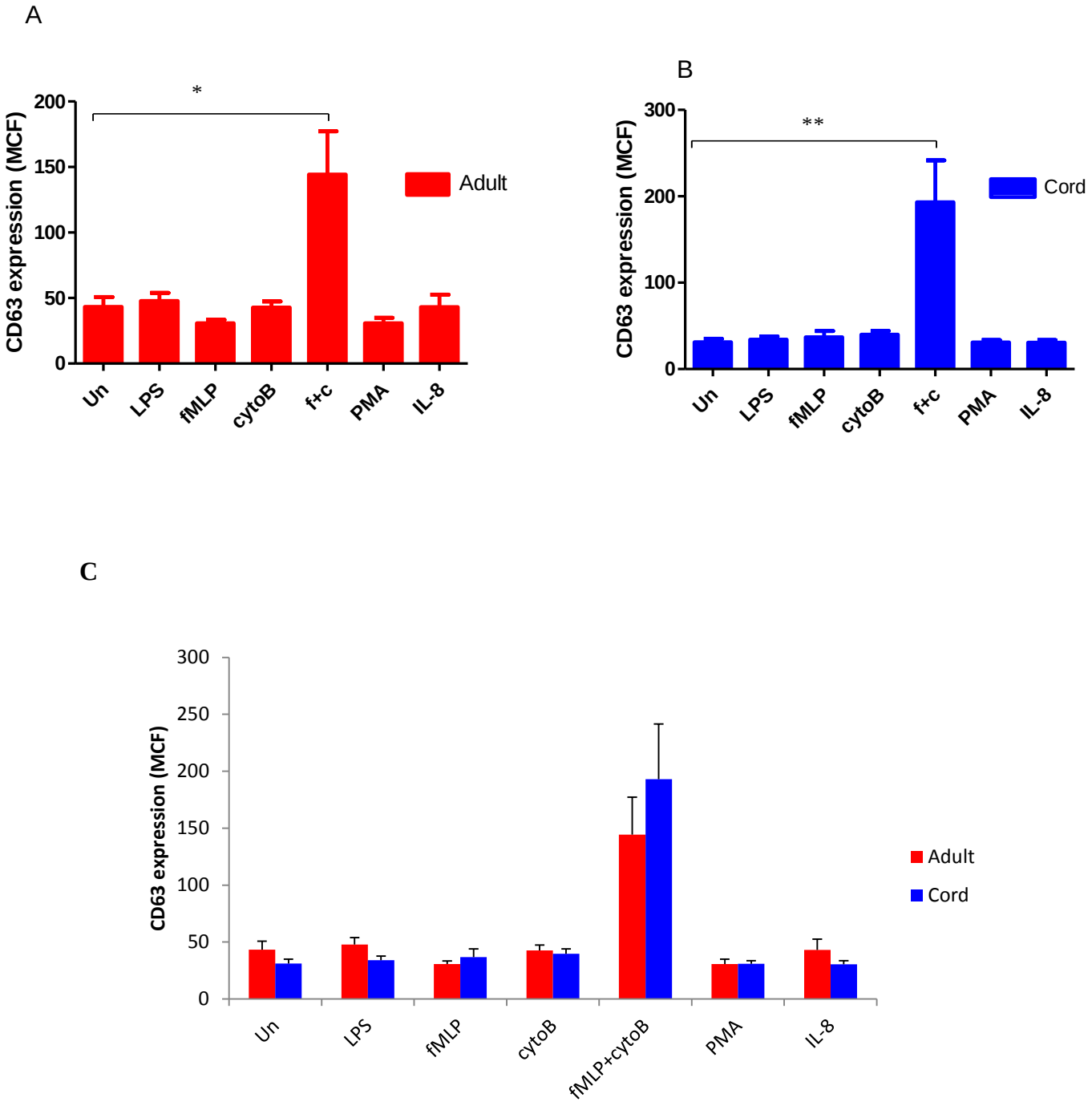


*Figure 3.14 Effect of neutrophil stimulation on expression of CD63 as measured by flow cytometry. CD63 expression was measured as mean cellular fluorescence (MCF) in unstimulated cells (un) and in response to LPS, fMLP, fMLP plus CytoB (f+c), PMA and IL-8 on neutrophils from adult blood (A) and cord blood (B). (C) Surface expression of CD63 on cord blood neutrophils compared to adult, expression shown as mean ± SEM * = p< 0.05; **= p <0.01 compared to unstimulated condition (n=10).*

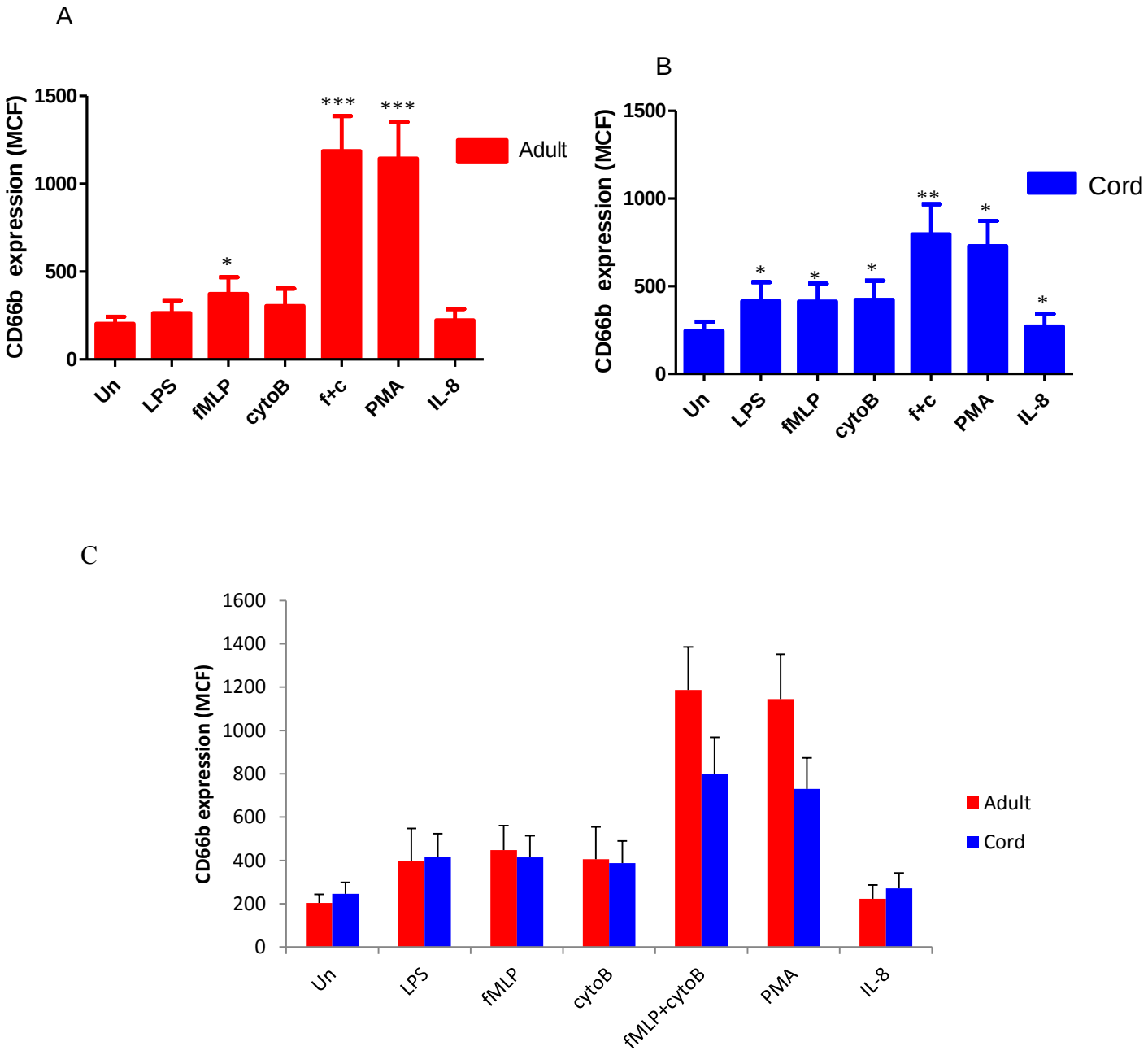


Figure 3.15 Effect of different stimulants on membrane expression of CD66b. Mean cellular fluorescence for APC-conjugated anti-CD66b staining was measured on adult (A) and cord neutrophils (B). (C) Comparison of mean expression of CD66b in both adult and cord. Mean and SEM shown for ten paired samples. Significant differences are shown (= p<0.05; ** =p<0.01 and ***= p<0.001 relative to unstimulated cells.*

3.2.4 Neutrophil expression of CD35 and CD43 in response to stimulation

The data in figure 3.16 demonstrate the mean fluorescence intensity of CD35 expression on unstimulated cord and adult neutrophils and in response to LPS, fMLP, CytoB, PMA, fMLP in combination with CytoB and IL-8. There was no difference in CD35 expression between adult and cord neutrophils in all conditions. In addition, incubation of cord and adult neutrophils with various agonists did not significantly change CD35 expression compared to unstimulated cells with the exception of adult neutrophils response to IL-8 which resulted in significant increase in CD35 expression p= 0.04. Stimulation of adult neutrophils with fMLP + cytoB or PMA, reduced the percentage of neutrophils expressing CD35 significantly to 40% (p =0.007) and 47% (p=0.016), respectively compared to unstimulated neutrophils 81% (figure 3.17).

On the other hand, the percentage of positive cord neutrophils expressing CD35 ranged from 74 to 79% amongst the unstimulated and stimulated cells (figure 3.17). Reduction in CD35 expression only reached significance when the cells were stimulated with (F+C), reducing the percentage of CD35 expressing cells to 45% (p =0.042). I also observed a slight reduction in the percentage of cord neutrophils expressing CD35 following incubation with PMA but statistically this was not significant. However, following stimulation with PMA, the mean cellular fluorescence for CD35 in cord neutrophils was reduced significantly from 30.7 in unstimulated cells to 18.9 in PMA stimulated cells (p= 0.0095). These stimulants also decreased MFI of CD 35 expression in both adult and cord neutrophils.

All stimulants caused significant reduction (p<0.01) in mean cellular fluorescence (MCF) of CD43 expression on adult neutrophils, particularly fMLP and PMA, which caused highly significant decrease in CD43 expression compared to unstimulated cells $p < 0.001$. The same

decreased CD43 expression trend was observed in cord neutrophils. IL-8 had the least effect on CD43 reduction, which was significant for the adult neutrophils (p = 0.02), but not for cord neutrophils (mean fluorescence of 40.7 compared to 46.2 for unstimulated cord neutrophils) figure 3.18. No significant difference was detected in CD43 surface expression between cord and adult neutrophils except when the cells were stimulated with PMA (p = 0.04). Similar reduction in the percentage of CD43 expressing cells was also observed (Figure 3.19), when adult and cord neutrophils were stimulated with the combination of F+C but reduction was statistically significant only in adult neutrophils (p = 0.008). This suggests that the enzymes releasing CD35 from the surface are different from those that release CD43, as different effects were observed following PMA stimulation.

CD15, also known as sialyl Lewis^x, is a leukocyte membrane bound carbohydrate involved in adhesion and interaction of E selectin and P selectin on endothelial cells. CD16 (FCγ receptor III) mediates the binding of immunoglobulin opsonised particles to phagocytes. As shown in figure 3.20 no significant difference was detected between cord and adult neutrophils expression of CD16 and CD15.

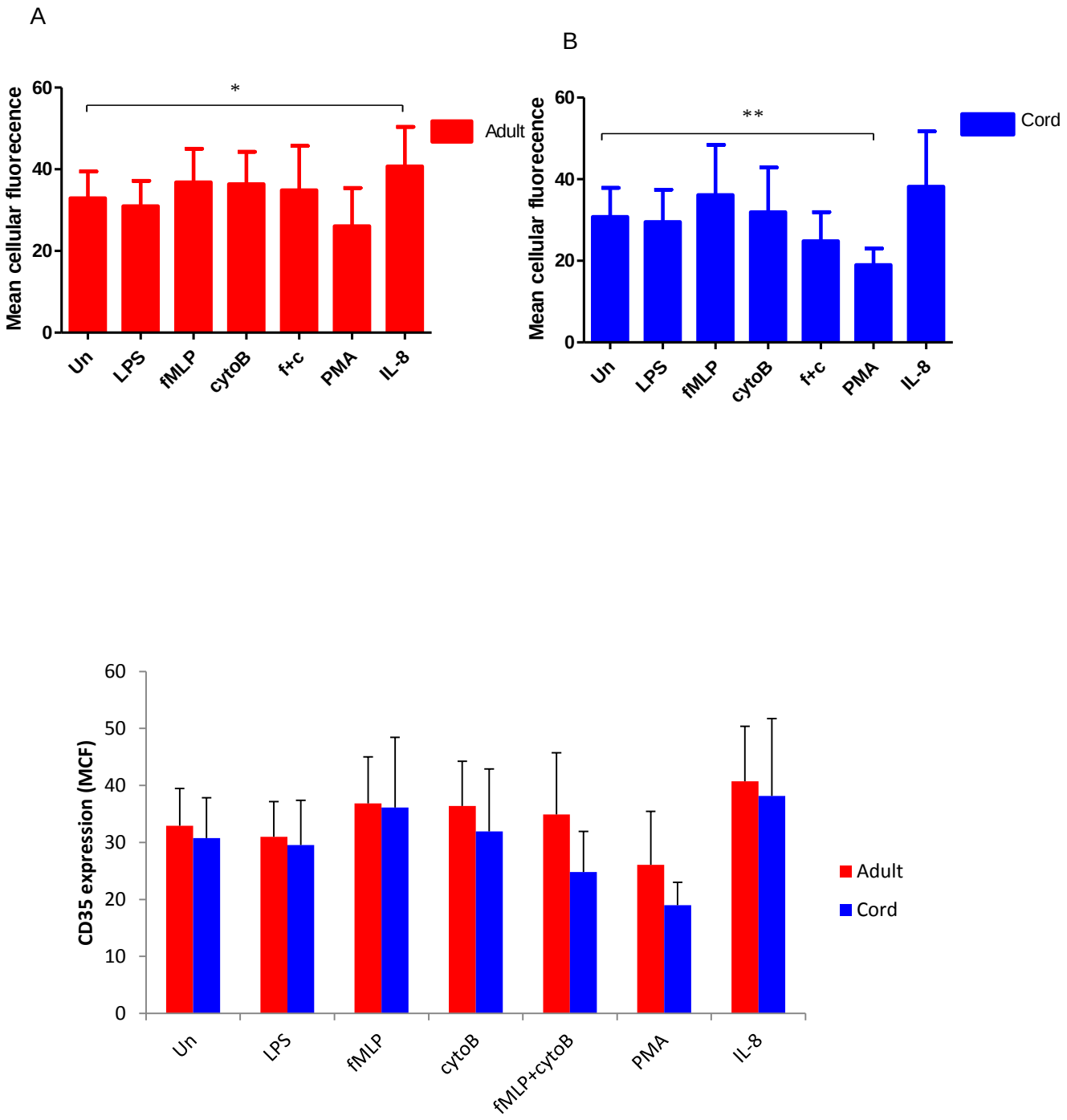


*Figure 3.16 Effect of different stimulants on membrane expression of CD35. Mean cellular fluorescence for APC-conjugated anti-CD35 staining was measured on cord (A) and adult (B) neutrophils. (C) Comparison of mean expression for both adult and cord. Mean and SEM shown for n=10, significant differences are shown *= p<0.05 and **= p<0.01 compared to unstimulated cells.*

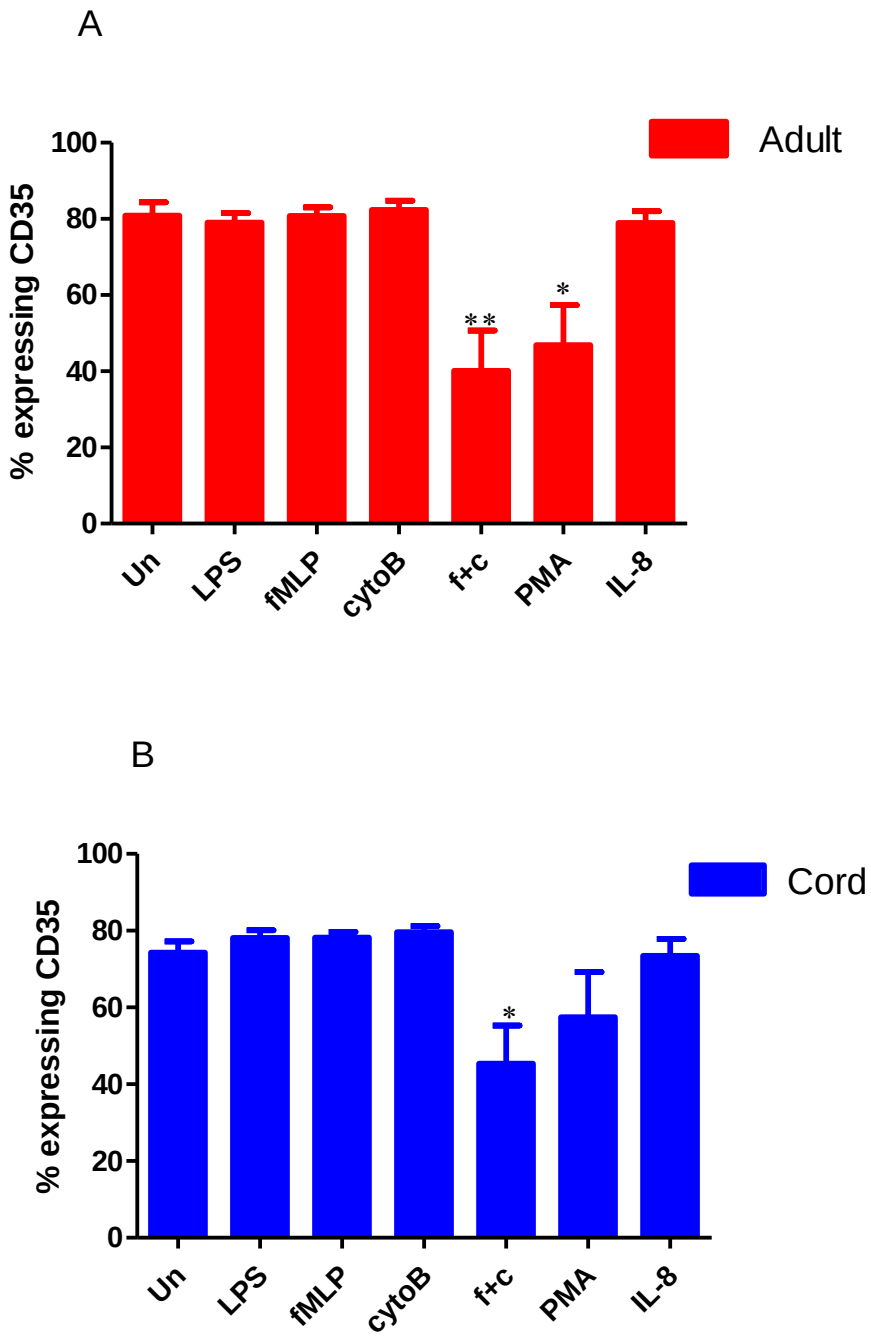


*Figure 3.17 the percentage of CD35 expressing cells in adult (A) and cord (B) neutrophils, above the background staining with isotype matched control. Mean and SEM are shown for ten paired samples, significant differences are shown ($p<0.05$ = *, $p<0.01$ = **) compared to the percentage of unstimulated cells.*

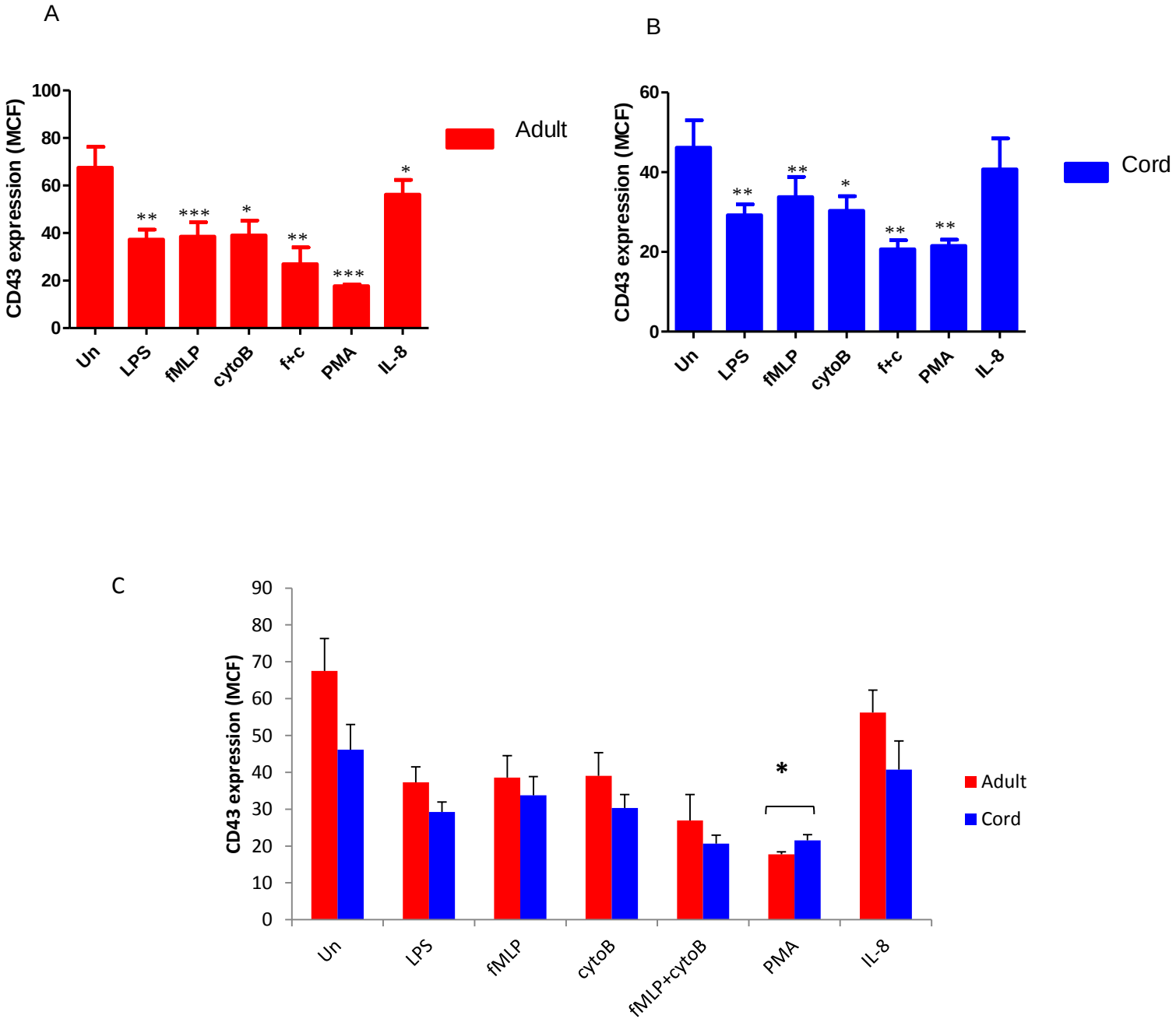


*Figure 3.18 Effect of different stimulants on membrane expression of CD43. Mean cellular fluorescence for PE-conjugated anti-CD43 staining of cord (A) and adult (B) neutrophils. (C) Comparison of mean expression for both adult and cord. Mean and SEM shown for ten paired samples. Significant differences are shown as * p=<0.05, ** =p<0.01 and * **=p<0.001.*

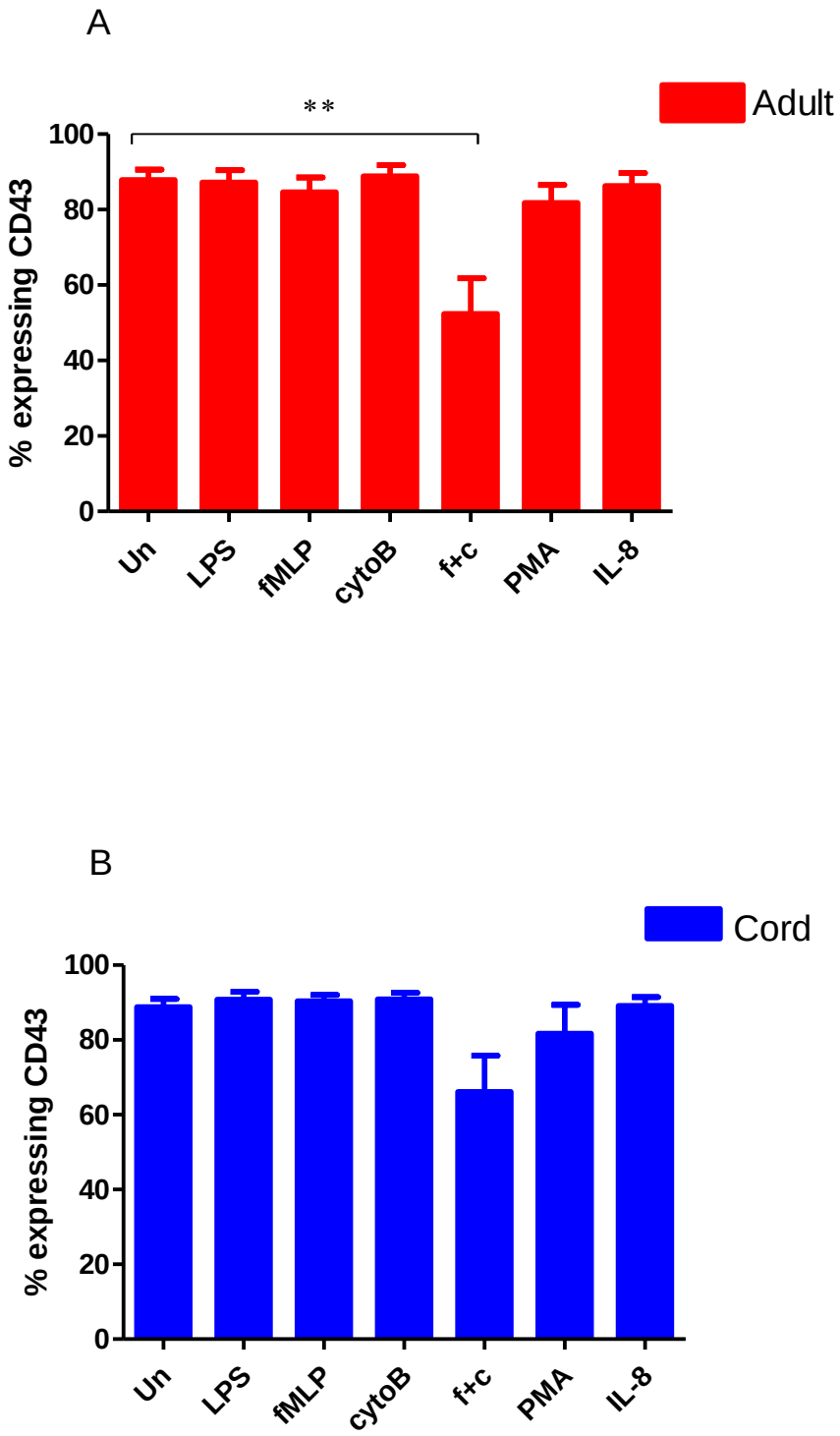


*Figure 3.19 Percentage of CD43 positive neutrophils in adult (A) and cord (B), above the background staining with isotype matched control. Mean and SEM are shown for ten paired samples, significant differences are shown (** = $p<0.01$).*

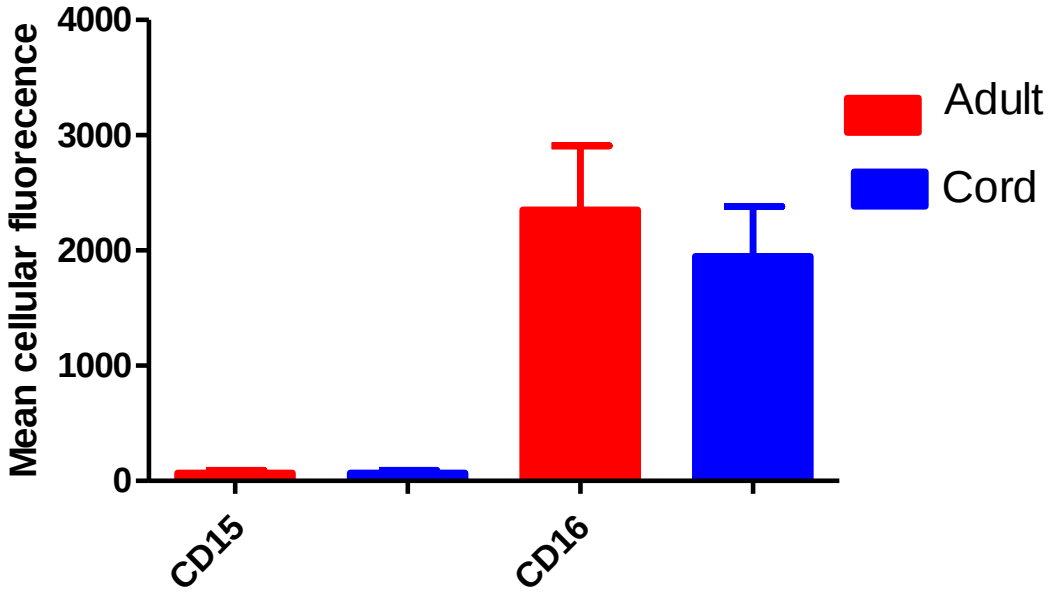


Figure 3.20 Mean cellular fluorescence of CD15 and CD16 on unstimulated cord and adult neutrophils. No statistically significant deference detected in the expression of two markers between cord and adult neutrophils.

3.2.5 Intracellular staining for CD177, PR3, SerpinB1 and other proteins

Parallel to extracellular surface marker expressions, intracellular levels of several proteins were also evaluated on unstimulated neutrophils. Only unstimulated cells were examined for these baseline level comparisons because of limited cell number availability. The mean intracellular fluorescence of PR3 for cord neutrophils was significantly higher than that of adult (MCF = 1347.3 units, 862.3 units respectively) $p < 0.05$ by unpaired t test. Both adult and cord PR3 intracellular levels were very high relative to the isotype matched control background staining (figure 3.21) and it does not have the bimodal nature observed for CD177.

Examination of intracellular CD177 confirmed the absence of CD177 in the negative two cord samples, as they were equivalent to background staining Like the results for surface expression, intracellular CD177 levels in cord neutrophils was significantly greater than that in adult neutrophils (*$p<0.001$;* unpaired t-test). The percentage of both cord and adult cells containing intracellular PR3 was 99% (indicating no subpopulations); whereas mean percentage of cells containing intracellular CD177 for cord neutrophils was 90% compared to 69% of adult cells, which always mirrored the patterns observed for surface expression for the same samples. This confirms that the surface expression subpopulations for CD177 expression are responsible for the bimodal PR3 expression on the surface of stimulated cells, not the presence of high and low PR3 subsets of neutrophils.

Mean cellular fluorescence of CD66b (a specific marker for the secondary granules) appeared to be higher in cords than adult, but failed to reach statistical significance (991.2 units and 781.2 respectively) figure 3.21 B. In contrast, mean CD63 (a specific marker for the primary granules) level appeared to be lower in cord neutrophils relative to adults, but this was not a

significant difference. This was of relevance as PR3 is thought to be contained primarily in CD63 positive primary granules, but has also been reported to be found in CD66b-positive secondary granules. The intracellular levels of CD35 and CD43 were also investigated as surface level reduction was examined for these markers, and other related complement regulators (C5a receptor (C5aR) and C3a receptor (C3aR)) were also examined. SerpinB1 has previously been shown to be expressed in high levels in neutrophils and may regulate the functional activity of PR3, so SerpinB1 levels were also measured. No significant differences were detected in MCF levels of C5aR, C3aR and Serpin B1 in unstimulated cord compared with adult neutrophils (figure 3.22). However, I did not study the effect of stimulation on the expression of these receptors. C5aR and C3aR levels were low, relative to isotype controls, while CD35 and CD43 were expressed at much higher levels.

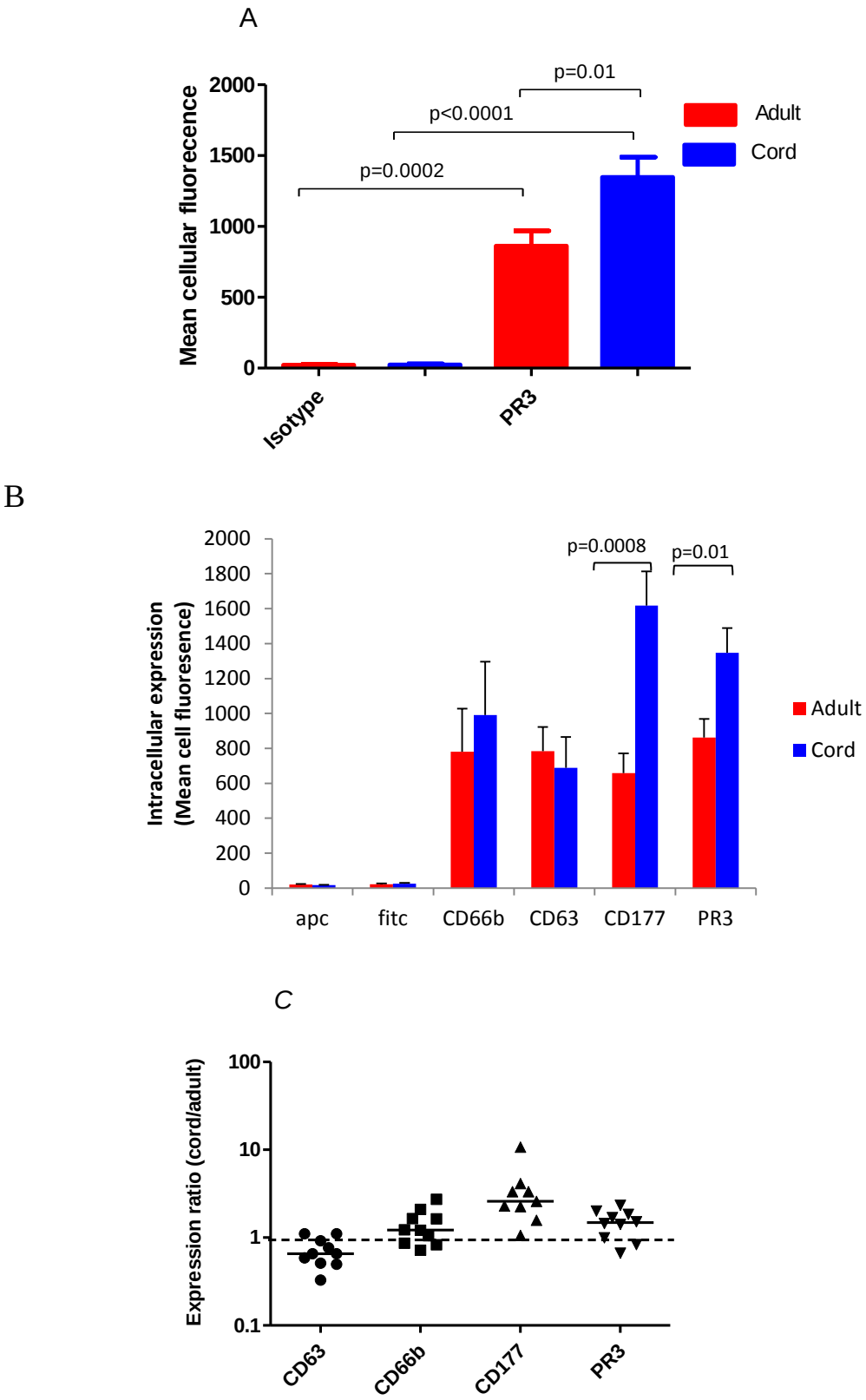
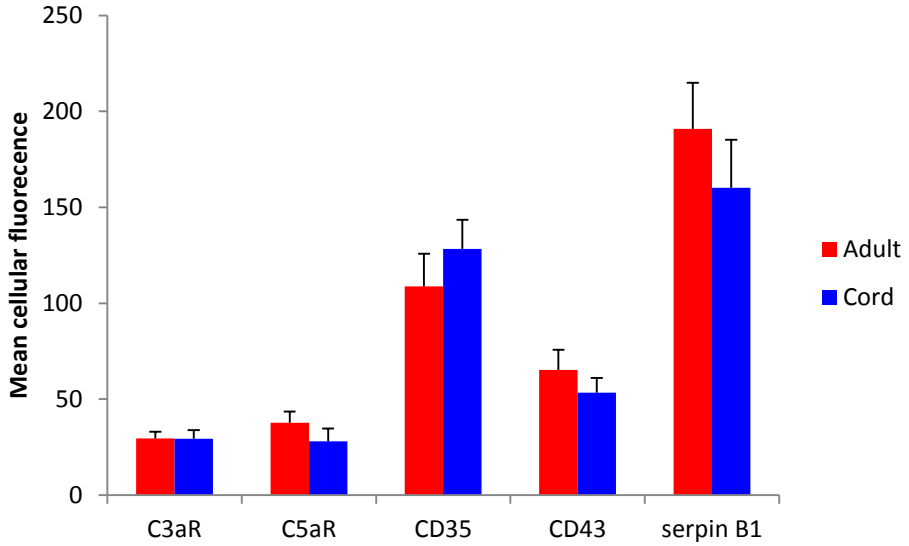


Figure 3.21 Intracellular levels of CD177, CD63, CD66b and PR3. (A) Level of PR3 compared to control in adult and cord neutrophils. (B) Comparison between Adult and cord neutrophils intracellular content of CD177, CD63, CD66b and PR3 mean cellular fluorescence as measured by flow cytometry. (C) Cord/adult ratio of CD177, PR3, CD63 and CD66b, n=10.

A)



B)

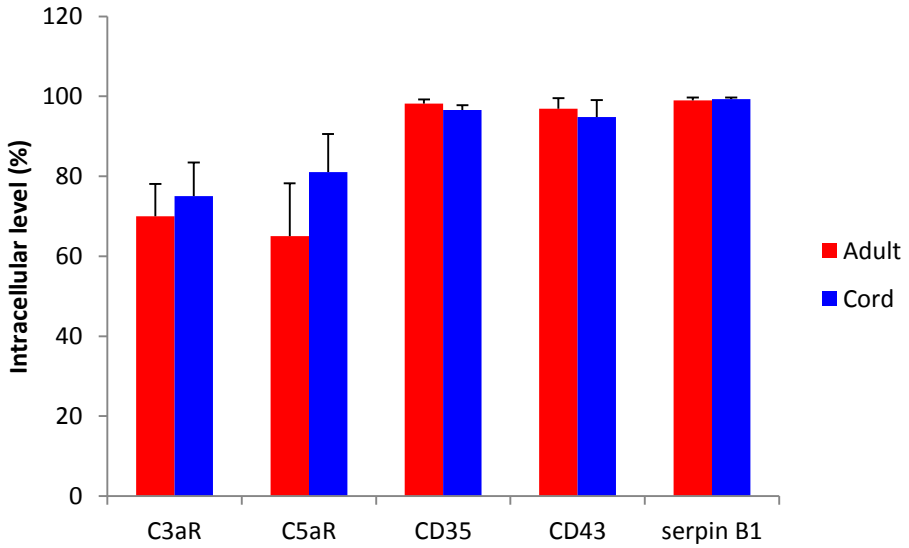


Figure 2.22 (A) Comparison of intracellular levels of complement receptors (C5aR and C3aR), CD35, CD43, and anti-proteinase SerpinB1) for unstimulated adult and cord neutrophils. (B) Percentage of positive cells expressing proteins above the background isotype control staining (n=10).

3.2.6 Determination of PR3 by ELISA

ELISA method was used to determine PR3 level in the supernatant and cellular fraction of adult and cord blood neutrophils in response to different stimuli compared to unstimulated controls. Unstimulated intracellular PR3 concentration in cord neutrophils (6.1 pg/cell, SEM 0.96) was significantly higher than that in adult neutrophils (3.6 pg/cell, SEM 0.46), $p = 0.03$. With the exception of PMA and fMLP combined with cytoB, all other stimulants caused minimal release of PR3 into the supernatant and no significant difference was detected in released PR3 from cord compared to adult neutrophils (figure 3.24). Total PR3 (combined released and intracellular fraction) was significantly higher in cord than in adult neutrophils ($p < 0.05$). Cytochlasin B and fMLP were able to release a mean of 18.9% of the total PR3 (cells plus supernatant) from adult neutrophils into the supernatant and 15.5% from cord neutrophil, p =N/S, whereas PMA stimulation only induced 5.5% of PR3 content from adult compared to 6.3% from cord cells. The total PR3 concentration in cord neutrophils was 2-fold greater than the adult concentration (mean 6255 ng/ml and 3711 ng/ml respectively; p =0.03) figure 3.23. PR3 release from CD177 positive cord neutrophils was higher than its release from the two CD177 negative cord neutrophil samples. However, CD177 negative cord samples were also able to release PR3, figure 3.25.

ELISA and flow cytometry (intracellular measurement of PR3) both consistently demonstrated higher PR3 concentration in cord neutrophils compared to adult neutrophils. For instance, the adult /cord ratio of PR3 level in ELISA was 0.59, which was comparable to the ratio of mean cellular fluorescence intensities for PE-conjugated anti-PR3 staining (0.64).

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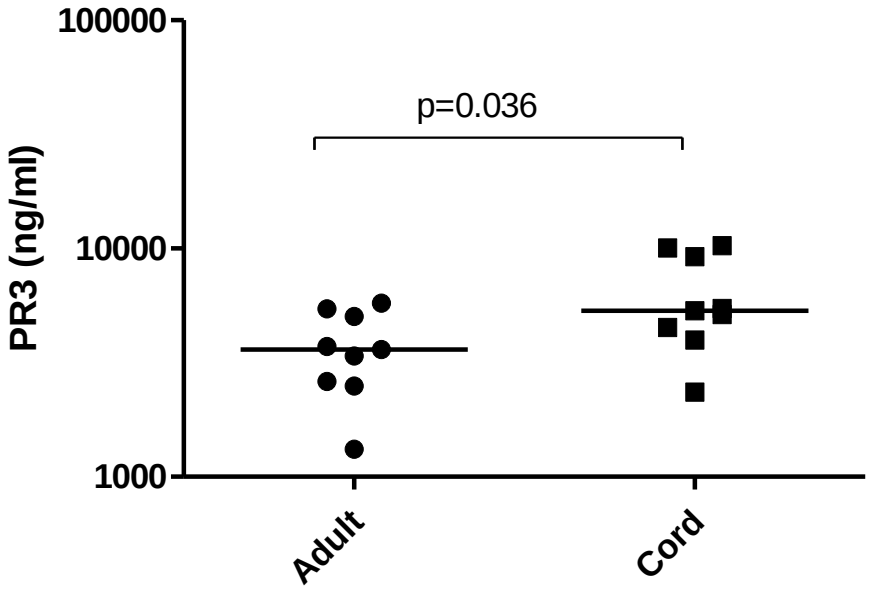
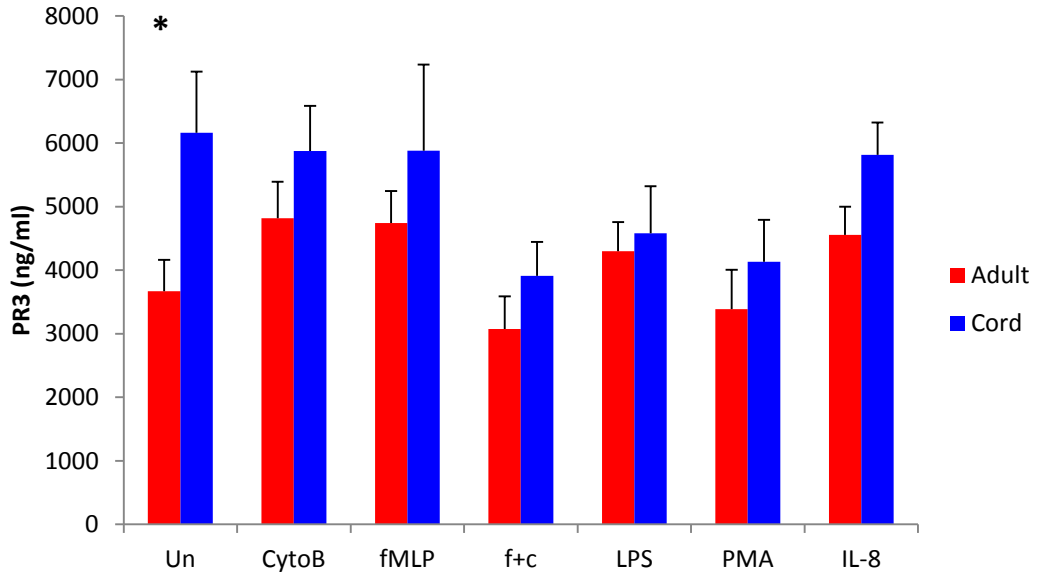
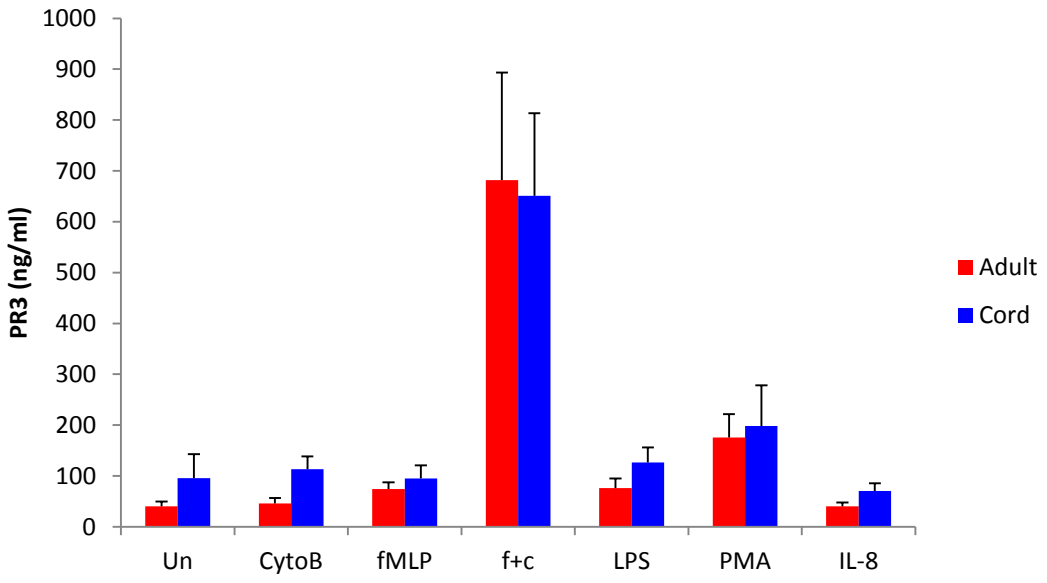


Figure 3.23 Scatterograph of total PR3 concentration present in both supernatant and cellular fractions of unstimulated neutrophils showing significantly greater PR3 concentration in cord compared to adult blood.

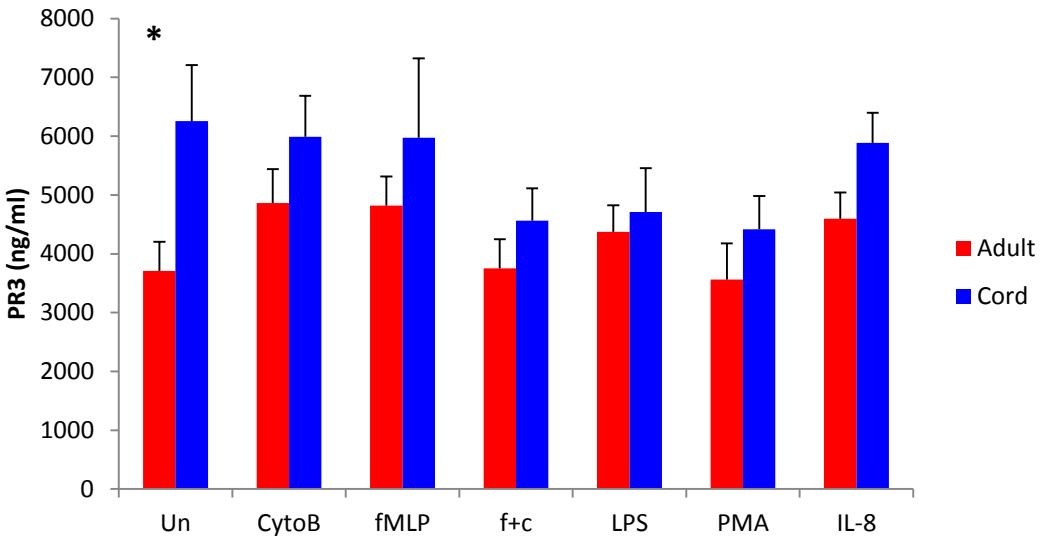
A Cell



B Supernatant



C Total



D Percentage release

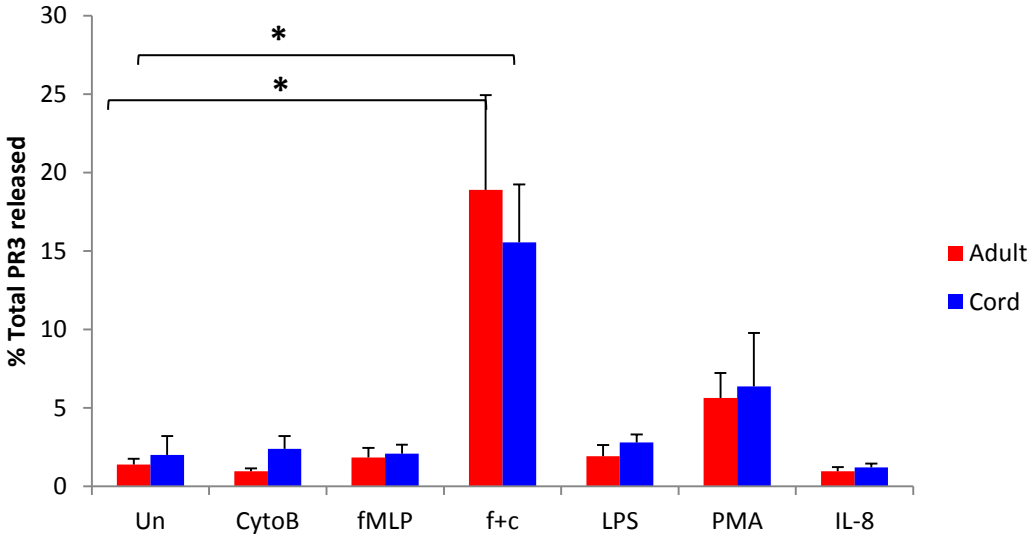
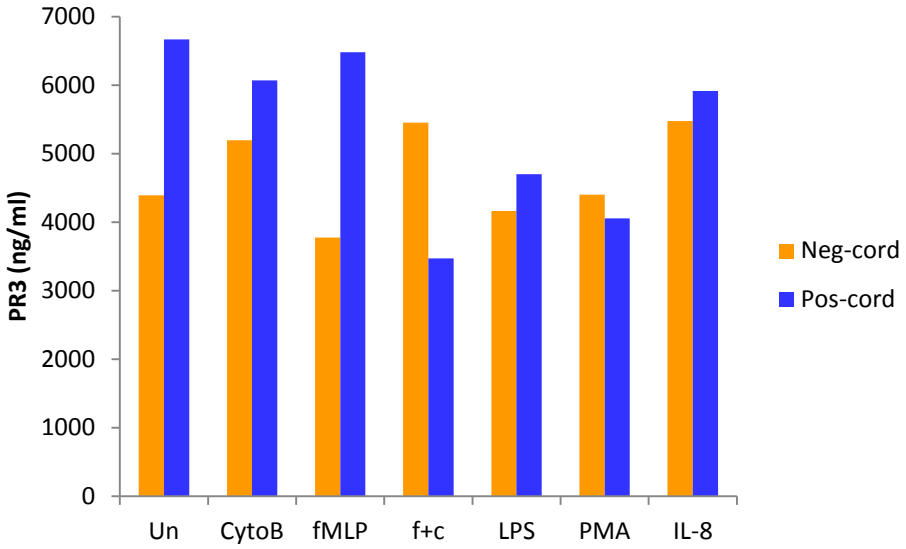


*Figure3.24. Mean PR3 concentrations from cord compared to that from adult neutrophils under a various stimulation conditions A) PR3 concentration retained in cellular fraction following activation. B) PR3 concentrations released into the supernatant. C) Total PR3 in both supernatant and cellular fractions. D) Percentage of total PR3 released into the supernatant. Error bars indicate SEM *= P <0.05.*

A Cell



B Supernatant

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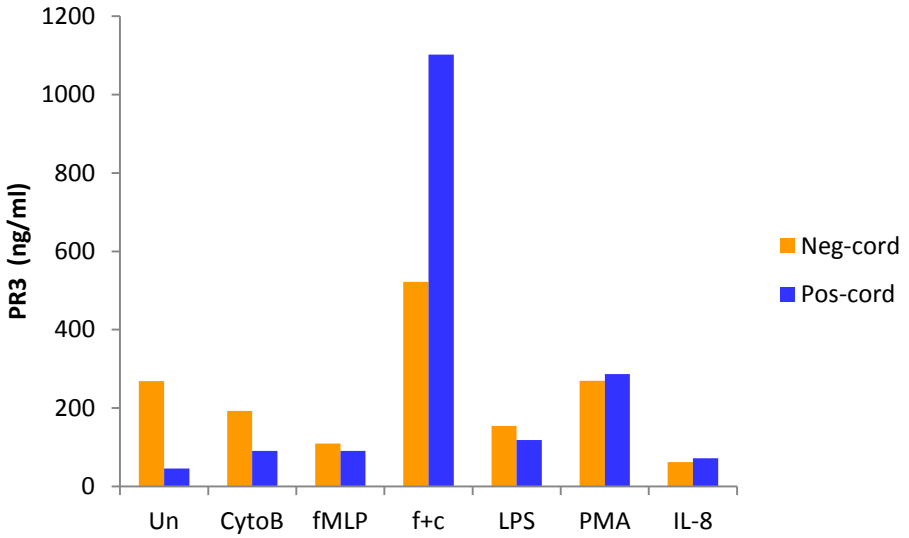


Figure 3.25 Mean PR3 concentrations from CD177 negative cord samples compared to that from CD177 positive cord neutrophils under a various stimulation conditions A) PR3 concentration retained in cellular fraction following activation. B) PR3 concentration released into the supernatant.

3.2.7 Cathepsin G functional activity

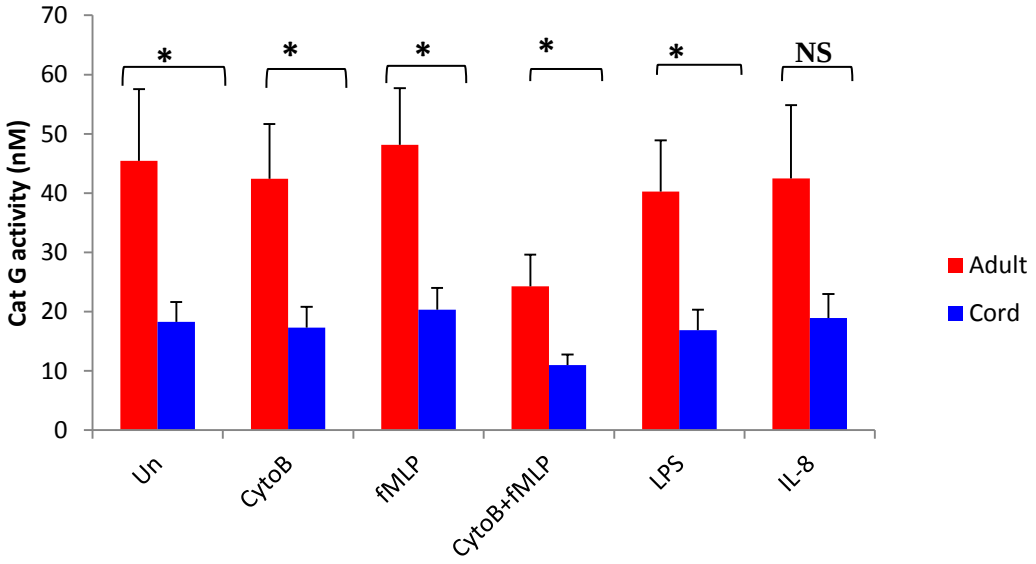
Isolated neutrophils from cord and adult samples were stimulated with a variety of stimulants as described in section 2.5.3. The cellular and supernatant fractions were separated by centrifugation and cellular fraction was released by distilled water and Triton X 100 detergent. Cathepsin G (CatG) activity was measured in both cellular and supernatant fractions following exposure to different stimuli, (n=10).

Unstimulated adult neutrophils contained significantly more cathepsin G (Cat G) in the intracellular compartment than cord neutrophils (mean 45.4 nM ± SEM 12.1 nM and 18.2 nM ± SEM 3.3 nM respectively, $p< 0.05$). Only a small fraction of Cat G was released from both unstimulated adult and cord neutrophil as evidenced by the Cat G activity in adult 10.7% and 13.7% for cord neutrophils. Total Cat G activity (combined intracellular and spontaneously released values) was significantly greater for unstimulated adult neutrophils (mean 53.9 nM ± SEM 13.3) compared to cord (mean 21.3±3.7 nM), $p= 0.042$. Similarly, the sum total Cat G activity (combined intacellular and released) in response to all stimulants was greater for adult neutrophils than cord neutophils (figure 3.26).

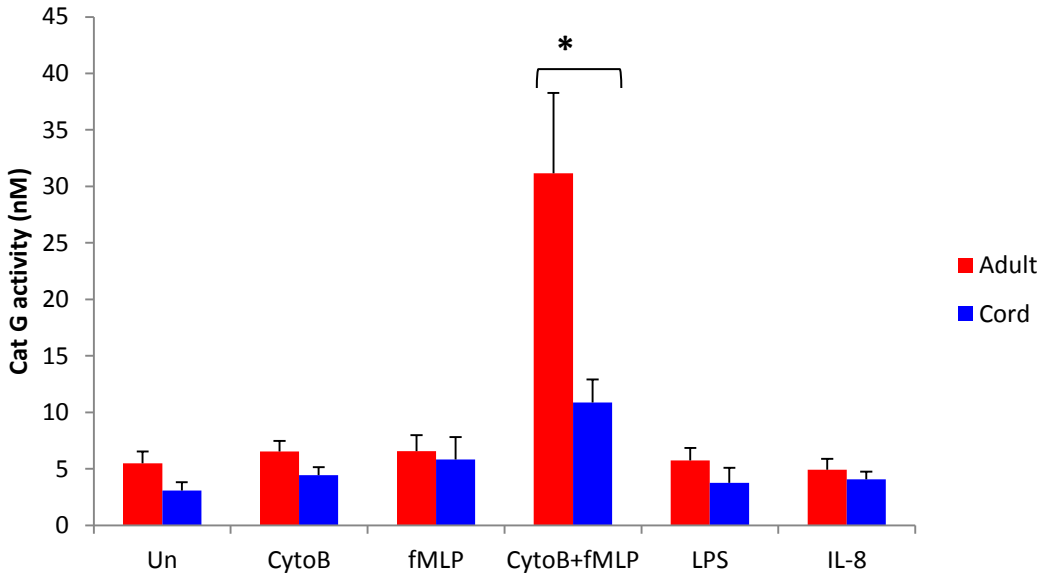
Significant differences in retained intracellular Cat G activity were obtained in the cellular fraction post-stimulation between cord and adult neutrophils for all conditions except IL-8 where no significant difference was observed in retained intracellular Cat G between adult and cord cells. The (F+C) combination was a much more potent inducer of Cat G release than all other stimulants, as no other condition resulted in a significant release of Cat G relative to unstimulated cells. F+C combination were able to release a mean of 56.2% of total

Cat G from the adult neutrophils into the supernatant and 49.7% from cord neutrophils, *P* insignificant. The Cat G activity measured in the supernatant was significantly greater from adult cells than from cord cells (mean 31.1 nM vs 10.8 nM; $p < 0.05$). The same pattern was found when elastase functional assay performed (submitted manuscript by other members of the laboratory; data not shown), except that F+C stimulation resulted in more than 60 % release of total cellular elastase for both adult and cord neutrophils. No significant differences were found in the percentage of Cat G released under any of the stimulation conditions, figure 3.26.

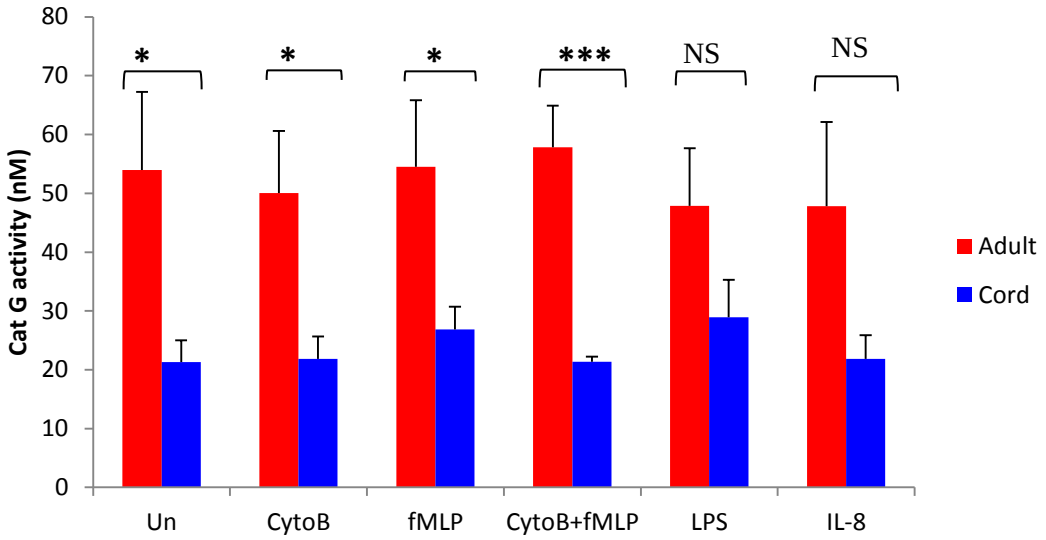
A Cells



B Supernatant



C Total



D Percentage release

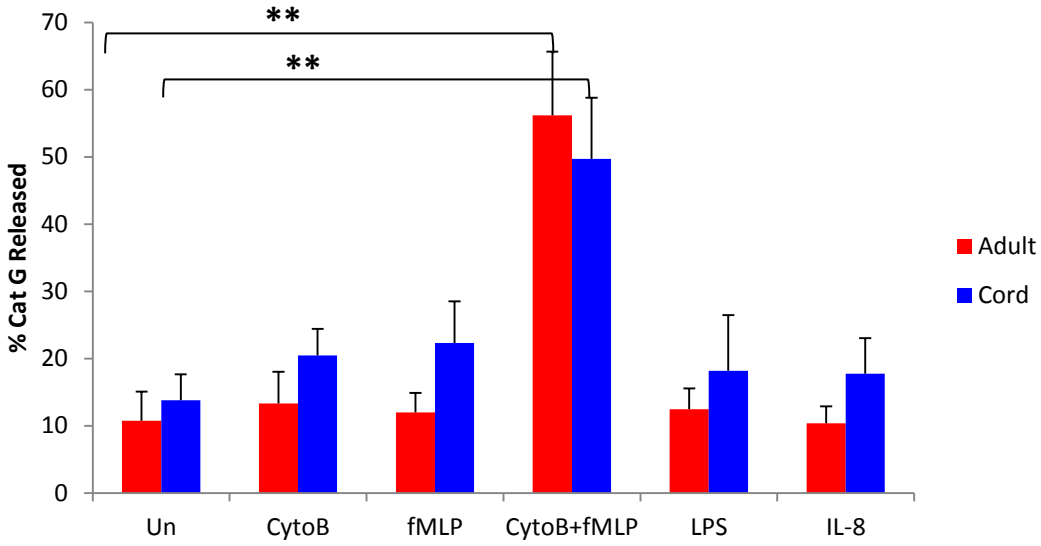


*Figure 3.26 Mean Cat G functional levels from cord compared to adult neutrophils under various stimulation conditions. A) Cat G activity retained in cellular fraction following activation. B) Cat G activity released into the supernatant. C) Total Cat G activity in both supernatant and cellular fractions (calculated from A + B). D) Percentage of total Cat G activity released into the supernatant. Only significant differences are marked * p=<0.05, ** =p<0.01 and * **=p<0.001.*

3.3 Discussion

3.3.1 CD177 and PR3

In this study, I analysed the surface expression of CD177 and mPR3 in cord and adult isolated neutrophils, and I assessed whether their expression on the plasma membrane of neutrophils depends on cell activation state. Based on expression of membrane-bound proteinase 3 (mPR3), two subsets of neutrophils can be identified: neutrophils that hardly express proteinase 3 ($mPR3^-$ neutrophils) after stimulation, and neutrophils that substantially express proteinase 3 ($mPR3^+$ neutrophils) after stimulation. Consistent with the results reported by others, the expression of mPR3 and CD177 were bimodal (Bauer *et al*., 2007; Brachemi *et al*., 2007; Von Vietinghoff *et al*., 2007). However, I found that isolated neutrophils from all of adults and some of cord blood samples in the absence of stimuli showed uniformly positive expression of mPR3 in agreement with Hu *et al*., 2009 who reported monomodal PR3 in 19/31 adult neutrophils (61%). In addition, I found that after incubation of cells with fMLP, PMA and fMLP in combination with cytoB (F+C), the pattern of mPR3 expression changed to bimodal, with a low-expression mPR3 and a high-expression mPR3 subset. This finding is also in accordance with Hu and co-workers who found that 12 of their 19 adult isolated neutrophil samples changed from monomodal to bimodal mPR3 expression following 15 minutes with TNFα (Hu *et al*., 2009).

In keeping with my results, Bauer *et al*. showed that CD177 and PR3 are expressed on the same subsets of neutrophils and both can be up-regulated in parallel suggesting that CD177 and mPR3 originate from the same intracellular storage (secondary granule) and shared the same mode of trafficking. They concluded that only PR3 present in secondary granules and secretory vesicles can be expressed on the membrane (Bauer *et al*., 2007).

It has been shown that percentage of mPR3^{+} and CD177-positive neutrophils display high inter-individual variation ranging from 0% to 100% of the total population of neutrophils for each person (Halbwachs-Mecarelli *et al*., 1995; Witko-Sarsat *et al*., 1999; Rarok *et al*., 2002; Schreiber *et al*., 2003). This percentage was stable in healthy individuals and did not change with G-CSF mobilisation of neutrophils (Goldschmeding *et al*., 1992; Wolff *et al*., 2003). Results of the present work are in agreement with these studies. I show inter-individual variability in cord as well as adult neutrophil CD177 expression and stability of percentage expression in each individual even after stimulation with different stimulants. A similar pattern of stability of CD177 expression ratio was demonstrated by Stroncek and co-workers who found that this ratio remained stable following the expression of CD177 antigen by myelocytes (Stroncek *et al*., 1998).This suggest that myelocyte CD177 expression status continues in subsequent myelopoietic stages: metamyelocytes, band and segmented forms and that CD177-negative myelocytes eventually produce CD177-negative neutrophils.

Genetic control of mPR3 expression is supported by monozygotic twin studies which demonstrated that the ratio of mPR3 expressing neutrophils in these twins is highly concordant (Schreiber *et al*., 2003). CD177 gene polymorphism was reported in Wolff *et al* study who found 6 allotypes of this gene, three of them were significantly associated with a small CD177 expressing subpopulation, indicating a genetic basis for CD 177 non-expression (Wolff *et al*., 2003).

Variation in membrane expression was noted also in PR3 where a study demonstrated that the proportion of freshly isolated neutrophils that express PR3 varies considerably between donors from 0-95% but is also extremely stable for each individual over prolonged periods of time (Halbwachs-Mercarelli *et al*., 1995) which is in accordance with our findings. Although

this work is not a longitudinal study, I did repeat the experiments with some individual adults 2 or 3 times throughout my project and the results were consistent each time. In this project the intracellular levels of PR3 showed some correlation with mPR3 levels in both adult ($r = 0.43$) and cord ($r = 0.54$) although the correlation was not statistically significant and this could be due to the limited number of samples.

Herein, I found that the mean percentages of CD177 and PR3 expression in neutrophils were higher in cord blood than in adult neutrophils. Stroneck *et al* showed that the size of positive CD177 subpopulation of neutrophils from umbilical cord was 91% which was significantly higher than that of neutrophils from both children and adult (56%). This percentage decreased by 1 year of age and remained stable thereafter (Stroneck *et al*., 1998). This suggests that the newborn status is to express a predominantly monomodal high and that expression is altered in certain subsets through an unknown mechanism. However, CD177-deficiency is probably genetic and expression is never induced in these individuals after birth.

Many studies have found that stimulation of isolated neutrophils with agonists such as PMA, TNFα, IL-8, fMLP, Cytochalasin B, TGF-β and GM-CSF up-regulate the expression of PR3 on CD177-positive neutrophil cell surfaces. (Csernok *et al*., 1994; Halbwachs-Mecarelli *et al*., 1995; Witko-Sarsat *et al*., 1999; Hellmich *et al*., 2000; Drewniak *et al*., 2008). In keeping with these studies I observed that some of the unstimulated neutrophils from healthy donors express PR3 on their surface and this surface expression is further augmented by several stimulants. The relatively greater effects of F+C on mean cell surface expression of CD177 and PR3 when compared with the effects of the rest of the agonists likely reflect the synergistic effect of this combination on neutrophil degranulation. Bauer *et al* reported that F+C was the most potent stimulant to the extent that mPR3 negative and CD177 negative neutrophils became positive but we could not reproduce such an effect on either mPR3 or on

CD177 expression. The group suggested that equal upregulation of CD177 and PR3 indicated that PR3 expressed on membrane originate from secretory vesicles and secondary granules rather than from primary granules which are less mobilised and did not contain CD177 (Bauer *et al*., 2007). Similarly, Abdgawad and colleagues showed that cell surface expression of mPR3 is dependent on CD177 and found a strong correlation between the percentage of mPR3 positive subpopulations and the percentage of CD177 positive subpopulations.

3.3.2 Effect of cytokines and sepsis on PR3 membrane expression

Previous study has reported that expression of mPR3 on positive neutrophils is higher in septic patients than in healthy individuals (Matsumoto *et al*., 2006). In sepsis, excessive neutrophil stimulation and degranulation resulted in highest mPR3 expression (approximately 70%) in positive neutrophils. Moreover, cytokines TNFα and IL-8 synergistically increased membrane expression of PR3 in neutrophils (Csernok *et al*., 1994). Membrane expression of PR3 was also reported to increase in another study on 46 patient with fatal sepsis where leukocytes showed a shift to the left (more band neutrophils) and expression had positive correlation with C Reactive Protein (CRP) (Von Vietinghoff *et al*., 2007). This suggests that exposure of neutrophil to cytokines and chemo-attractants could mediate the observed increases in mPR3 on neutrophil during sepsis. Also, priming of neutrophil with LPS, TNFα and PAF followed by activation with IL-8, resulted in striking synergistic increases in cell surface expression of PR3 (Campbell *et al*., 2000). Stimulation of neutrophils with TNF-α, which brings them to a pre-activated state, may translocate PR3 to the plasma membrane and raise the expression level up to two-to three fold of that on resting neutrophils (Reumaux *et a*l., 1995). Elevated levels of CD177 transcription in neutrophils were previously found in

patients with bacterial infections, but not in patients with viral hepatitis (Gohring *et al*., 2004).

Intracellular storage pools of PR3 are reportedly located not only in primary but also in secondary granules and secretory vesicles (Witko-Sarsat *et al*., 1999). Activation of neutrophils by TNF is adequate to mobilize secretory vesicles and possibly tertiary granules (Bouaoina *et al*., 2004; Chakrabarti *et al*., 2006).

On the other hand, when I measured CD177 expression, unstimulated and stimulated cord neutrophils were shown to express significantly higher levels of CD177 on their surface compared to adult as shown in figure 3.4. Gender and age difference in expression of CD177 was also previously described. For example, CD177 expression was higher in women of reproductive age than men and in pregnant women than in healthy non pregnant female donors (Caruccio *et al*., 2003; Taniguchi *et al*., 2004). CD177 drops in older women but not in older men (Matsuo *et al*., 2000), which may be related to low oestrogen level in this age group and probably indicates that finding elevated level of CD177 in cord neutrophils here might be due to the presence of more estrogen in pregnant women which influence their infants. However, this suggestion cannot explain the absence of CD177 expression in some newborn infants in our study group. Absence of CD177 in these two newborns could be inherited, although evaluation of CD177 level in their parents was not done. This is supported with previous reports which demonstrated that absence of CD177 was found in approximately 3% of Caucasians, 5% of African Americans and 11% of Japanese (Matsuo *et al* ., 2000, Stroncek *et al*., 2004). One causes of this deficiency has been reported as an mRNA splicing defect (Kiessel *et al*., 2002).

In the present work I found that the level of mPR3 was notably higher on unstimulated neutrophils in CD177-negative cord samples compared to the level of background (isotype control). Hu *et al*., 2009 found 3 healthy donors from 31 volunteers with CD177-negative neutrophils which expressed mPR3 after stimulation with TNFα. Therefore, neutrophils from these three samples, despite their negative CD177 status, were able to expresses membrane PR3 on their surface. These results, in agreement with my findings indicate that expression of PR3 on the neutrophil surface can be mediated by other mechanisms independent of CD177 as a receptor.

Activation of cells by fMLP combined with cytoB considerably increased mPR3 expression on adult neutrophils but expression was not altered in CD177 negative cord neutrophils. This suggests that the role of CD177 in cell surface expression of mPR3 on unstimulated CD177 negative cord cells could be compensated by yet unknown mechanisms. In an attempt to investigate the underlying mechanism, Campbell *et al* showed that ionic interactions are important in the binding of PR3 to the plasma membrane and PR3 could be eluted from the membrane of neutrophil following cellular activation. PR3 can bind stably to anionic and neutral membranes, but binds strongly to negatively-charged bilayers. Moreover, hydrophobic residues in PR3 also bind to the membrane with high affinity (Campbell *et al*., 2000; Hajjar *et al*., 2006). In keeping with Hajjar findings, PR3 could be inserted into the membrane lipid bilayer via the hydrophobic region (Goldmann *et al*., 1999). However, other workers did not believe that mPR3 binding to neutrophil surface occurs only in a charge-dependent manner (Owen, 2008) and it was demonstrated that mPR3 could not be eluted off the membrane by drastic PH changes (Witko-Sarsat *et al*., 1999; Korkmaz *et al*., 2008).

Others suggest that PR3 membrane binding is possibly mediated by proteins other than CD177 such as FcγRIIIb (CD16), or β2 integrin (CD11b/CD18) and CD66b (Matsuo *et al*., 2000; David *et al*., 2003; David *et al*., 2005; Fridlich *et al*., 2006).

Membrane bound expression on mPR3 low neutrophils has also been observed in other studies (Halbwachs *et al*., 1995; Schreiber *et al*., 2004; Van Rossum *et al*., 2007). These results suggest that CD177 is not an exclusive receptor of mPR3, and the binding sites for CD177-independent mPR3 expression are still open for discussion. However, CD177 seems to be necessary for bimodal PR3 expression pattern since I found absence of PR3 bimodal expression pattern in the two negative CD177 cord samples even after stimulation.

Quantification of intracelluar PR3 was also performed using ELISA in adult and cord neutrophils. Unstimulated intracellular PR3 concentration in cord neutrophils (6.1±0.95 pg/cell) was significantly higher than that in adult neutrophils (3.6±0.46 pg/cell $p \leq 0.05$). ELISA study and flow cytometry analysis both demonstrate higher PR3 concentration in neonatal neutrophils than adult neutrophils. The fact that the two very different methods give us similar findings adds significant strength to our findings. The ELISA method is a summation of all the PR3 either released by all the cells or remaining in the cell pellet whereas flow cytometry measures PR3 concentrations in individual cells, before and after stimulation. Both methodologies suggest that PR3 concentrations are approximately double in cord neutrophils than adult neutrophils. This was the first report of PR3 ELISA in cord neutrophils whereas adult levels were in agreement with Witko-Sarsat *et al*., 1999 and Campbell *et al*., 2000.

3.3.3 CD63 and CD66b

CD63, a member of the tetraspanin superfamily, is an activation marker in neutrophils and one of the membrane proteins of azurophilic granules, which has been shown *in vitro* to be expressed on the cell surface following neutrophils activation in the presence of fMLP following cytochalasin B priming. PMA and fMLP alone are known to induce minimal translocation of CD63 (Cham *et al*., 1994). CD63 is also found in platelets (Kuijpers *et al*., 1991). Lopez *et al*. found CD63 expression increased on synovial fluid neutrophils of rheumatoid arthritis patients (Lopez *et al*., 1995).

In this work, I did not detect any significant difference in neutrophil CD63 expression between cord and adult in unstimulated neutrophils. I have demonstrated that cell surface expression of CD63 in response to different stimuli did not change from the baseline of unstimulated cells in both adult and cord neutrophils except with F+C. This combination brought about greater expression of CD63 in comparison with unstimulated cells in adults reflecting their potent effect on neutrophil degranulation, in agreement with earlier reports (Dewald and Baggiolini, 1986; Niessen and Verhoeven 1992; Cham *et al*., 1994). The combination of F+C also has great effect on up-regulation of CD63 on cord neutrophils not previously reported before this work. Up-regulation of CD63 in response to F+C was higher in cord than in adult neutrophils, but the difference was not statistically significant.

Secondary granules are mobilised easily upon stimulation early during inflammation. They appear later in development and are smaller, and more numerous, than azurophilic granules. They are de-granulated *in vitro* by low concentration of PMA or fMLP even in the absence of

cyto B (Cham *et al*., 1994). CD66b is present in membrane of specific granules (Molinedo *et al*., 1999; Molinedo *et al*., 2010).

In this project, these agonists had similar effect on CD66b expression on neutrophils, which is a marker mainly located in the specific granules. Stimulation of adult cells with fMLP alone causes significant increase of CD66b compared with unstimulated neutrophils whereas PMA and F+C stimulation induced even more highly significant expression of CD66b in both cord and adult neutrophils. Up-regulation of CD66b membrane expression in response to both of PMA and F+C was higher in adult than in cord neutrophils, but the difference was not statistically significant. These results are in accordance with previous reports and probably are explained by the capacity of PMA to elicit release of specific and tertiary granule contents and induction of all three major granules secretion by F+C (Molinedo *et al*., 1991). Our findings are in keeping with those of Niessen and Verhoeven 1992 who showed that F+C is a potent activator of specific granule degranulation using CD67 rather that CD66b as a specific granules marker. Effect of PMA on degranulation of specific granules was reported in many studies (Smith and Peters 1982; Dewald and Baggiolini 1986; Niessen and Verhoeven, 1992). However, in this study we did not find any differences between cord and adult neutrophils in CD66b expression.

The expression of CD66b in preterm neonates was comparable with that of adults (Payne *et al*., 1993), gestational age has been reported to have no effect on the percentage of CD66b positive cells (Weinschenk *et al*., 1998) and pregnancy has no apparent effect on CD66b expression (Sacks *et al*., 1998; Naccasha *et al*., 2001). However, pregnant women, who delivered prematurely, had a higher intensity of CD66b expression on their neutrophils (Gervasi *et al*., 2001).

Neonatal neutrophils may have fewer specific granule numbers and also possibly abnormal morphology. Newborn neutrophils have reportedly less lactoferrin content, but release of lactoferrin on stimulation is reportedly comparable to adult (Ambruso *et al*., 1984).

Mollinedo and co-workers found that cell surface expression of CD11b and CD66b were up-regulated upon neutrophils activated with PMA, which degranulate specific and tertiary granules. Their expression was also up-regulated by fMLP which induces secretion of the three major granules in the presence of cytoB. Moreover, fMLP treatment alone induced up-regulation of cell surface expression of primary granule marker CD63 but not CD66b (Mollinedo *et al*., 2010).

3.3.4 CD35 and CD43

CD35 antigen is normally expressed on bands and segmented neutrophils. It is present in a large intracellular pool that could be easily trans-located to the plasma membrane with activation (Ahearn and Fearon, 1989), likely secretory vesicles.

Foetal, preterm neonatal, and term neonatal granules expressed CD35 in an intensity that was similar to adult granulocyte (Smith *et al*., 1990) in agreement with our observation. Similarly, Abughali *et al*. quantified CD35content of neonatal granulocytes and found no difference in CD35 level from adults (Abughali *et al*., 1994). Smith *et al*. found no significant change in CD35 expression between term and adult after fMLP stimulation whereas expression in response was significantly less in preterm neutrophil (Smith *et al*., 1990)

Contrary to this conclusion, another study found that the expression of CD35 on resting neonatal neutrophils was significantly higher than in adult neutrophils but stimulation of

neutrophil with fMLP and IL-8 increased CD35 expression more markedly in adults (Lothian *et al*., 1997)

We observe that neutrophils from term neonates expressed significantly less CD35 on the plasma membrane in response to stimulation than neutrophils from adults. The percentage of CD35 expressing neutrophils were significantly down regulated in response to PMA and F+C in adult cells whereas only F+C caused significant reduction in the percentage of CD35 expressing cord neutrophils. In this study, neutrophils from adults expressed significantly higher CD35on their surface in response to stimulation with IL-8 than expression in unstimulated in agreement with Lothian *et al* 1997 while it has no significant effect on cord neutrophils.

CD43 (Sialophorin/leukosialin), is highly represented on neutrophils and other leukocytes (Fukuda and Tsuboi. 1999). CD43 on neutrophils has both anti-adhesive and pro-adhesive functions. CD43 was shown to inhibit E selectin dependent adhesion of circulating neutrophils to endothelium while it does not interfere with their integrin-mediated adhesion (Mambole *et al*., 2008; Matsumoto *et al*., 2008).

In my study, all stimulants caused significant reduction in CD43 expression on adult neutrophils, particularly PMA. The current work is in agreement with Rieu *et al*. study which demonstrated that membrane expression of CD43 was down-regulated by up 80 % in response to cell activation with PMA or F+C while TNFα followed by fMLP stimulation resulted in up to 40% decrease of CD43 expression (Rieu *et al* ., 1992). Other previous studies also have shown similar down regulation of CD43 expression by PMA (Halbwachs-Mecarelli *et al*., 1996).

The same response in term of CD43 expression was observed in cord neutrophils, except when stimulated with IL-8 which lead to reduction in CD43 expression, but the down-regulation was not statistically significant. This finding agreed with Remold-O'Donnell and Parent 1994 work who found that PMA, and to much lesser extent, fMLP and IL-8 decreased neutrophil expression of CD43.

In addition, Bazi and Strominger 1994 demonstrated that CD43 is enzymatically cleaved from the surface of PMA-activated granulocytes. CD43 shedding has been shown to be mediated by neutrophil elastase (Cham *et al*., 1994). Remold-O'Donnell and Parent, 1995 found that neutrophils CD43 was cleaved in a dose-dependent manner by low level of neutrophil elastase. Cathepsin G can also cleave CD43 although it has lower affinity and 10 times higher concentration than elastase is required whereas PR3 could not cleave CD43.

3.3.5 CD16

CD16 expression on unstimulated cord neutrophils was equivalent to its expression in adult neutrophils in this work. This result is in keeping with Bikou *et al* report that the expression of CD16 in leukocytes and CD35 in myeloid cells in cord blood were comparable to their expression in adult neutrophils (Bikou *et al*., 1997). Moreover CD16 expression was reportedly low in elderly particularly in presence of bacterial infection (Butcher *et al*., 2001).

Takahishi *et al* found that the mean percentage of CD16 positive neutrophils was significantly lower in cord (73.3%) than in adult blood (95.9%). Difference may be related to mode of delivery since 5 out of the seven neonates included in their study were born

vaginally and only 2 were born by Caesarean section, whereas the 10 cord samples in my study were obtained after elective Caesarean section. Different methodology was followed in blood collection and purification and collected sample volume was smaller (3 ml of heparinised whole blood) (Takahishi *et al*., 1994). Activation of neutrophil could probably be related to delivery mode or manipulation of sample resulting in the lower expression reported in this study. Activation of neutrophil is known to induce CD16 shedding from neutrophil membrane via proteolytic shedding (Middelhoven *et al*., 2001). Cord neutrophils from vaginally delivered neonates were shown to be primed by labour stress while cord neutrophils obtained following Caesarean section were not metabolically primed (Khalfan *et al*., 1995). In this study CD16 expression was measured only in unstimulated neutrophils.

In addition, total leukocyte and neutrophil counts were higher in cord blood after vaginal delivery compared with Caesarean section. However, there were no statistical differences in the expression of CD16 or CD35 receptors on neutrophils between the delivery groups in one study (Grönlund *et al*., 1999).

Shedding of membrane receptors may be required to limit cell responsiveness to external ligand or to dissociate cells from ligand involved in the transmigration process. However shedding may also represent a mechanism for the production of soluble receptors that convey signals to other cells in a manner analogous to cytokines (Tedder, 1991).

Furthermore, I compared the expression of CD15, another specific granule marker that co-localised with lactoferrin (Buescher *et al*., 1990). Its expression in cord neutrophils was similar to its expression in adult neutrophils in resting condition but I did not study the expression in response to stimuli.

3.3.6 Cathepsin G and elastase

Comparative study of functional activity of cathepsin G in cord and adult neutrophils described in this chapter has not been previously reported. However, a few studies have compared elastase content in adult and neonatal neutrophils. Some of these studies assessed free elastase activity in umbilical cord plasma. They suggested that plasma elastase elevates soon after birth probably due to neutrophil activation during delivery and gradually declined afterwards (Tsaka and Herkner 1990; Philip *et al*., 1994; Adeyemi and Abdulle 1998). To avoid neutrophil activation during delivery in this thesis, all cord samples were collected from neonates born by elective Caesarean section.

Our findings demonstrate that cord neutrophils contain significantly less cathepsin G and elastase than adult neutrophils in agreement with previous work on neutrophil elastase activity in our laboratory which showed that cord neutrophils have less elastase activity than adult neutrophils. Due to lack of known substrate that is specific for PR3, I could not investigate the functional activity of PR3.

However, a similar proportion of protease is released by cord and adult neutrophils in response to stimulation. Therefore, in response to stimulus, the total elastase or cat G released by cord neutrophils will be proportionately less than the amount released from adult neutrophils. This could be an additional contributing factor to cord neutrophils limited bactericidal ability and hence to neonates susceptibility to infection.

Small amount of Cat G is known to leak into extracellular fluid when azurophilic granule contents are released from neutrophil in response to physiological stimuli (Owen *et al*., 1995).

Stimulation with combination of fMLP and cytoB resulted in release of most of Cat G from neutrophils. Elastase activity assay was previously studied in our laboratory. However, in this project I repeated elastase activity experiments in parallel to Cat G analysis as both of these enzymes are released from the same subset of granules and reproducibility of elastase results arguably could be a helpful guide in interpretation of Cat G results. Only unstimulated cells or cells receiving maximum stimulation (F+C) were examined as previous work in the laboratory showed that these were the only condition to significantly result in release from neutrophils. In accordance with previous results, Cat G release was only significantly released with F+C. Similar response noticed in the activity of these enzymes to F+C stimulation is explained by localisation of both enzymes in the same granule subset, namely primary granules. However, some studies suggested that cathepsin G found in the primary granules of neutrophils , is also on the surface of neutrophils (Maison *et al*., 1991; Owen *et al*., 1995; Owen and Campbell 1998).

Owen *et al*., 1995 found minimal expression of serine proteases (Cat G and elastase) on unstimulated neutrophil which was increased remarkably 30-fold in response to PMA stimulation. More relevant biologic stimulants had less ability to induce membrane expression of these proteases, with fMLP causing a modest two fold increase in their expression. LPS had mainly a priming effect as it induced minimal upregulation when used alone but when followed by fMLP induced more expression of Cat G and elastase on neutrophil surface. They concluded that active neutrophils express persistently active serine proteinases on their cell surfaces that are accessible to both natural and synthetic substrates (Owen *et al*., 1995).

The important immunological role of neutrophil elastase and Cat G was demonstrated in several studies. Mice deficient in elastase and /or Cat G were susceptible to fungal infections despite normal neutrophil development and recuritment (Tkalcevic *et al*., 2000). Neutrophil elastase deficient mice have a defect in their ability to clear Gram negative, but not Gram-positive organisms in some studies (Belauauaj *et al*., 1998; Hirche *et al.*, 2008), but recently elastase and Cat G were shown to be equally important in eradication of Gram-positive bacteria. Evidence from a study on Cat G knock out and Cat G/elastase double knock out mice showed these mice had a reduced ability to clear *S pneumoniae* serotype 19 pneumonia (Hahn *et al*.,2011). Similarly, Cat G -/- mice were increaseingly susceptible to staphylococcus infections (Reeves *et al*., 2002). In contrast, one study suggested that Cat G deficieny could be compensated by other proteases, as they found that Cat G -/- animals were phenotypically normal when challenged with Gram-negative and Gram-positive bacteria (Maclover *et al*., 1999).

High levels of proinflammatory cytockines were found in wound fluid taken from Cat G deficient mice suggesting that Cat G may play a role in inactivating proinflammatory molecules at the site of wound injury. The abilty of Cat G to cleave and inactivate cytokines IL-1, TNF, and IL-8 could accelerate wound healing and reduce neutrophils influx to the site of inflammation. Therefore, this could also be the reason for delayed wound healing in mice deficient in Cat G (Maclover *et al*., 1999).

Known functions of Cat G are diverse including antimicrobial activity, elastase-like non-specific degradation of extracellular matrix components such as proteoglycan, collagen, laminin, fibronectin and even elastin (Baggiolini *et al*., 1979) and regulation of inflammatory

responses through degradation of complement components and immunoglobulins (Campbell *et al*., 2000). In addition Cat G has been implicated in control of blood pressure by converting angiotensinogen and angiotensin I to angiotensin II (Tonnesen *et al*., 1982; Ramahas and Pateston 2002). There is intriguing evidence that Cat G plays a direct role in neutrophil accumulation at sites of inflammation. Since inhibitors of Cat G diminish neutrophil migration *in vitro* (Lomas *et al*., 1995). Cat G, as well as elastase, can also cleave CD43, whereas proteinase 3 has no CD43 cleaving activity (Remold-ódonnell and Parent, 1995). Cat G also cleaves CD14, a receptor for LPS, and reduces its cell surface expression (Le-Barillec *et al*., 2000).

3.4 Summary

In this chapter I have presented flow cytometry data on different markers and enzymes of neutrophils obtained from cord and adult blood. Detailed flow cytometry analysis of cord blood neutrophils had not been previously published specifically for proteinase 3 and the function activity of Cat G. I have demonstrated that neutrophils obtained from cord blood express more PR3 and CD177 on the cell surface than neutrophils obtained from adult volunteers, but the amounts of surface CD63, CD66b, CD35 and CD43 expression were not significantly different. I have additionally shown that cord blood neutrophils contain lower Cat G and elastase functional activity than adult neutrophils. A similar percentage of neutrophil Cat G is released from cord and adult neutrophils with different pro-inflammatory stimulations.

Chapter Four

Neutrophil proteases in the lungs of infants at risk of developing Chronic Lung Disease of Prematurity

4.1 Introduction

In the preceding chapter, I demonstrated that neutrophils from healthy full term cord blood contain more PR3 than adult neutrophils. Neutrophil proteases are implicated in pathogenesis of chronic lung disease (CLD) in premature infant but PR3 was not previously studied in this context. This chapter will therefore focus on the role of proteinase 3 in the development of CLD of premature infants.

In recent years, we have seen substantial improvement in the treatment and thereby survival of infants born prematurely. Consequently, prevalence of long-term complications has increased in those infants who are now surviving extreme preterm birth (Horbar *et al*., 2002).

Neutrophil proteinases have a pivotal role in the multifactorial and complex pathogenesis of CLD. Imbalance between neutrophil proteinases and their inhibitors is of crucial importance to the disturbance of lung development seen in CLD. The best described agent of proteolytic lung injury was neutrophil elastase although more recent studies suggest that in the new CLD elastase is of reduced importance. Similar to elastase, PR3 have comparable proteolytic activity which is considered one of the complex pathogenic factors resulting in lung injury. PR3 role in pathophysiology of other lung diseases including cystic fibrosis and emphysema has been studied before suggesting its involvement in these diseases. Hence PR3 was implicated in proteinase- mediated airway damage and thereby in pathophysiology of airway inflammatory disorders.

In this chapter I have measured neutrophil PR3 in bronchoalveolar lavage fluid samples obtained from infants at risk of developing CLD. The results were compared with other proteinases levels in the same samples to see whether a relationship could be found between the level of PR3 and MMP-9 or elastase in BAL fluid.

4.2 Results

4.2.1 Patient characteristics

Three groups of mechanically ventilated infants were studied (table 4.1, adapted from Davies *et al*., 2010); premature infants (<32 weeks gestation) who developed CLD (group 1, 20 newborns), preterm infants who developed and recovered from neonatal respiratory distress syndrome (group 2, 17 newborns) and a term control group included 6 babies who were ventilated for non-respiratory reasons. Control group neonates were born with gastroschisis and underwent primary closure of the abdominal wall defect during the first few hours of life and in many cases were ventilated for a number of days following surgery. Infants were previously recruited for BAL study by Dr Davies from neonatal unit at the University Hospital of Wales and were investigated for elastase activity and MMP-9 levels and their inhibitors.

Table 4.1 Patient characteristics

	Chronic Lung Disease	Resolved RDS	Term control
Number of Patients	20	17	6
Number of Samples	80	34	21
Gestational age (weeks)	27 (25^{+4}-29^{+3})	28^{+6} (27-29^{+2})	Term
Birth Weight (grams)	835g	1120g	2550g
Male: Female	11:9	10:7	3:3
Vaginal :Caesarean Delivery	10:10	6:11	5:1
Antenatal steroids>24 hours	16/20 (80%)	12/17 (71%)	0/6 (0%)
Patent Ductus Arteriosus	13/20 (65%)	6/17 (35%)	0/6 (0%)
Surfactant therapy	20/20 (100%)	17/17 (100%)	0/6 (0%)
Prolonged rupture of Membrane(>24 hours)	4/20 (20%)	1/17 (6%)	0/6
Length of ventilation (days)	7 day (5.5-11 day)	1 day (1-4.5 day)	3 day

4.2.2 Neutrophil PR3 concentration in BAL supernatant

Sufficient number of BAL samples were available to study PR3 concentrations released by neutrophils in BAL fluid. Human PR3 belongs to the same serine protease family as elastase and Cat G. PR3 is found in primary and secondary granules, and secretory vesicles. Due to the expression of the high affinity receptor (see previous chapter), PR3 can also be expressed on the surface of CD177-positive neutrophils from where it can be released into surrounding environment upon neutrophil activation (Csernok *et al*., 1990; Halbwachs-Mecarelli *et al*., 1995; Witko-Sarsat *et al*., 1999).

PR3 ELISA was performed on BAL fluid of CLD infants, resolved RDS and term controls. PR3 was mesurable in all BAL samples, but the peak concentration of PR3 was significantly greater in infants who developed CLD, with median of 6049 ng/ml compared to preterm infants with resolved RDS or term controls (3440, 740.2 ng/ml respectively) ($p = 0.0096$, $p = 0.0076$, respectively by Mann-Whitney U test) as demonstrated in figure 4.1. There was no significant difference in peak PR3 concentration between preterm infants whose RDS resolved and term infants.

When longitudinal data for each individual infants was analysed during different ages for samples taken, the episodic spikes for increased PR3 concentrations can be appreciated. The timing of PR3 spikes was extremely variable, with the initial PR3 spike being after the second day of age as shown in figure 4.2,4.3 and 4.4 for each sesperate groups.

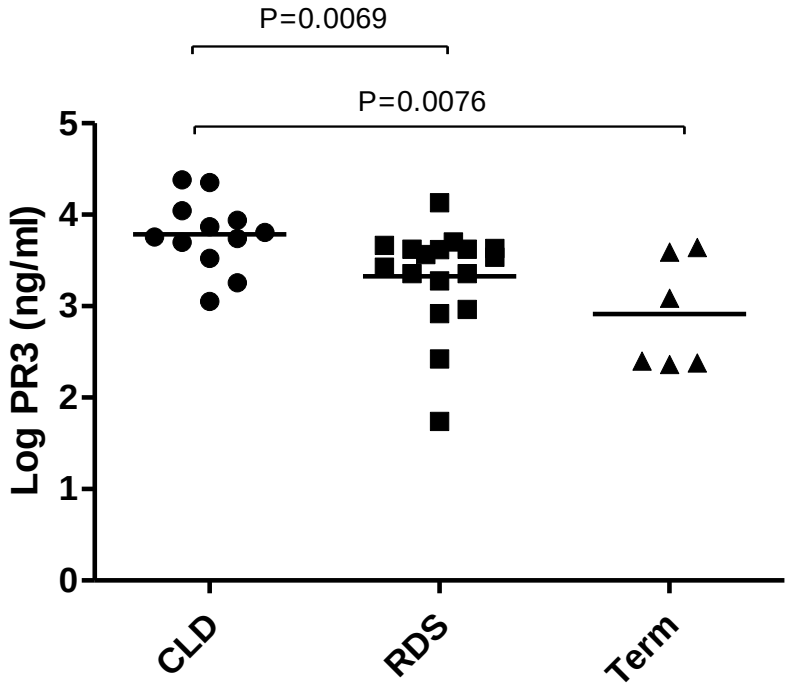
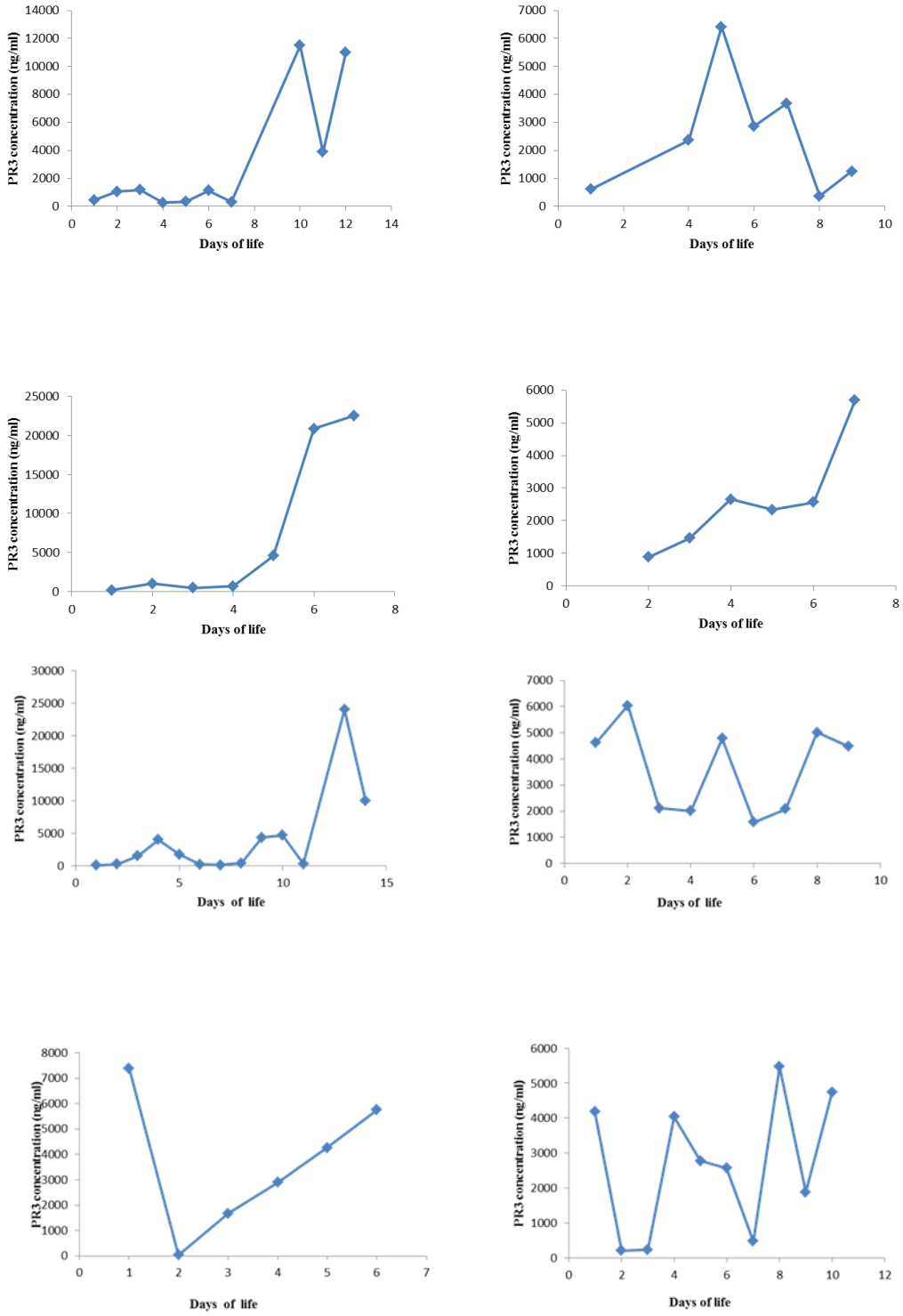


Figure 4.1 A scatterplot of the peak PR3 concentration for infants with CLD, resolved RDS and term (control) infants, demonstrating significantly higher PR3 peak level in infants developing CLD. Median values are marked with a horizontal line and significant differences are shown.

Figure 4.2 Longitudinal data showing PR3 concentrations for CLD babies, each graph represents an individual infant (only babies with more than 3 BAL samples are shown).



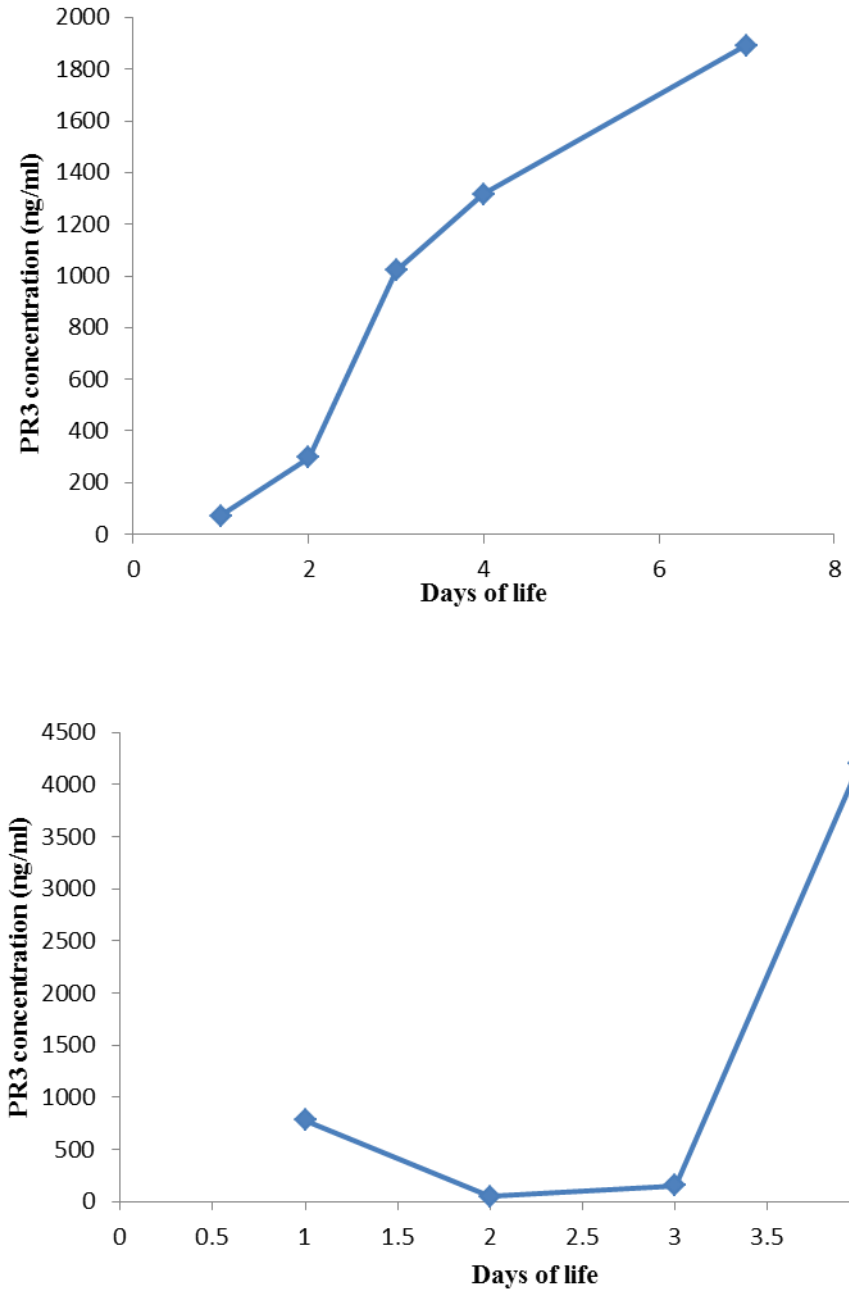
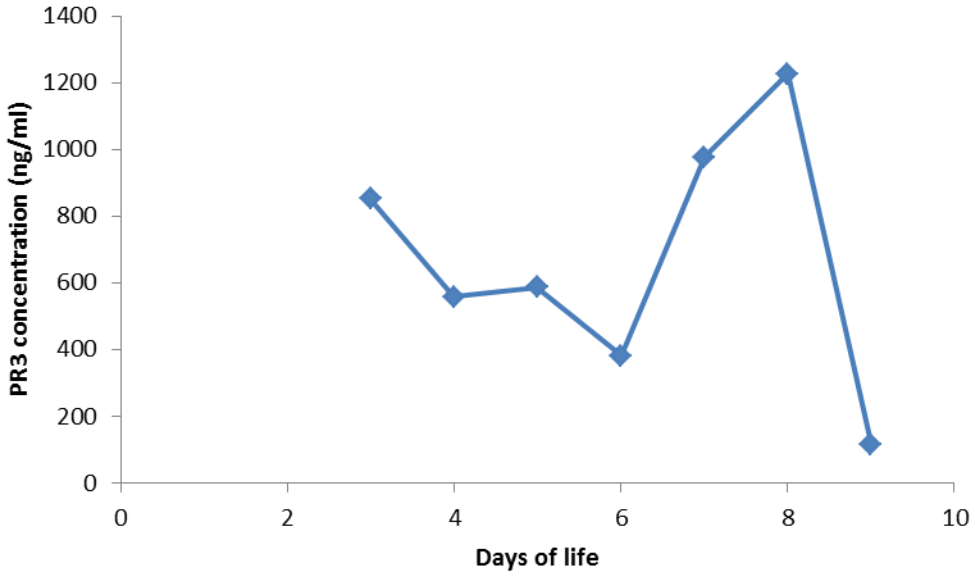


Figure 4.3 Graphs of longitudinal PR3 concentrations in resolved RDS babies, each graph represent an individual infant (only babies with more than 3 BAL samples are represented).



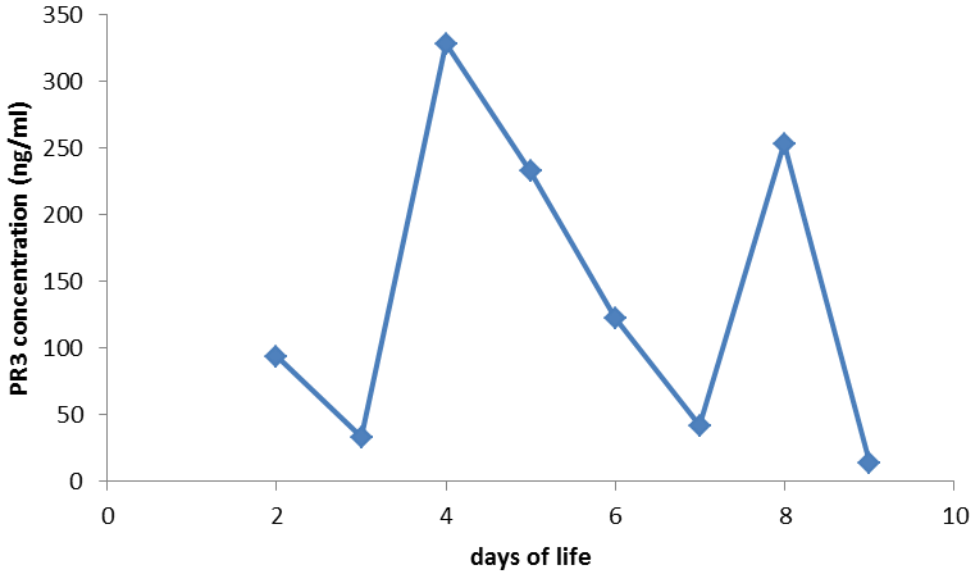


Figure 4.4 Graphs showing PR3 concentration in two separate term BAL samples. Each graph represents an individual infant (only babies with more than 3 BAL samples are represented).

4. 2.3 MMP-9, PR3 and Elastase in BAL samples

The concentration of MMP-9 and elastase activity in the same CLD, RDS and term BAL samples has been previously studied in our laboratory by Dr Davies (Davies *et al*., 2010). Herein, I compare the previous results for MMP-9 and elastase activity with PR3 concentration on the same selected samples for CLD, RDS and term control infants.

The relationships between the peak PR3 concentrations and corresponding peaks in MMP-9 concentrations, combined with measured elastase activity are shown in figures 4.5, 4.6 and 4.7 for study arms, CLD, resolved RDS and term control, respectively. These figures demonstrate the episodic nature of spikes for both MMP-9 and PR3 concentrations and also show the relationship between their peak variability in some samples. In general, it was unusual to observe elevation of both PR3 and MMP-9 concentrations or presence of elastase activity in the first two days of life. Comparison of proteases levels showed different patterns; occasionally PR3 closely mirrored the levels of MMP-9 (CLD patients number 8, 17, 19, 22 , 34 and RDS patient 11) while other times the PR3 levels appeared to change independently of MMP-9 levels (CLD patients 10, 39, 46, RDS patients 25 and term infants 35 and 37).

What is clear is that despite being considered a predominantly azurophilic granule protease, PR3 levels were totally independent of elastase levels. Furthermore, the amount of PR3 in the BAL samples far exceeded the levels of any other protease examined for these patients.

Figure 4. 5. Graphs of PR3/MMP9 concentrations over first days of life

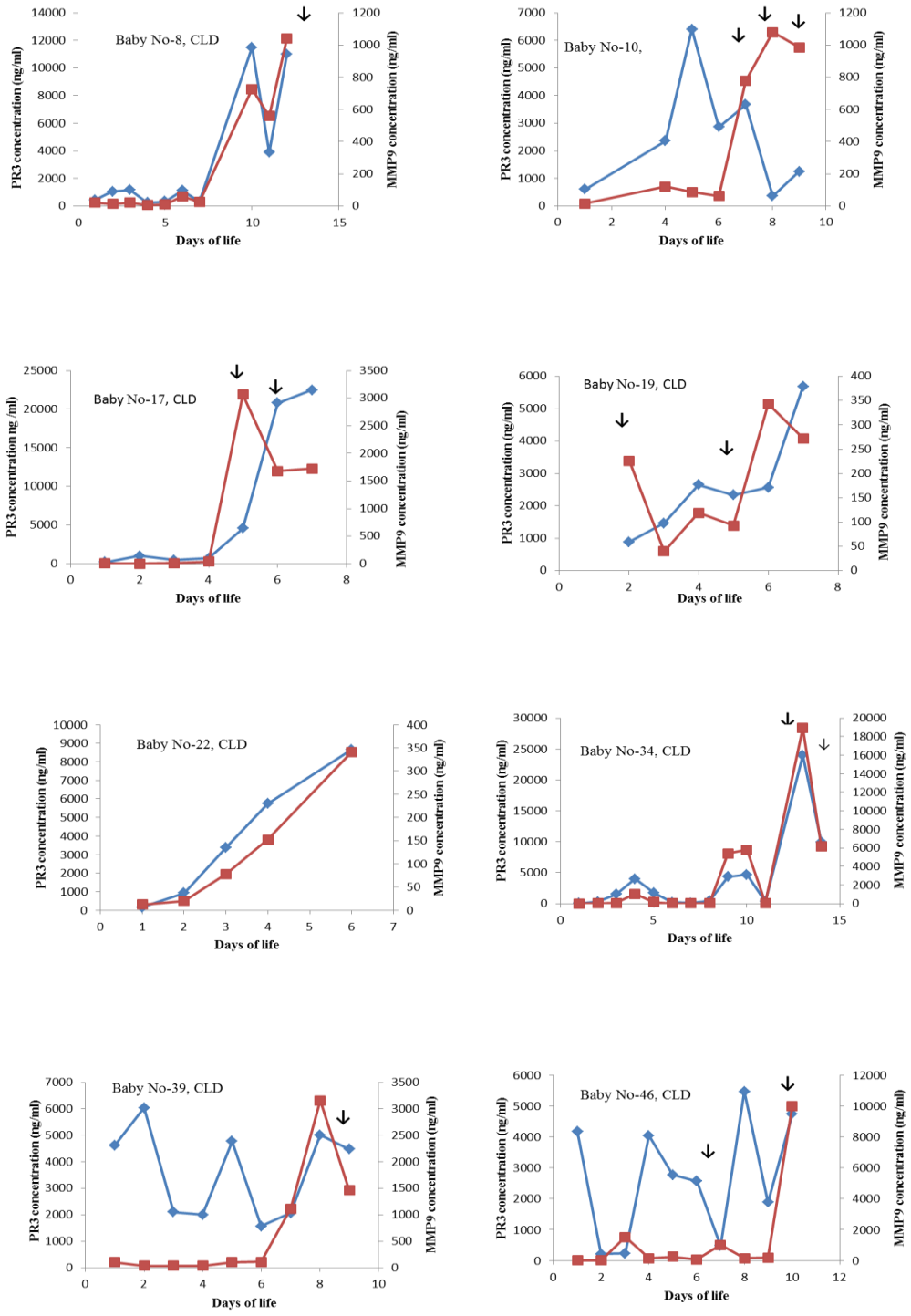


Figure 4.5 Graphs show longitudinal data PR3 (blue line) and MMP-9 (red line) concentrations for CLD babies. Each graph represents the same baby over specific period. Vertical arrows represent samples in which elastase activity was detectable.

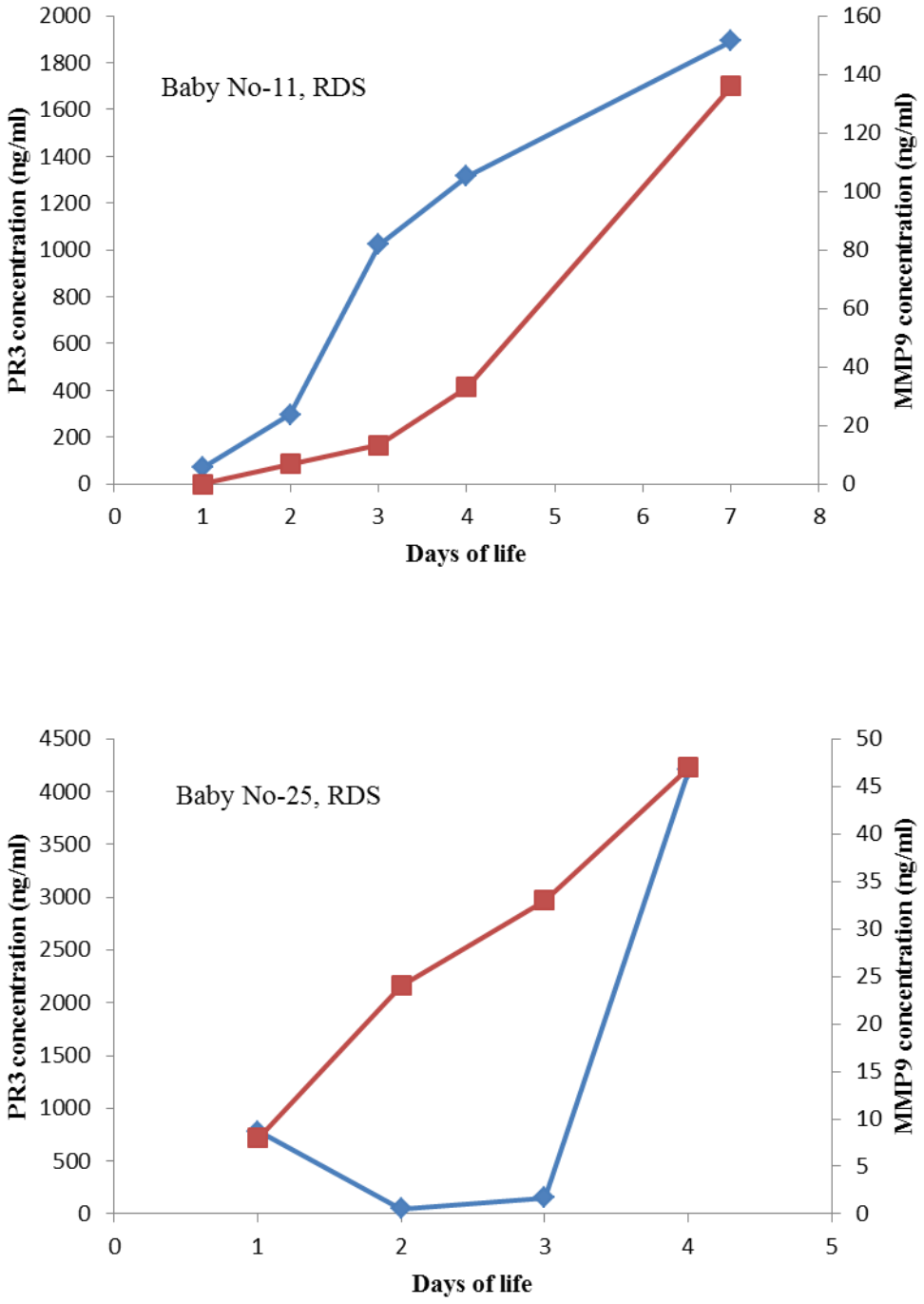


Figure 4.6 Graphs showing relationship between PR3 concentration (blue line) and MMP-9 concentration (red line) for individual RDS babies. No elastase activity was detectable in any patient in this group.

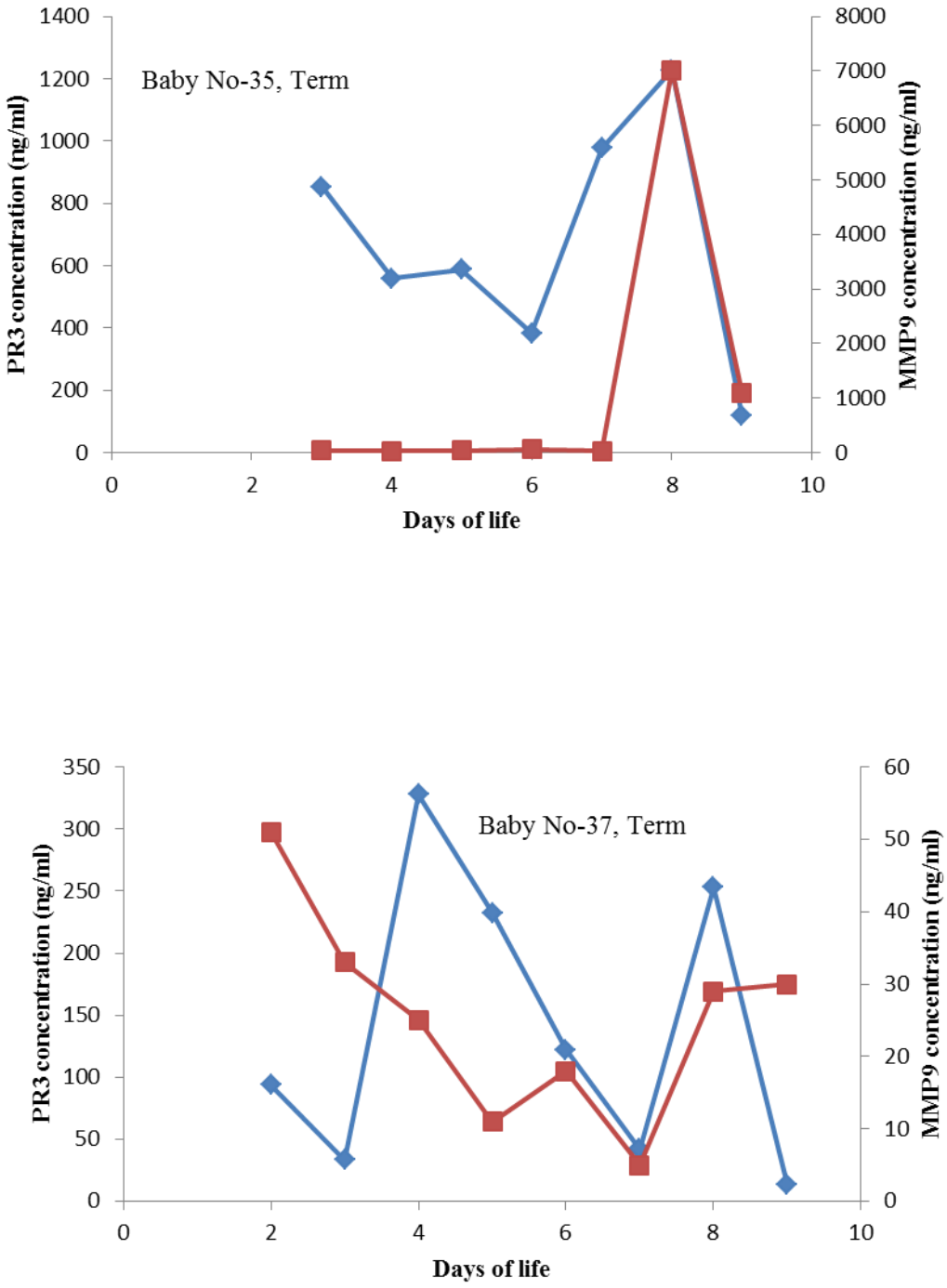


Figure 4.7 Graphs show longitudinal data PR3 (blue line) and MMP-9 (red line) concentrations for term (control) infants. No elastase activity was detectable in any patient in this group.

4.2.4 Peak concentration of PR3, MMP-9 and Elastase

Figure 4.8 (A) demonstrates the peak concentrations of PR3 and MMP-9 in the BAL fluid samples from the same CLD infants. The peak levels of PR3 concentrations were significantly higher than the corresponding peak MMP-9 concentration on the same CLD newborns (median PR3, 196.2 μM; MMP-9 4.16 μM, $p = 0.001$). As shown in figure 4.8 (A), Elastase activity was low and only detectable in a small number of BAL CLD samples (in at least one BAL fluid samples collected from10/20 CLD newborns).

In addition, significant difference was also observed between the peaks of both proteases in BAL samples from resolved RDS babies as demonstrated in figure 4.9 (A) (median PR3 147.7 μM; MMP-9 of 1.4 μM, $p < 0.0001$). No elastase activity was reported by Davies *et al* for any of the samples that I was able to co-investigate in this same sub-set of RDS BAL fluid samples examined here , but overall, elastase activity was demonstrated at some point in only 2/17 resolved RDS newborns in the original sample series.

Elastase activity was not demonstrated in serial BAL fluid samples obtained from term control group whereas significant difference was detectable between peak PR3 and MMP-9 level, figure 4.10 (A) (median PR3 44.8 μM vs MMP-9 of 1.2 μM, $p = 0.02$).

4.2.5 Correlation between proteases levels

Irrespective of the longitudinal relationship of the sample collection, each sample was analysed for a correlation between PR3 and MMP-9 levels to determine if there was a relationship between the levels. A significant correlation was found in CLD samples between PR3 and MMP-9 (figure 4.8B) with a Spearman correlation co-efficient of 0.65, $p<0.0001$.

A similar positive correlation was also shown between PR3 and MMP-9 concentrations taken from infants with resolved RDS (r =0.42, p =0.01) (figure 4.9 B). As shown in figure 4.10 B, no correlation was found between the two proteases in the term control samples (r =0.3, p = 0.1).

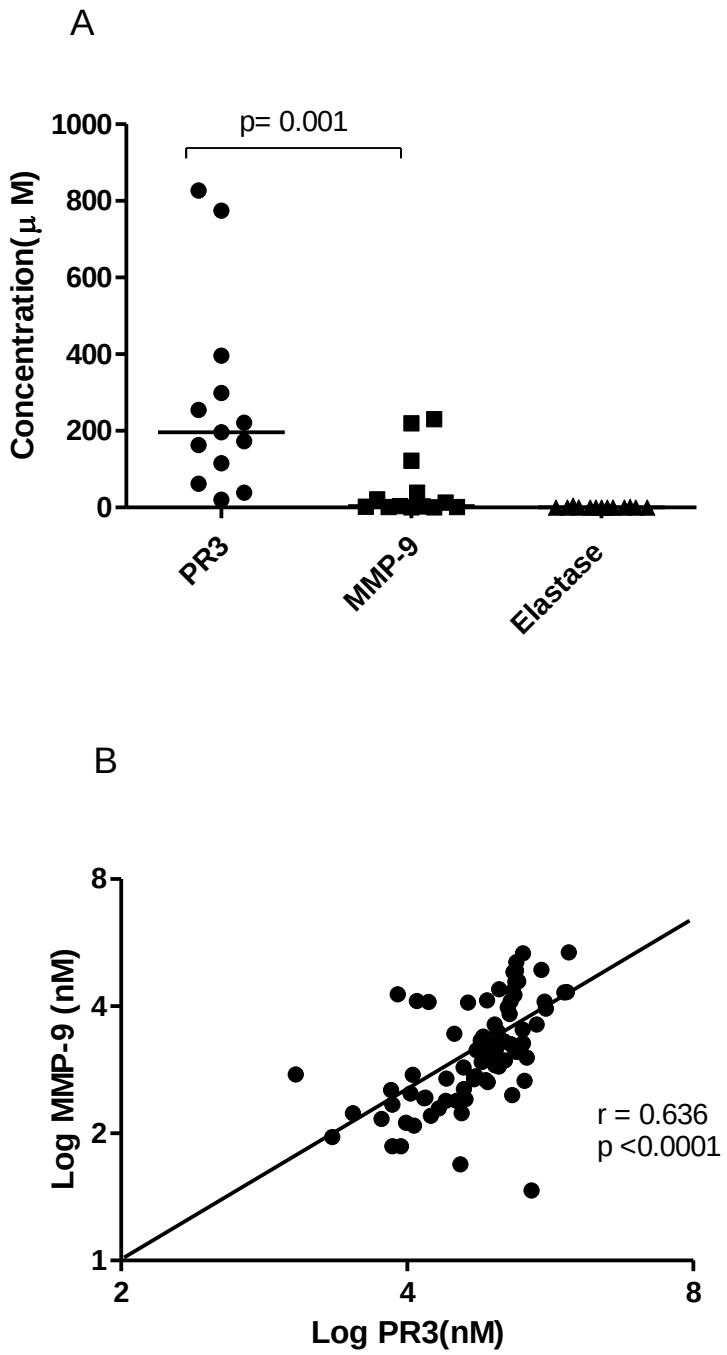


Figure 4.8 figures demonstrate the relationship between PR3, MMP-9 and Elastase. A) Scatter plot of peak concentrations of PR3, MMP-9 and elastase in CLD infants, horizontal lines indicate median value and significant difference is shown. B) The figure shows the correlation between log MMP-9 and log PR3 in CLD BAL fluid.

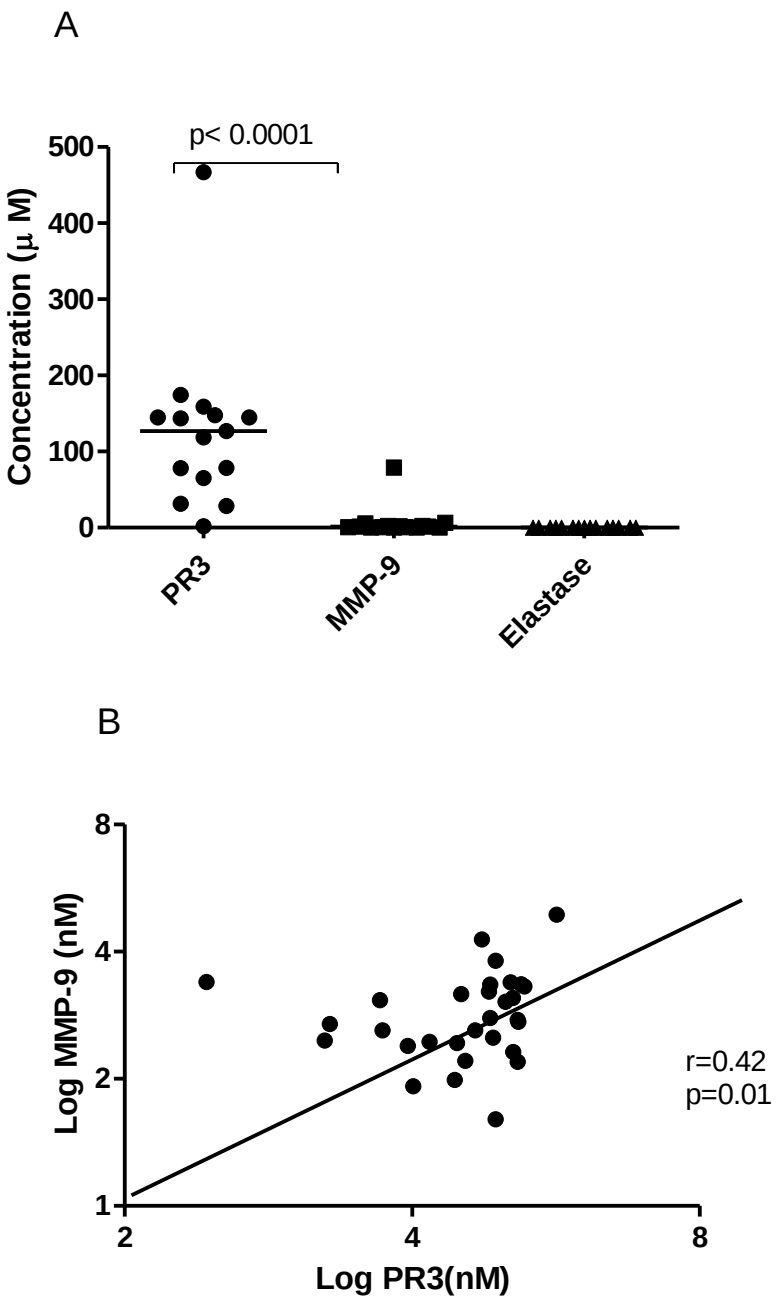


Figure 4.9 figures demonstrate the relationship between PR3, MMP-9 and Elastase. A) Scatterplot of peak concentrations of PR3, MMP-9 and elastase in RDS infants, horizontal lines indicate median value and significant difference is shown. B) The figure shows the correlation between log MMP-9 and log PR3 in RDS BAL fluid samples.

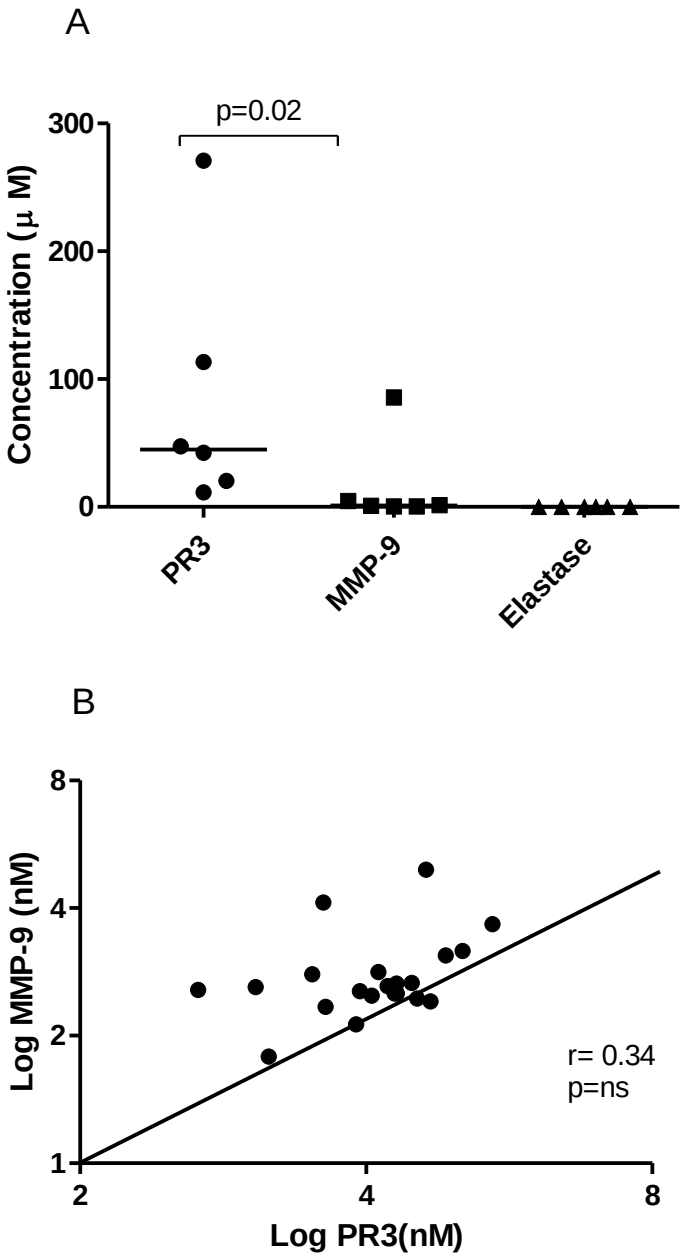


Figure 4.10 figures demonstrate the relationship between PR3, MMP-9 and Elastase. A) Scatterplot of peak concentrations of PR3, MMP-9 and elastase in term infants, horizontal lines indicate median value and significant difference is shown. B) The figure demonstrates the relationship between log MMP-9 and log PR3 in term BAL fluid (ns=not significant).

4.3 Discussion

This study is a continuation to previously published work from our laboratory which investigated the relationship between proteases and their inhibitors in ventilated neonates and provided detailed analysis of the protease-inhibitor balance in infants at risk of developing CLD (Davies *et al*., 2010).

The study group included 36 newborns all of them have gestational age less than 32 weeks. The median gestational age of infants who develop CLD was 27 weeks compared to 28^{+6} weeks for infants whose RDS resolved. The study included six full term infant as controls. All preterm born neonates included in the study were intubated for RDS received exogenous surfactant therapy and most of them had antenatal corticosteroid given 24 hours before delivery. Ventilation setting was adjusted to deliver low pressure, low volume ventilation according to the neonatal unit protocol and all infants born with respiratory distress received postnatal antibiotic until blood cultural were shown to be negative. On the other hand, term infants were ventilated post-operatively after surgical repair for gastroschisis and were selected for control group rather than term newborns ventilated for respiratory infection or birth asphyxia in order to avoid the confounding effect of infection or hypoxia on physiological lung function. No specific lung abnormality is linked to gastroschisis and at birth the lung should be healthy.

Many studies that have examined lung lavage samples for protease analysis on infants have used tracheal aspirate fluid (TAF); however, this largely reflects upper airway secretions. By using bronchoalveolar lavage fluid this study analyses proteolytic changes from the lower airways, which is particularly important in new CLD where large oversimplified alveoli are seen.

No previous study has examined the concentration of PR3 in neonatal lung lavage and its role in the development of CLD has not been studied before. In this chapter I have measured PR3 concentration in CLD, resolved RDS and term control infants although I could not run PR3 ELISA in all full set of the previous study samples because some of them have been fully used for previous investigations by other students. The detection of PR3 level in almost all samples in the three study groups is different from episodic pattern of elastase activity results reported from the same sample set (Davies *et al*., 2010).

Davies *et al* measured elastase activity in CLD, resolved RDS and in control term. Neutrophil elastase activity was detected only in 18% of total CLD BAL fluid samples (23/129), compared to 7% of the total BAL fluid samples from RDS groups (3/46) whereas no elastase activity was detected in the 23 samples obtained from control term infants. Furthermore, elastase activity was episodic in nature and was present only in some of the samples from each infant. The timing of the activity spikes was also extremely variable, with the initial spike of elastase activity seen at a median of 7 days after birth. They demonstrated that the rise of elastase activity was positively correlated with the increase in BAL fluid neutrophils counts. Alpha 1- anti-trypsin- elastase (AAT-E) complex was detected in 90% of CLD samples compared with 47% of RDS group. This may explain high PR3 concentration in BAL in the CLD and RDS groups in my study since α 1 anti-trypsin (AAT) is an inhibitor of elastase as well as PR3 but the affinity of elastase to bind α 1 anti-trypsin is higher than its affinity to PR3 (Korkmaz *et al*., 2005).

MMP-9 protein peak concentrations were significantly higher in CLD group than in RDS infants. The same episodic pattern for elastase activity was demonstrated in MMP-9 concentration and its rising pattern started after the second day.

The results of this chapter demonstrated that infants developing CLD have a significantly greater peak of PR3 concentration than infants with resolved RDS and term control. Longitudinal PR3 concentration in BAL samples of majority of the newborns closely parallels the pattern of MMP-9 concentration levels in the same infants i.e. the episodic release of these two proteases coincides in many of the BAL samples. No significant difference in PR3 concentration was detectable between RDS and term neonates.

In this study I provide evidence that neutrophil PR3 is present in significant levels in bronchial lavage aspirates and its presence seemingly reflect a role in pathogenesis of CLD in neonates.

PR3 is one of the three main neutrophil serine proteases found in neutrophil azurophilic granules along with cathepsin G and elastase. They share some functions, for example, their ability to catalyse the cleavage of proteins into smaller peptides or amino acids. Kao *et al*., 1988 demonstrated that, similar to elastase, PR3 could induce experimental emphysema, when instilled in hamsters probably due to its ability to degrade elastin and matrix structural proteins. Serine proteases can also contribute to prolongation of inflammation through their activation and inactivation of zymogens, protease inhibitors, cytokines, growth factors and cell surface receptors (Owen and Campbell 1999; Witko-Sarsat *et al*., 2000; Wiedo *et al*., 2005; Pham, 2008).

Enzymatic activity of released proteases in lung is tightly regulated by anti-protease inhibitors that include AAT and elafin. A disturbance in the balance between serine proteases and these inhibitors is thought to play a role in neutrophil dominated lung inflammation. Protease-inhibitor imbalance is due to excessive neutrophil degranulation and subsequent release of proteases. The relation between elastase and its inhibitors, AAT and serpin was studied before on these samples and AAT-elastase complex was detected in 90% of CLD

samples compared to 47% of the RDS group. Davis *et al*., 2010 also found that the peak percentage of AAT present as complex is greater in the CLD group suggesting that even though elastase activity may be controlled it is still ongoing and would support the hypothesis that at the pericellular level elastase released lung injury could be occurring. Elastase is inhibited more readily with AAT due to this inhibitor higher affinity to bind elastase than PR3 which may explain the high level of PR3 concentration in BAL, relative to elastase. It is not clear whether PR3 was free or bound to inhibitors, since I did not study the relationship between PR3 and any of its inhibitors because of inadequate volume of the samples. However the relationship between PR3, elastase and elafin has been studied before and it was demonstrated that the efficiency of PR3 inhibition by elafin is much lower than that by other inhibitors of PR3 and elafin concentration is lower than AAT in the lung. Moreover, AAT behaves as an irreversible inhibitor of PR3, whereas the PR3-elafin complex is transient and dissociable, such that it could progressively be transferred to ATT (Ying and Simon 2001). One important observation is that, secretory leukoprotease inhibitor (SLPI) a small protein in the lungs can inhibit elastase and Cat G, but not PR3 (Rao *et al*., 1993). SLPI is present in epithelial lining fluid of respiratory tract at molar concentrations almost half of that of elastase but two thirds of SLPI recovered is non-functional. It plays an insignificant role in protecting the normal respiratory epithelium against elastase except in upper airways where SLPI accounts for 80 to 90 % of elastase inhibitory activity (Vogelmeier *et al*., 1991). Interestingly PR3 can selectively degrade oxidised and native SLPI (Rao *et al*., 1993).

PR3 has some biochemical features that are not shared by elastase; in particular its plasma membrane localisation (Halbwach-Mecarelli *et al*., 1995).The hydrophobicity of PR3 and its subsequent membrane association may play a role in the resistance to proteolysis in bronchial

lavage fluid. Moreover, other leukocytes in addition to neutrophils, particularly monocytes may release PR3 adding another source to the high concentrations of PR3 especially in CLD and RDS groups (Witko-Sarsat *et al*., 1999).

Only one study highlighted the role of PR3 in lung inflammation, but this study was in cystic fibrosis patients of an older age group. Witko-Sarsat and colleagues, measured elastase and PR3 in sputum of CF patients using ELISA and demonstrated that PR3 levels were higher than elastase concentration in CF whereas PR3 concentration was found to be lower than elastase in non CF bronchiolitis. PR3 contained in CF sputum is enzymatically active and the evidence of PR3 activity is supported by demonstrating resistance to inhibition by SLPI. On the other hand, lysates from purified blood neutrophils isolated from control or from CF patients contained approximately five times less PR3 than elastase.

Cord blood neutrophils in my study revealed more PR3 in neonates than in adult neutrophils although Witko-Sarsat *et al*. reported that neutrophil lysates or purified azurophil granules contained five times more elastase. This suggests that PR3 released not only from primary granules but also from vesicles as well as from specific granules which are easily mobilised.

The role of matrix metalloproteinases in the development of CLD remains unclear, MMP-9 is found in the gelatinase granules from where it is release at a lower stimulation threshold than elastase from primary granules upon neutrophil activation (Lominadze *et al*., 2005). Several studies investigated MMP-9 and it is role in CLD, but they give conflicting results. Most studies have demonstrated no difference in MMP-9 concentrations between infants who developed CLD and infants who have RDS (Cederqvist *et al*., 2001; Danan *et al*., 2002;

Ekekezie *et al*., 2004; Sweet *et al*., 2004). Dik *et al*. reported that MMP-9 concentrations were actually reduced in CLD infants compared to infants whose RDS resolved (Dik *et al*., 2006). Tambunting *et al*. studied the role of MMP-9 in the development of CLD in baboon model and demonstrated that the CLD animals had high concentrations of MMP-9 compared to controls (Tambunting *et al*., 2005). In this project I found that PR3 concentrations in BAL fluid from CLD group had a strong and highly significant correlation with the concentrations of MMP-9 measured on the same samples (r 0.65 and $p = <0.0001$).

In general, levels were found to start from relatively baseline, then to have sudden episodic spikes at variable time points after birth, but frequently occurred late following prolonged period of intubation. The episodic spikes for the various factors tended to be coincident and I hypothesised that infection was the most likely explanation. Infection has been linked previously with the development of CLD (Davies *et al*., 2006) and may be also an important factor in the release of neutrophil proteases.

Proteinase 3 may lead to lung injury with other proteases including MMP-9 and elastase. Better understanding of the complex interaction between proteases and their inhibitors would facilitate effective prevention of proteolytic lung injury. Given the importance of infection in causing the episodic proteases spikes, appropriate approach to reduce CLD would be treatment of chest infection, good infection control and surveillance.

4.4 Summary

In this chapter I have found that the peak level of PR3 in BAL fluid obtained from CLD infants was significantly higher than that in infants who had RDS and term control babies. PR3 concentration showed episodic spikes and displayed high variability between individuals. Similar findings had been demonstrated for MMP-9 concentrations. Significant correlation was demonstrated between longitudinal data of MMP-9 and PR3 levels in CLD infants and also in infants who had resolved RDS, which is in contrast to the expected relationship between elastase and PR3 being contained in the same granule subset (primary).

In conclusion, my data suggest that PR3 could play a major role in proteinase-mediated airway damage and in the pathogenesis of lung diseases in neonates.

Chapter Five

Terminal complement components concentration and complement functional activity in cord and adult sera

5.1 Introduction

In the previous two chapters, the focus was on the neutrophil as a one of the cellular component of the innate immune system. This results chapter will therefore focus on the quantitative and functional aspects of complement system as an example of humoral arm of innate immunity that have important role in eradication of pathogens.

The complement system is a key player in innate immunity and its role in combating invasive pathogens is more prominent in newborns owing to their immature adaptive immunity. Hereditary deficiency of terminal complement components has been described in many ethnicities and studies have shown that affected individuals are prone to infections, particularly from Neisseria meningitides (Figueroa and Densen 1991; Pettigrew *et al.*, 2009). Moreover, the virulence of many Gram-negative and -positive bacteria was attributed to their ability to evade complement utilising different strategies (Pausa *et al*., 2003; Silverman *et al*., 2005; Rooijakker and Van Strijp 2007; Zipfel *et al*., 2007; Schneider *et al*., 2009). Newborn serum has low opsonic activity and C9 deficiency which limits the capacity to form MAC and hence may predispose to severe invasive infections (Høgåsen *et al.*, 2000).

Complement studies in neonates have focused on quantitative assays and also on functional abilities of term and preterm newborn serum, mainly measured as erythrocyte haemolytic activity and bacteriocidal effect on epidemiologically important Gram positive and negative pathogens. Invasive *Ureaplasma* infections primarily occur in preterm neonates with an immature immune system or in immune compromised patients such as those who are hypogammaglobulinaemic or receiving immunosuppressive therapy. This suggests an essential role for a healthy immune system to fight off U*reaplasma* infection. However,

nothing is known about the interaction between newborn complement system and *Ureaplasma parvum.*

Methods used for quantitative assay of complement components include immunoelectrophoresis, electroimmunediffusion, radial immunodiffusion, and immunonephlometry, but sensitivity and specificity of these methods are lower than ELISA (Høgåsen *et al*., 2000). Complement measurement varies with method and with genetic differences between populations (Sonntag *et al*., 1998b). Variation in precision and relative accuracy between these methods has been described but is not fully explained. For instance, the areas of precipitation in radial immunediffusion are ill- defined in occasions (Adinolfi and Beck 1975). Results may also depend on anti-serum specificity and source (Buffone and Lewis 1977).

The aim of this work was to optimise sandwich ELISA methods using the most sensitive antibodies from commercial and non-commercial sources to accurately measure terminal complement components in cord and adult serum. Amino acid hydrolysis was used to accurately quantify highly purified C6, C7, C8 and C9. I also compared the haemolytic activity of cord and adult serum and ability to kill serovars of U *parvum* before and after correcting low complement C6-C9 concentrations in cord serum.

5.2 Results

5.2.1 Comparison of different monoclonal capture antibodies for purified C6 and native serum C6

In order to optimise our C6 ELISA, Hycult, Quidel and non-commercial monoclonal antibodies from Prof. B. Paul Morgan's (BPM) laboratory, Cardiff University were each diluted in coating buffer and used as capture antibodies to coat Nunc Maxisorb 96 well plates. Hycult antibody binding was more efficient in terms of capturing purified Quidel C6 as well as C6 in human serum than Quidel anti-C6 antibody. The non-commercial BPM has the lowest ability among antibodies to capture C6, particularly in serum (Figure 5.1 A and B). Subsequently, experiments were carried out to identify the best working concentration of the most sensitive anti-C6 (Hycult) antibody (Figure 5.1 C). The ELISA plate was coated with different concentration of Hycult antibody and 4μg/ml was chosen as the optimal working concentration of the most sensitive antibody to measure C6 level in cord and adult serum as well as in pooled adult sera.

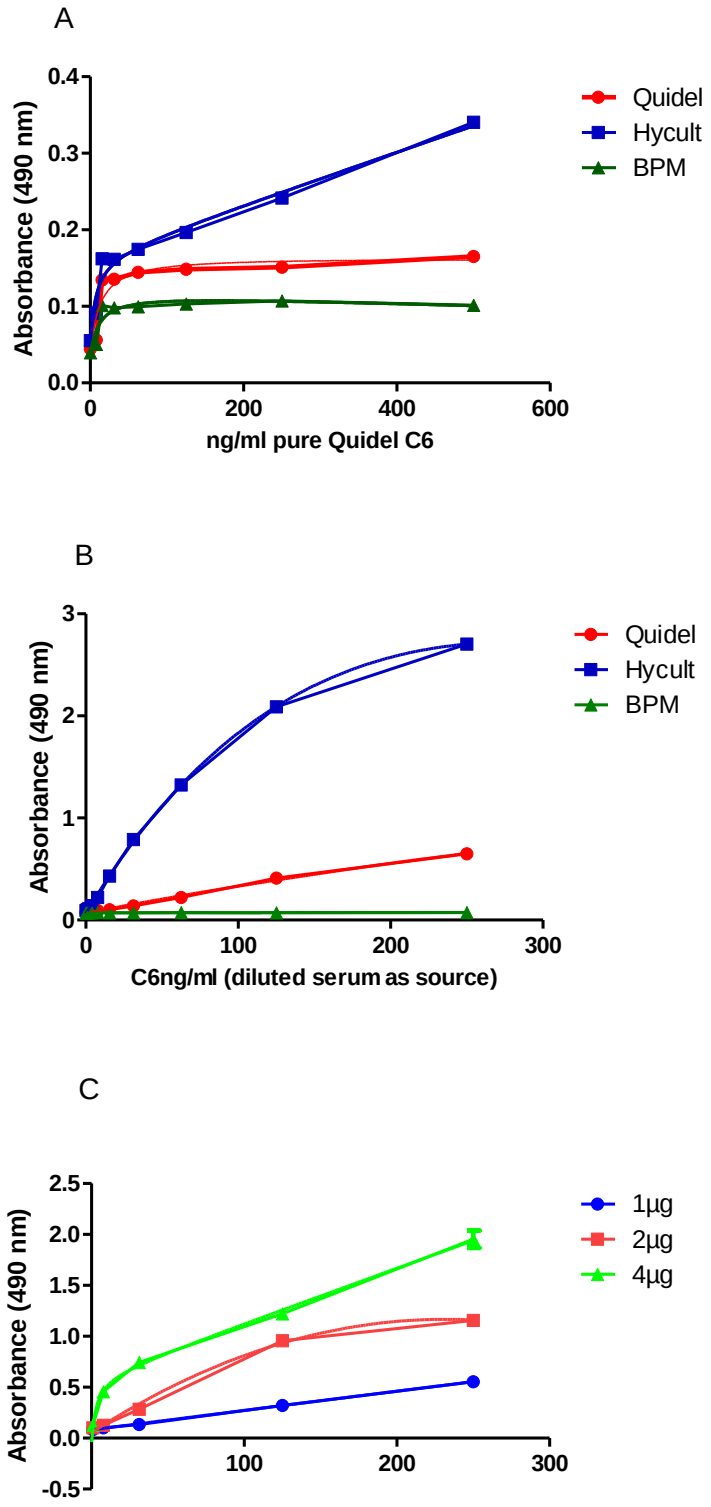


Figure 5.1 ELISA detection using different monoclonal antibodies (mAbs) to capture C6.Tested capture antibodies were Hycult Biotechnology, Quidel and non- commercial BPM. A) Capture purified C6; B) capture native C6 in serum and C) determination of the optimum working concentration of most sensitive (Hycult) antibody for coating of wells.

5.2.2 Complement component C7 ELISA

Choosing the suitable capture antibody for C7 was an essential point during our assay development, therefore the suitability of different antibodies for capturing C7 was tested. The sensitivity of Hycult anti-C7 antibody to capture purified C7 and native C7 was more than the sensitivity obtained with anti-C7 Quidel antibody. Non-commercial BPM anti-C7 antibody was the least sensitive antibody (figure 5.2 A and B). Polymorphism has been reported in C7 which is a central protein of terminal components cascades, and "N" and "M" alleles have been reported (Würzner *et al*., 1995). Hycult anti-C7 antibody WU 4-15 is a monoclonal antibody that recognizes only the M allele for C7 molecules.

In this study WU-4-15 antibody was used initially for detection of C7 from cord and adult serum sample. I observed that WU-4-15 could detect C7 in all cord and adult sera except one adult serum where no C7 was detected. Since this individual had full lytic complement function, further investigation of this antibody's specificity revealed that it did not detect other C7 alleles as shown in figure 5.3. This made WU-4-15 inappropriate for further use and the experiments shifted to the less optimal Quidel anti-C7 antibody. To enhance the signal for Quidel anti-C7 antibody, it was determined that capturing the monoclonal antibody on plates coated with 4 μg/ml of rat anti-mouse IgG1 monoclonal antibody enhanced its ability to capture C7 and improved measurement (figure 5.2 C). Therefore Quidel anti-C7 antibody was used to measure the levels of C7 in cord and adult sera. Figure 5.3 B shows that no significant correlation was found between the C7 concentrations in adult serum obtained by Hycult and Quidel antibody ($r = 0.4$; $p = 0.15$), which suggests that the Quidel anti-C7 antibody does not share a bias for a single C7 allele.

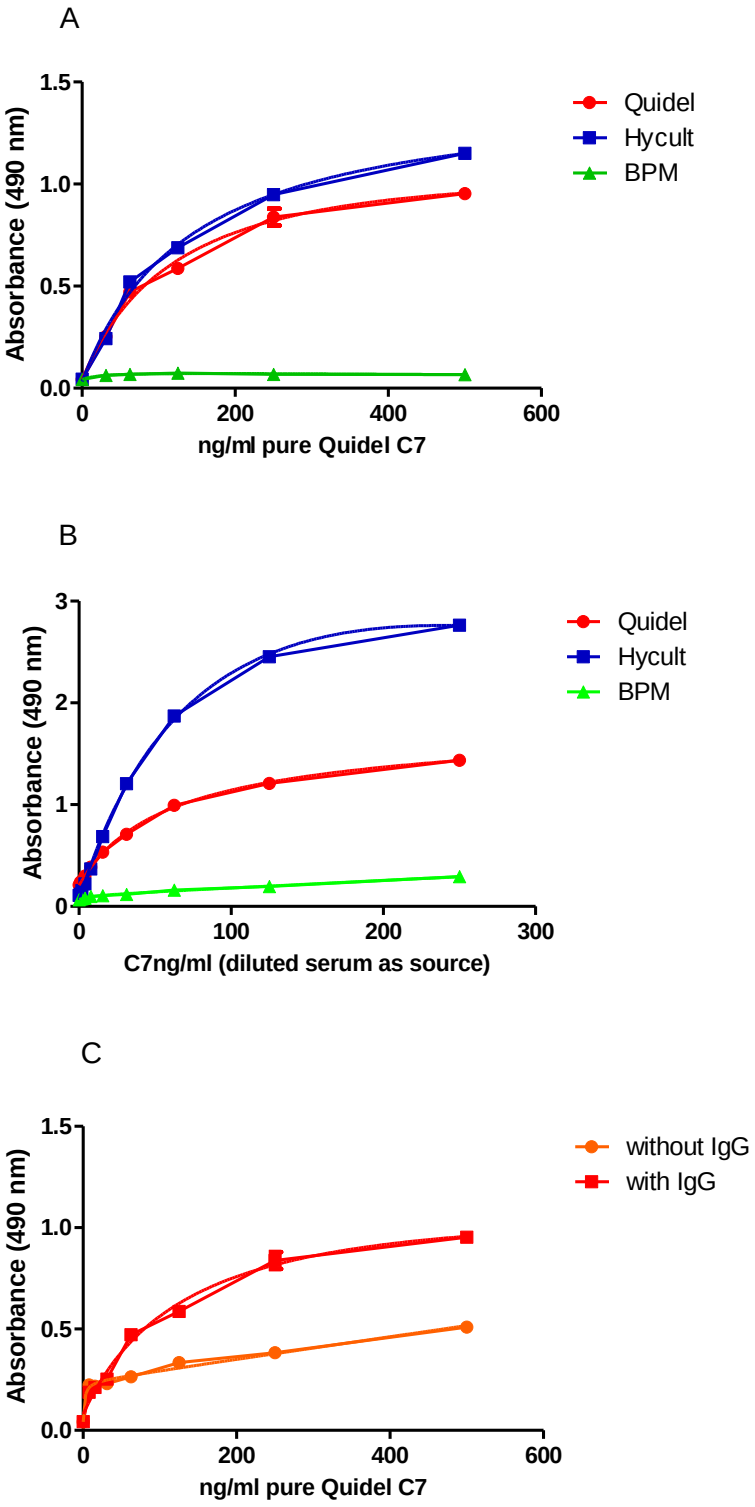


Figure 5.2 comparison of different monoclonal antibodies (mAbs) ability to capture C7.Tested capture antibodies were Hycult Biotechnology, Quidel and non- commercial BPM. A) Ability of tested antibodies to capture purified C7 (B) ability to capture native C7 in serum and (C) Effect of adding rat anti-mouse IgG antibody on the sensitivity of Quidel monoclonal antibody.

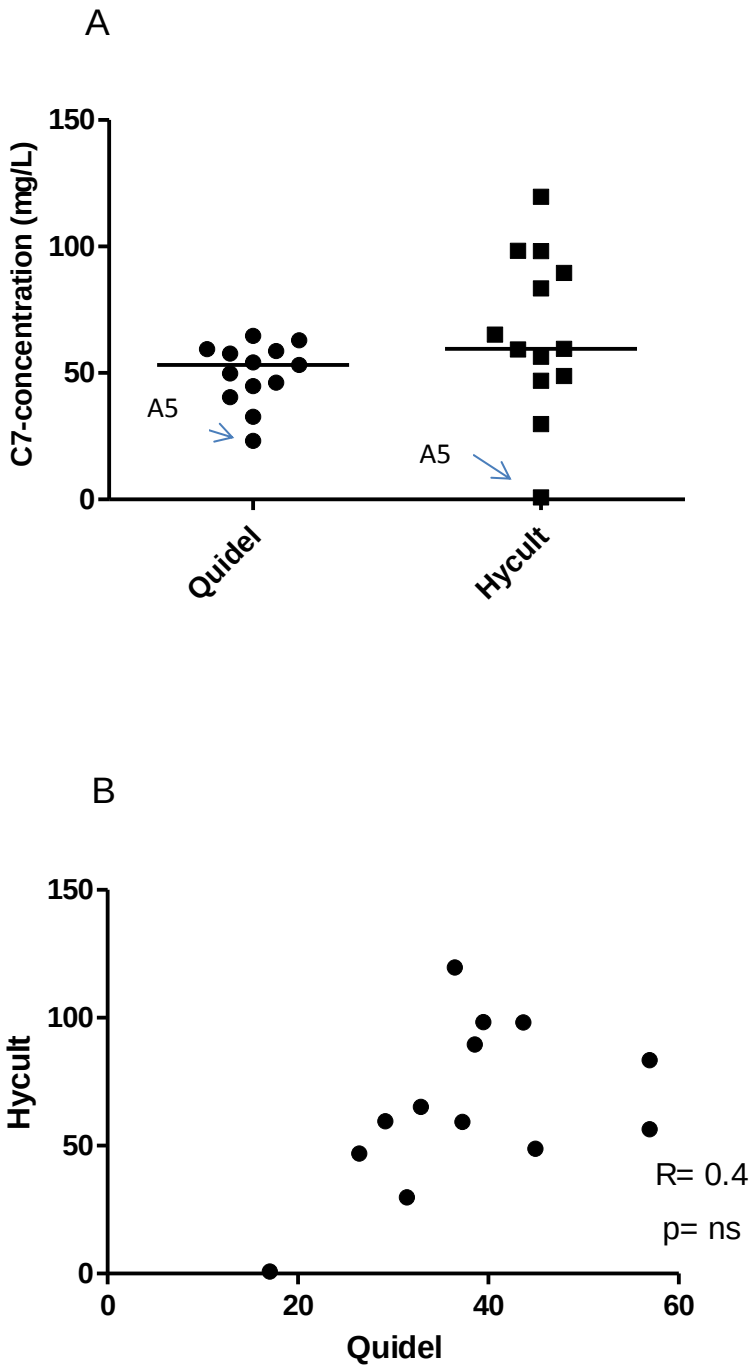


Figure 5.3 Level of C7 in adult serum, (A) C7 concentrations in adult sera using two different monoclonal antibodies Hycult and Quidel. Arrow points to adult VxF5 serum which probably has C7 N allel not detectable by Hycult antibody. It has also the lowest value when measured by Quidel antibody. (B) Correlation between Quidel and Hycult C7 concentrations in adult sera.

5.2.3 Optimizing of the capture C8 ELISA

As a first step in the development of C8 ELISA, two monoclonal antibodies were used as capture antibodies for purified C8 and serum C8. Figure 5.4 shows that non-commercial BPM anti-C8 antibody was the most efficient of the antibodies in capturing C8 for ELISA. Optimisation of the best working concentration was performed in serum samples and two micrograms/ml was the optimal working concentration for BPM anti-C8 to capture purified and serum C8.

The ability of the selected antibodies on the basis of their sensitivity in capturing their corresponding complement components in purified and native form were also tested to assess their ability to capture C6, C7 and C8 within exogenous C5b-9 complex. Figure 5.5 shows that selected mAbs were also capable to capture the corresponding components even when the components are already assembled in the terminal complex form.

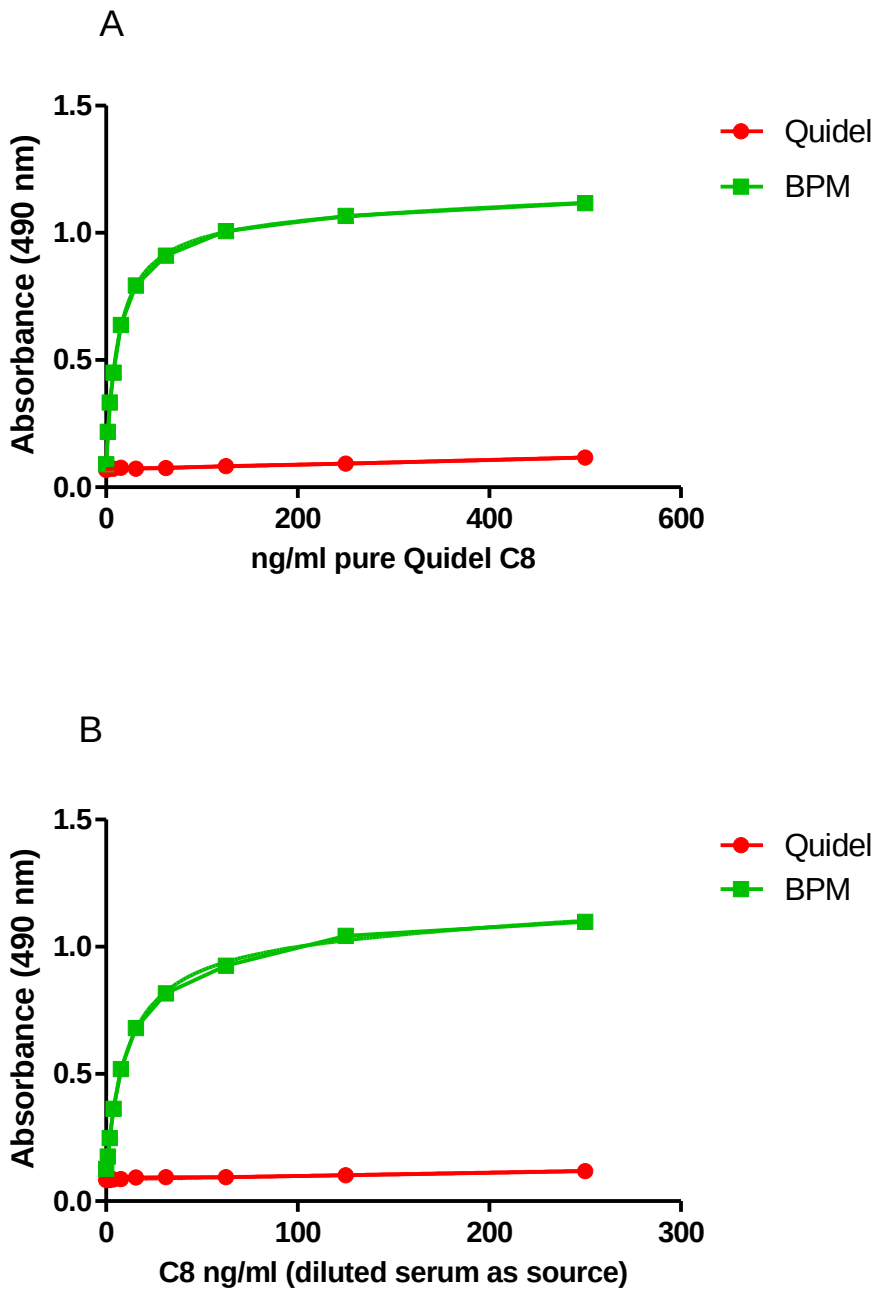


Figure 5.4 Ability of Quidel and non-commercial BPM monoclonal antibodies to detect C8 by ELISA. (A) Ability of tested antibodies to capture purified C8. (B) Ability to capture native C8 in human serum.

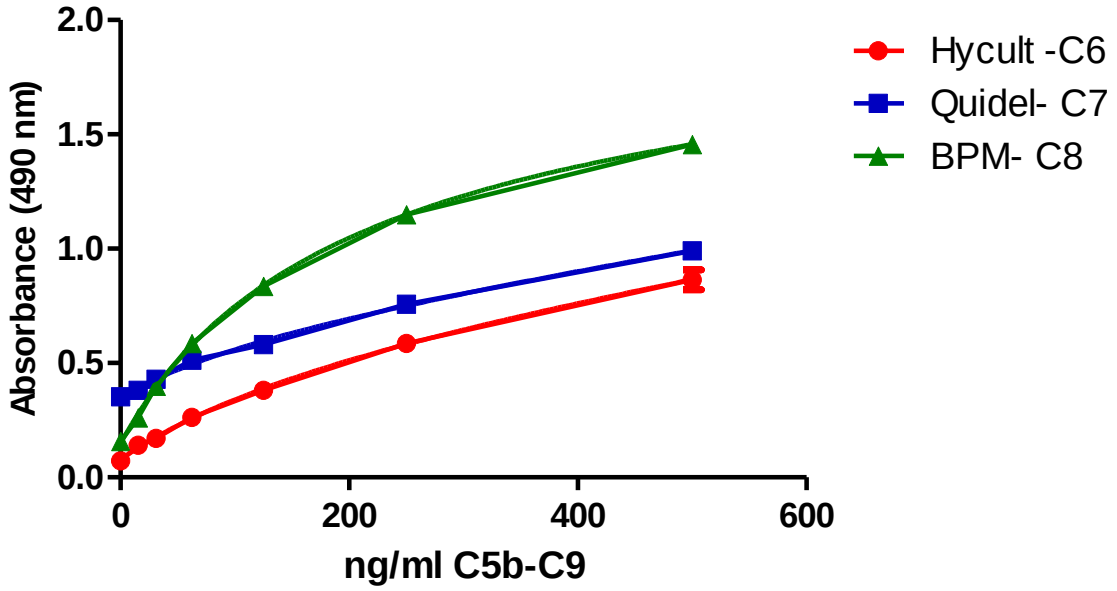


Figure 5.5 ELISA detection using monoclonal antibodies (mAbs) to capture their corresponding components C6, C7 and C8 within C5b-9 complex. A doubling dilution of mAbs was used to coat the wells at 4µg/ml for Hycult C6, 2µg/ml for Quidel C7 and 2µg/ml for non-commercial anti-C8 (BPM).

5.2.4 Optimising ELISA for C9

The ability of five preparations of monoclonal antibodies to recognise Quidel purified C9, native serum C9 as well as C9 in C5b-9 complex was compared. Antibodies were used to coat Nunc Maxisorb 96-well plates at a concentration 2-4μg/ml. One polyclonal anti-C9 antibody was used to detect bound C9 (diluted 1/1000) which was subsequently visualised by Jackson Laboratories' HRPO-conjugated minimum cross-reactivity Donkey anti-goat antibody and OPD. Antibody A223 performed the worst as a capture antibody for purified and native C9 as well as for C9 within the complex, figure 5.6 and 5.7. The affinity of aE11 antibody was similarly weak in capturing purified C9 and C9 in serum but was able to detect C9 within C5b-9 complex. In addition, immobilising A223 and aE11 antibodies with rat anti-mouse IgG1 monoclonal antibody did not enhance the ability of these antibodies to capture purified C9 and C9 within the serum, as had been achieved for the anti-C7 above. Antibodies WU13-15 and non-commercial B7 were the best capture antibodies for purified C9 and C9 within the complex. Adding rat anti-mouse IgG antibody did not enhance the sensitivity of these antibodies to capture purified C9. On the other hand, anti-mouse IgG enhanced the ability of X197 to capture purified C9. B7 and X197 antibodies were the best capture antibodies for native C9 in serum followed by WU 13-15. Non-commercial B7 was uniquely the most efficient capture ELISA antibody for C9 in all examined conditions, therefore the best working concentration for B7 was sought and 2μg/ml was found to be the optimal concentrations for measuring C9 levels in cord and adults samples. It is important to note that WU-13-15 and aE11 are sold as neo-epitope C9 antibodies and that they are not supposed to recognise native C9. To this end the ability of WU-13-15 to capture purified (and therefore potentially altered) C9 was much greater than the ability to detect serum C9, whereas aE11 was only effective at recognising C9 in the context of sC5b-9 complexes, figure 5.6 and 5.7.

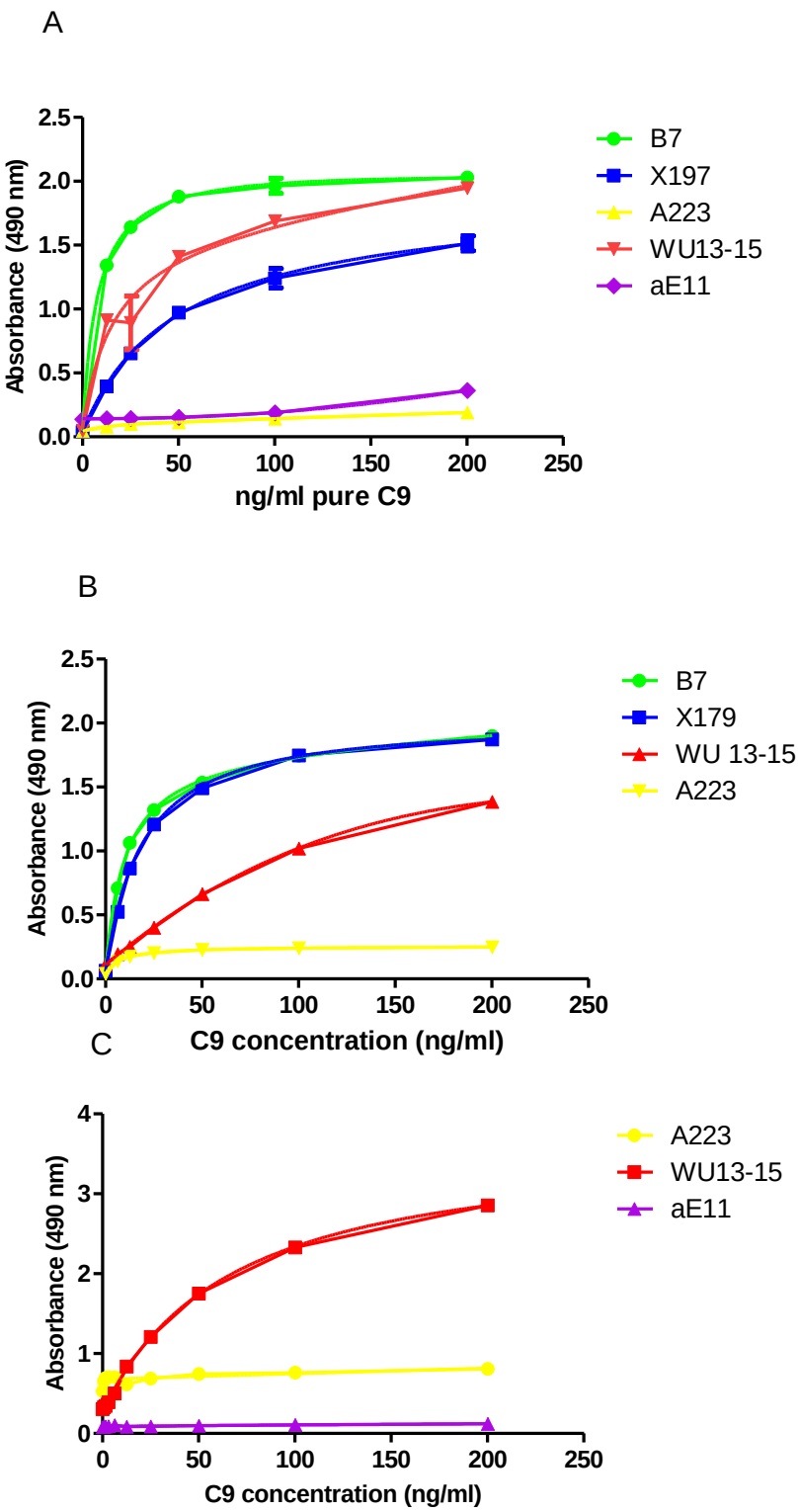


Figure 5.6 ELISA detection using monoclonal antibodies (mAbs) to capture C9.Tested capture antibodies were Quidel-anti-C9 (cat.A223), Hycult Biotechnology anti-C9 (clone WU13-15, X179 and aE11) and locally available anti-C9 (clone B7). (A) Ability of these mAbs to recognise Quidel purified C9. (B and C) Effect of adding rat anti-mouse IgG monoclonal antibody on the ability of these (mAbs) to detect purified C9.

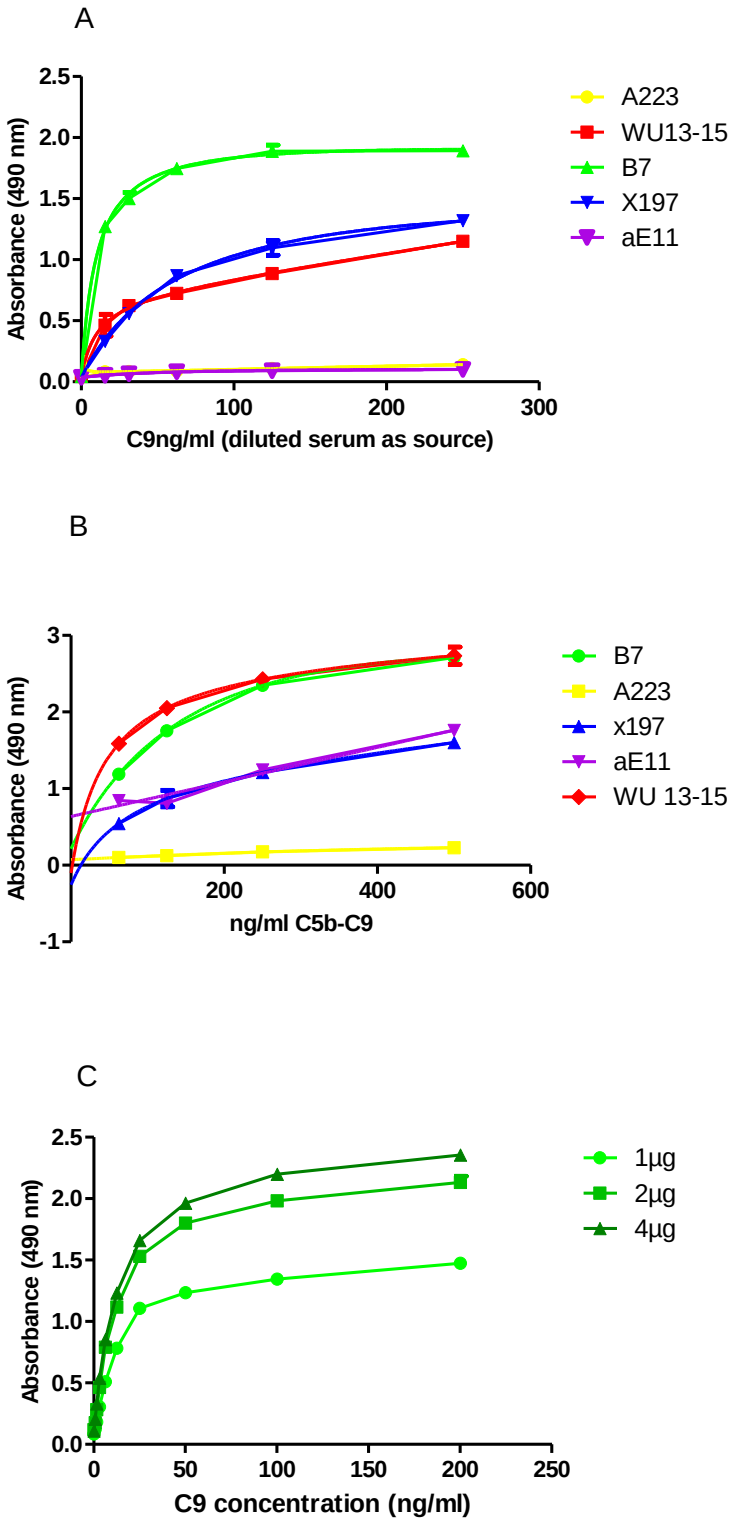


Figure 5.7 compares of different monoclonal antibodies (mAbs) for capturing C9. (A) Ability of mAbs to capture native C9 in serum. (B) Their ability to capture C9 within C5b-9 complex. (C) Determination of the optimum concentration of the most sensitive C9 capturing antibody B7, doubling dilution of mAbs was used to coat the wells starting at 1µg/ml to 4µg/ml for non- commercial monoclonal antibody (B7).

5.2.5 Levels of Terminal components in cord and adult serum

All complement component levels measured, except C7 were lower in cord serum compared to adult levels (figure 5.8). Complement levels were expressed as mean with lower and upper 95% confidence intervals (CI). Adult C7 levels were 42.29 mg/L (36.67-47.92 mg/L), comparable to neonatal C7 levels of 43.03 mg/L (37.24 -48.81mg/L), while neonatal C6 (12.84mg/L, CI 9.88-15.79 mg/L) and C8 (14.11 mg/L, 95% CI 11.22-16.99 mg/L) levels were 25-34% of adult C6 (37.49, CI 27.1 - 47.4 mg/L) and C8 (55.17 mg/L, 45.32-65.01 mg/L) levels (p<0.001). Neonatal C9 levels were very low (6.4% of adult level) (2.98 mg/L, CI 1.63 – 4.32 mg/L) compared to adult levels (45.89 mg/L CI 36.48- 55.29 mg/L; p<0.01). Complement concentration values were corrected with amino acid hydrolysis correction factors of the provided standards (determined at Quidel) which were 0.89, 0.69, 1.705 and 0.95 for C6, C7, C8 and C9 respectively. Optimised ELISA methods was also applied on international pooled adult serum to obtain reference adult levels of C6-C9.When compared with cord serum samples the results showed the same statistically significant differences figure 5.9. The reference adult values for C6, C7, C8 and C9 expressed as mean (± 95% CI) were 30.89 mg/L (CI 17-44.7 mg/L), 45.67 mg/L (CI 41.7-49.6), 46.36mg/L (CI 45.1-47.5) and 35.45 mg/L (CI 33.1-37.8) respectively. Cord/pooled serum percentages were 41.55%, 94.34%, 30.43% and 8.37% for C6, C7, C8 and C9 respectively.

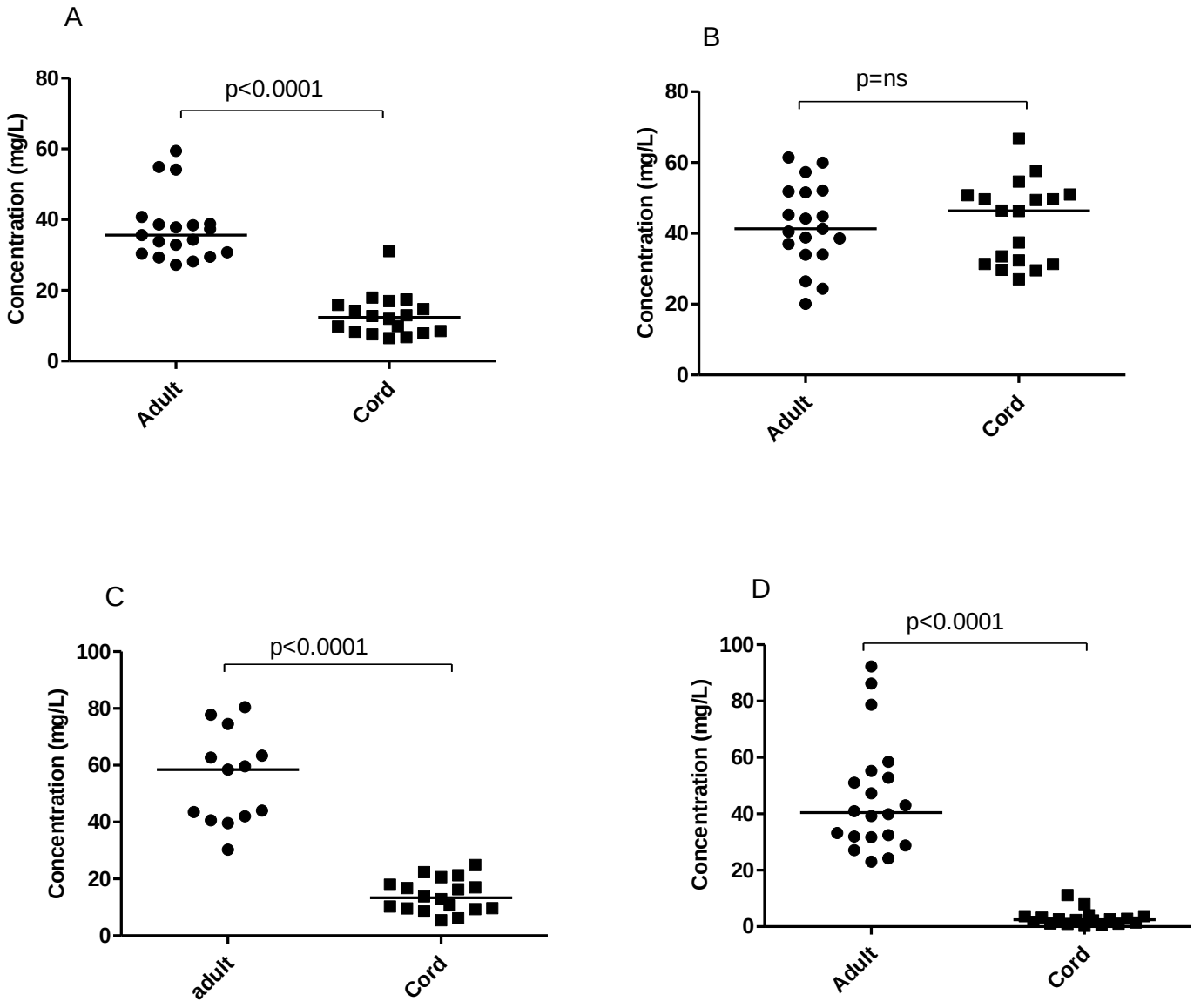


Figure 5.8 Scatter plot showing levels of terminal complement components in cord and adult sera, C6 (A), C7 (B), C8 (C) and C9 (D). Mean levels differences were highly significant, (p <0.0001) for C6, C8 and C9 whereas no statistically significant difference was found for C7 level in cord and adult serum. Horizontal lines represent the mean.

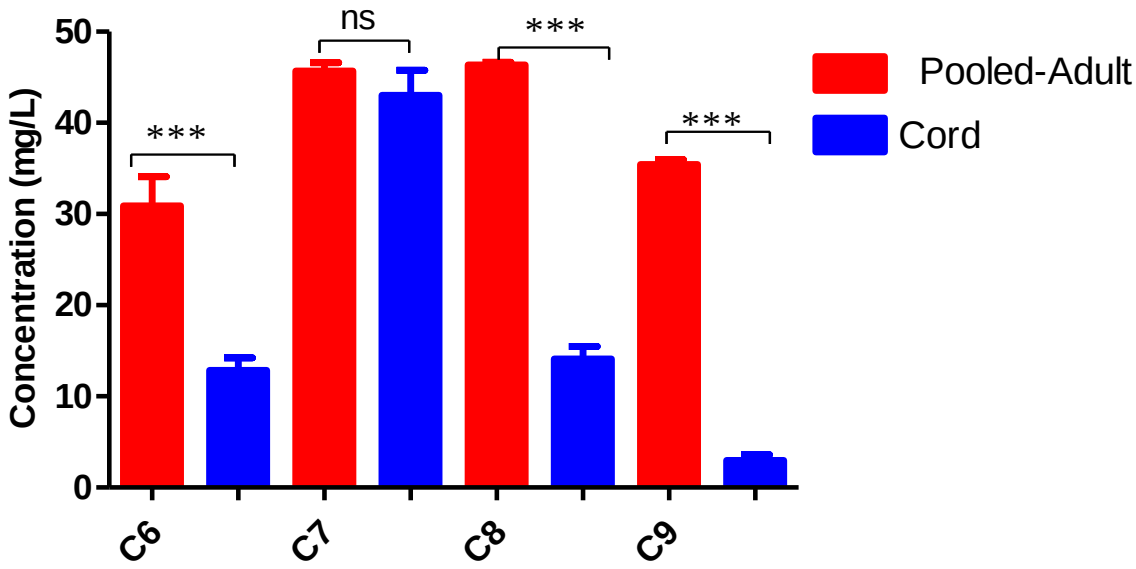


Figure 5.9 Levels of terminal complement components in cord and international pooled adult sera. Levels shown as mean ± SEM. (***) denotes highly significant difference, p <0.0001.

5.2.6 Haemolytic activity of cord serum and the effect of C9 supplementation

Haemolytic activity in cord sera was found to be markedly lower than adult levels. Achievement of 50% haemolysis level required an average serum dilution of 1/22 for adult compared to 1/12 for cord (p <0.05). However, when cord sera were supplemented with purified C9, the most deficient terminal component, to a concentration of 50 mg/L. The resulting neonatal CH50 values were equivalent to adult values despite low C6 and C8 levels in cord sera (Figure 5.10).

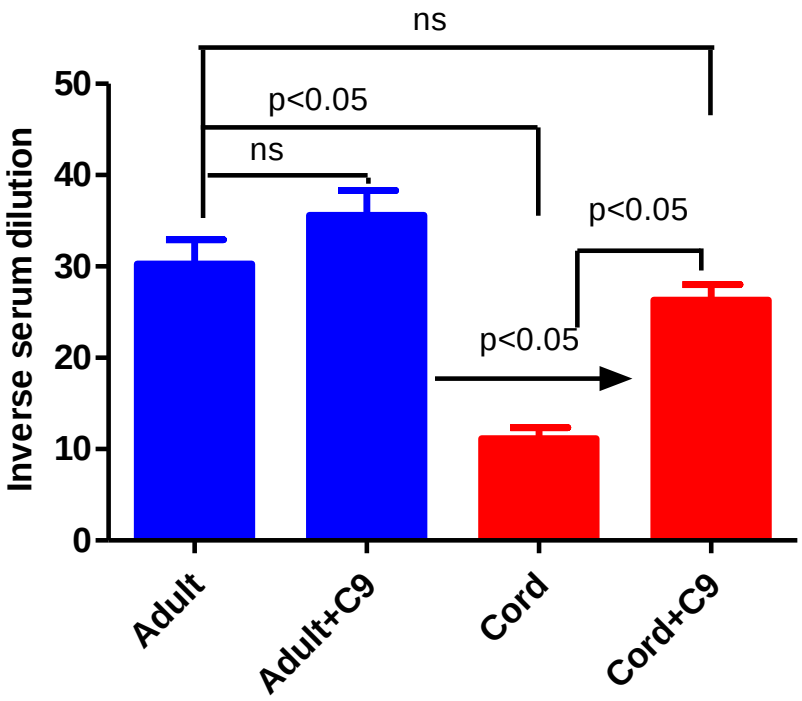


Figure 5.10 Haemolytic activities of cord and adult sera. Inverse dilution of cord and adult sera to achieve 50% haemolysis of antibody-sensitised sheep erythrocytes are shown. Limited CH50 in cord sera were restored by adding purified C9 (final concentration 50 mg/L). Significant differences are shown in the figure, ANOVA test, n=20).

5.2.7 Analysis of the complement killing assay of *Ureaplasma parvum*

The killing assay described in material and methods chapter, section 2.3.5 enabled the determination of magnitude of *Ureaplasma* killing by six adult sera and 14 cord sera. Occasionally, wells were observed in which colour change was absent although subsequent wells further down concentration were positive (figure 5.11). To keep consistency in determining 1 CCU (the final well in which colour change occurs), negative wells were counted as positive if a single negative well was subsequently followed by two or more positive wells. In addition, to rule out any bias in spontaneous negative wells within the titration, all killing assay experiments were repeated in duplicate on independent days. As degree of killing was calculated from the relative bacterial growth following exposure to matched sera with complement intact (NHS) or following heat-inactivation (HI) controls, poor growth following exposure to HI control could result in an artificially low killing. Therefore, only experiments where bacteria exposed to HI serum that grew to at least 10^3 CCU were considered valid.

5.2.8 Cord and adult sera variation in SV1, 3, 6 and 14 killing by complement pathways

The ability of 50% cord serum to kill representative strains of each *U parvum* serovar is shown in figure 5.12. When all activation pathways were available, 2/14 cord sera reduced the CCU of DFK-1 (SV1) by greater than 1000-fold, 6 /14 reduced CCU by 10 to 100-fold, while no killing (<10-fold killing) was observed for 6/14 (figure 5.12 A). Twelve out of 14 cord sera reduced HPA5 (SV3) CCU by greater than 1000-fold, (1/14/) reduced CCU by less than 1000-fold and the final cord (1/14) reduced CCU by 100 fold (figure 5.12 B).

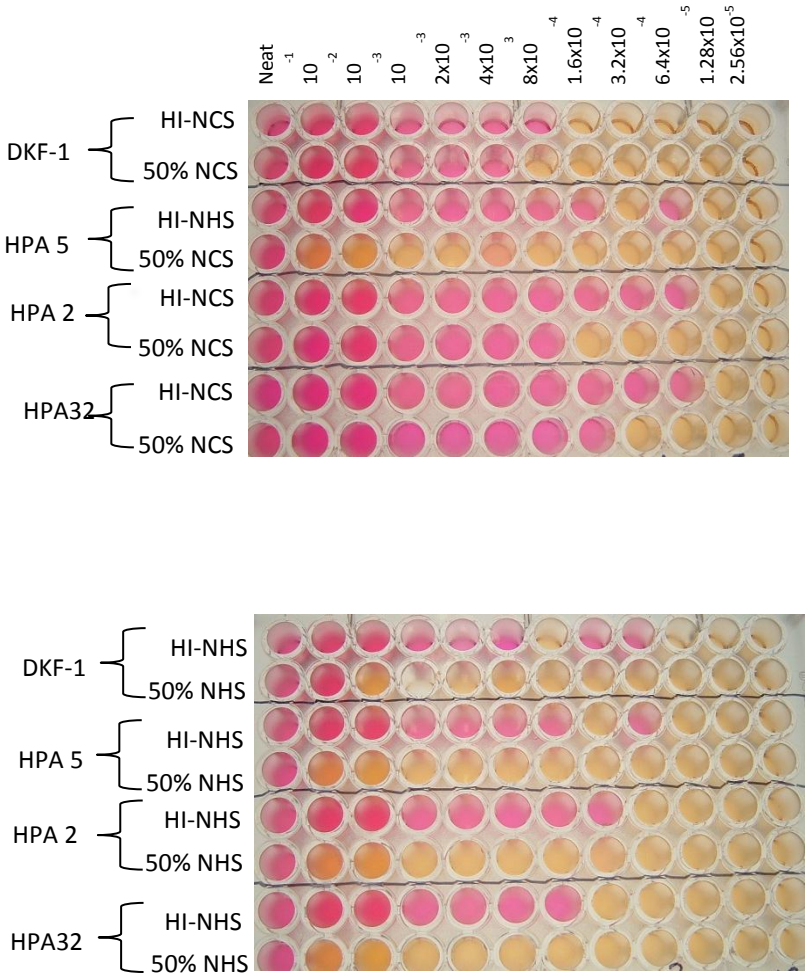


Figure 5.11 Complement killing assay to determine the bactericidal activity of cord and adult sera. The four representative serovars of U. Parvum were DKF-1 (SV1), HPA5 (SV3), HPA2 (SV6) and HPA32 (SV14). (A) The killing capacity of 50% normal cord serum (NCS). (B) Killing of all serovars by normal human serum (NHS). Pink wells represent growth of Ureaplasma whereas yellow wells represent absence of growth. Killing was determined by calculating the fold decrease in CCU from NHS relative to the heat-inactivated normal human serum (HI-NHS) control.(yellow colour wells indicate no killing while pink colour indicates killing).

A single serum decreased HPA2 (SV6) CCU by greater than 10-fold, and the remaining 13 sera decreased numbers by less than 10 –fold (i.e. not significant killing; figure 5.12 C). No killing of HPA32 (SV14) was seen for any of the cord sera, figure 5.12 D. Therefore, no significant killing of SV6 or SV14 could be mediated by cord sera.

In comparison, bactericidal effect of adult sera (figure 5.13) showed greater degree of killing where a single adult serum reduced HPA5 (SV3) CCU by greater than 1000 fold, while the five remaining adult sera decreased the CCU by greater than 10,000 fold. All adult sera were able to reduce DKF1 (SV1) CCU by greater than 10-fold relative to heat-inactivated controls; the greatest bactericidal activity was observed up to 10,000 fold killing. A single adult serum decreased HPA2 (SV6) CCU by greater than 10 fold whereas the remaining five sera reduced numbers by less than 10 fold. All six adult sera failed to kill HPA32 (SV14) as evidenced by less than 10 fold reduction in CCU. This indicates that adult sera were equally as poor at killing SV6 and SV14 as were cord sera.

In Summary, complement-mediated *Ureaplasmacidal* effect of adult sera was greater in SV1 than cord sera whereas both sets have almost equal bactericidal ability to eradicate SV3. Servars 6 and 14 were resistant to killing by cord as well as adult serum. Figure 5.14 shows the mean Ureaplasmacidal capacity of cord and adult sera against all serovars, when results are combined for comparison.

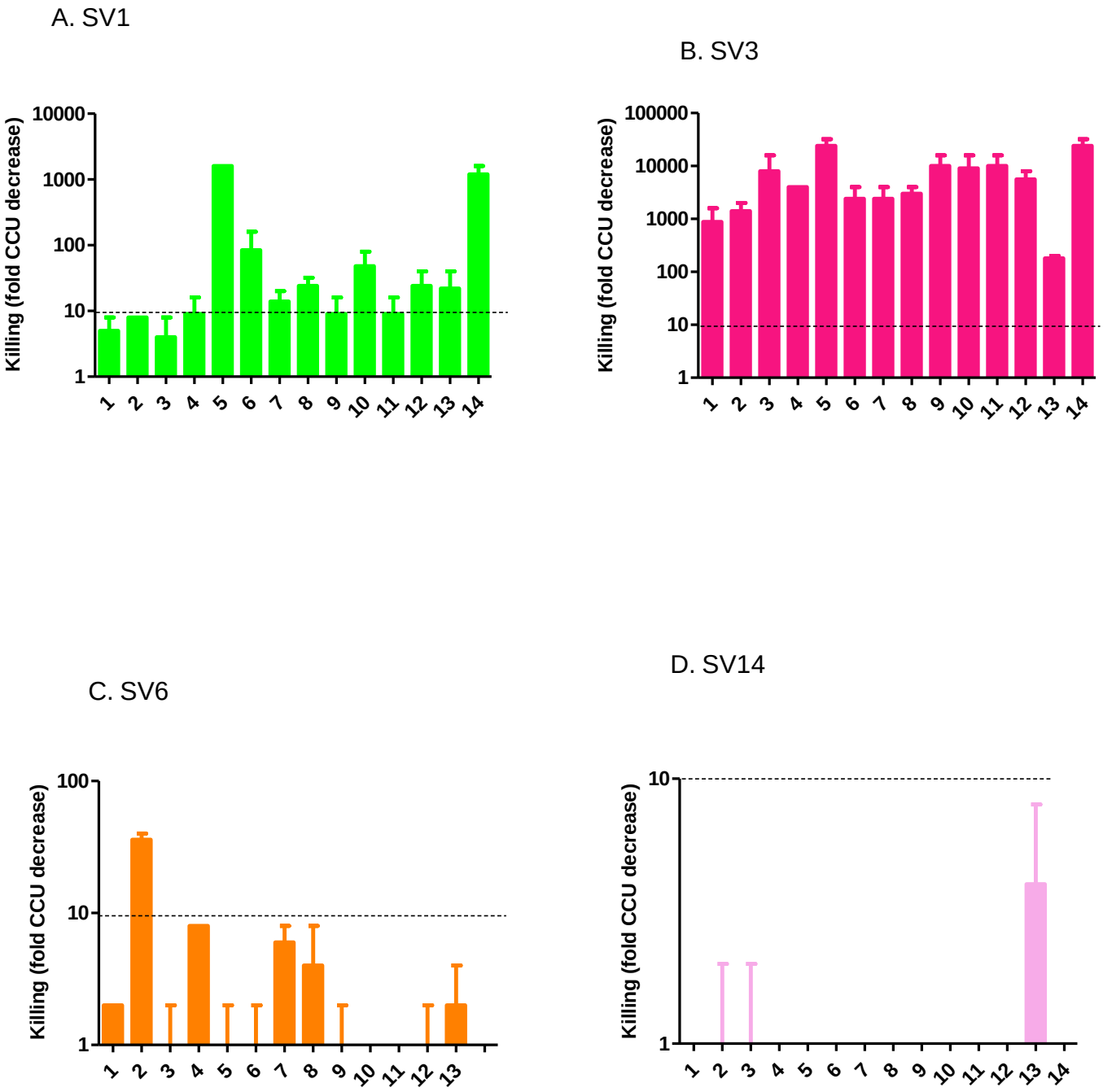


Figure 5.12 -The Killing capacity of cord sera against the four serovars of U. Parvum. (A) SV1 isolate DKF1, (B) SV3 isolate HPA5, (C) SV6 isolate HPA2 and (D) SV14 isolate HPA32. Each number in the x axis indicates individual cord serum.

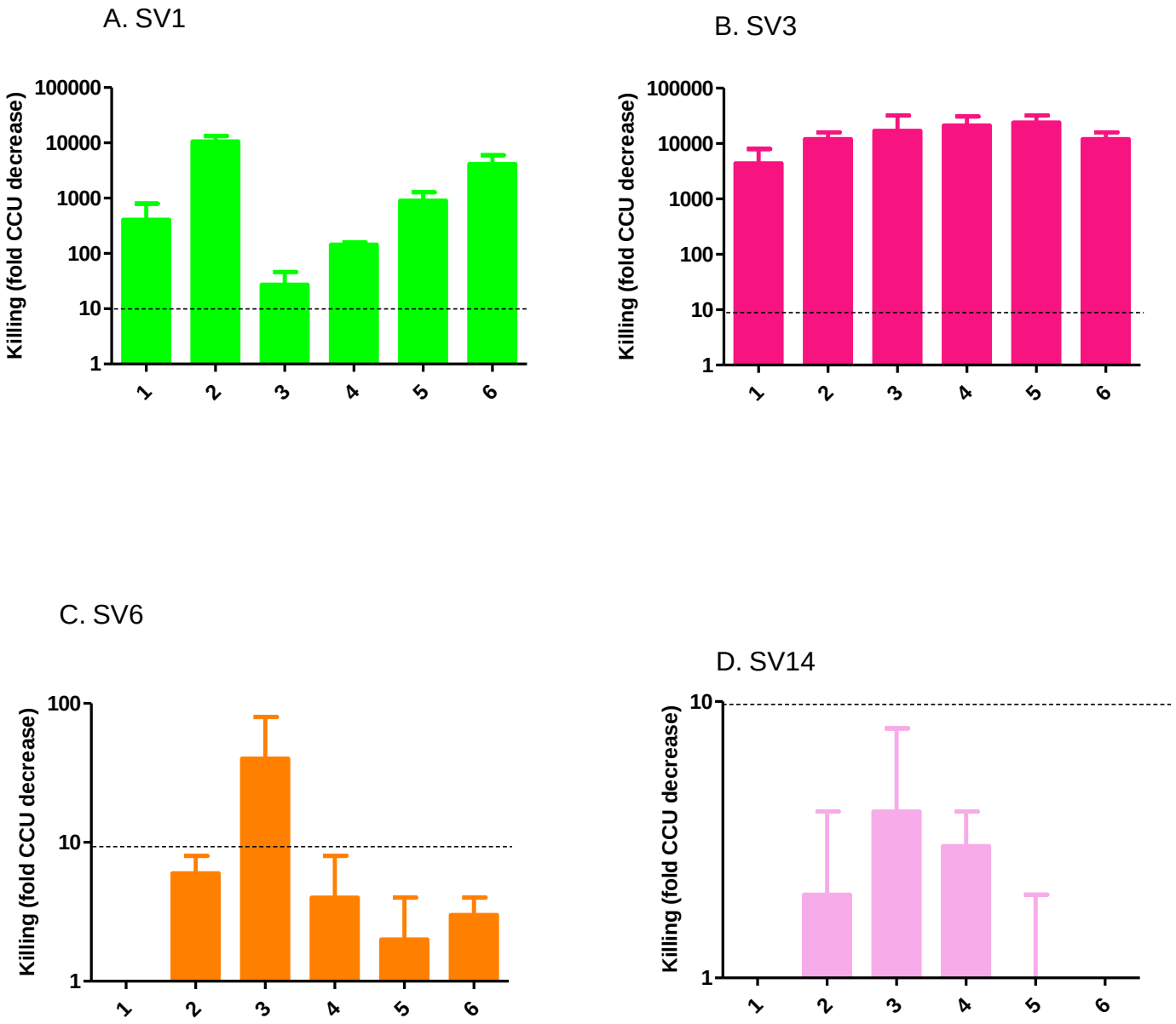


Figure 5.13 The Killing capacity of adult sera against four serovars of U. Parvum. (A) SV1 isolate DKF1, (B) SV3 isolate HPA5, (C) SV6 isolate HPA2 and (D) SV14 isolate HPA 32. Each number in the x axis indicates individual adult serum.

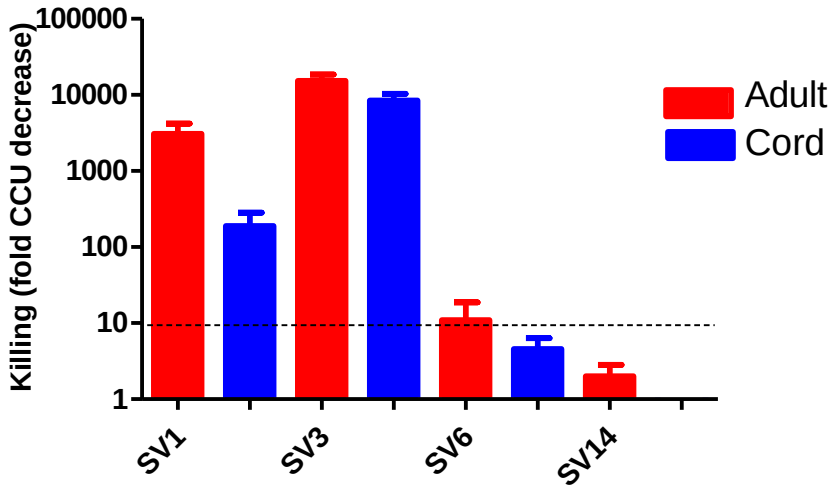


Figure 5.14 The Killing capacity of cord compared to adult sera against the four serovars of U. Parvum. (A) SV1 isolate DKF1, (B) SV3 isolate HPA5, (C) SV6 isolate HPA2 and (D) SV14 isolate HPA 32.

5.2.9 Titration of serum for killing capacity

I determined the capacity of different serum concentration (titrated serum) for complement-mediated *U parvum* killing assay. To see if complement component concentration played a role in the degree of ureaplasmacidal activity at different serum dilutions, a series of killing experiments for six adult and seven cord sera were performed at 2-fold dilutions from 50% to 6.25% (figure 5.15). The fold-killing of different concentrations of adult and cord sera for SV1 and SV3 are shown. The bactericidal effect of cord and adult sera on SV1 was directly proportional to the concentration of serum used. A 10-fold decrease in SV1 killing was seen for adult sera when concentration decreased from 50% to 25% and no significant killing was observed at 12.5% or lower. In line with lower complement component concentration, killing of SV1 by 50% cord serum was similar to the bactericidal effect of adult serum 25% concentration. No significant killing was observed at 25% or lower cord concentration (figure 5.15A). The level of adult and cord serum killing of SV3 was almost the same at 50% or 25% serum concentration but killing capacity dropped equally by about 10-fold at 12.5% and then became insignificant at 6.25% in adult serum while at this concentration cord serum was still able to kill SV3. In conclusion, unlike SV1 killing, cord sera killing of SV3 were equivalent to adult killing, until diluted to 6.25% when it was higher than adult sera (figure 5. 15 B).

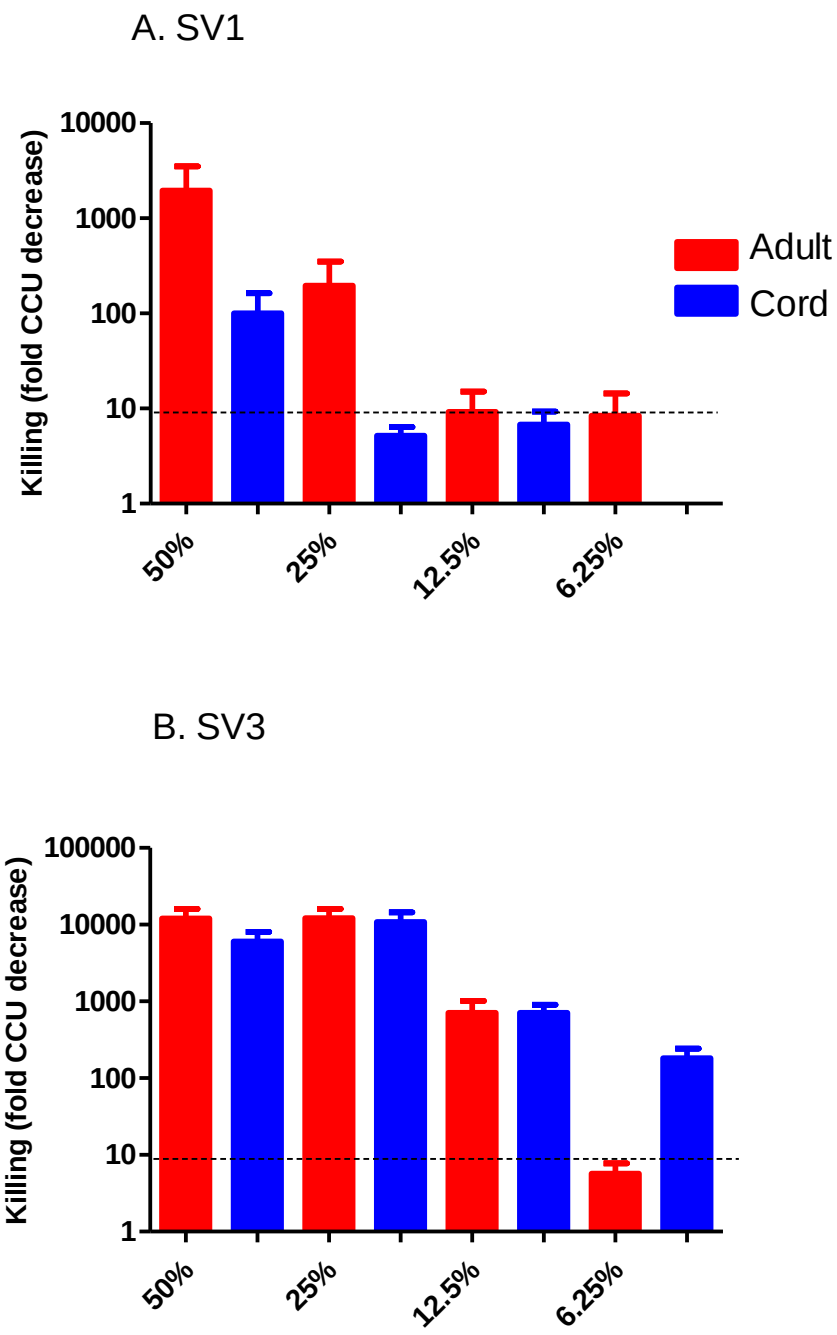
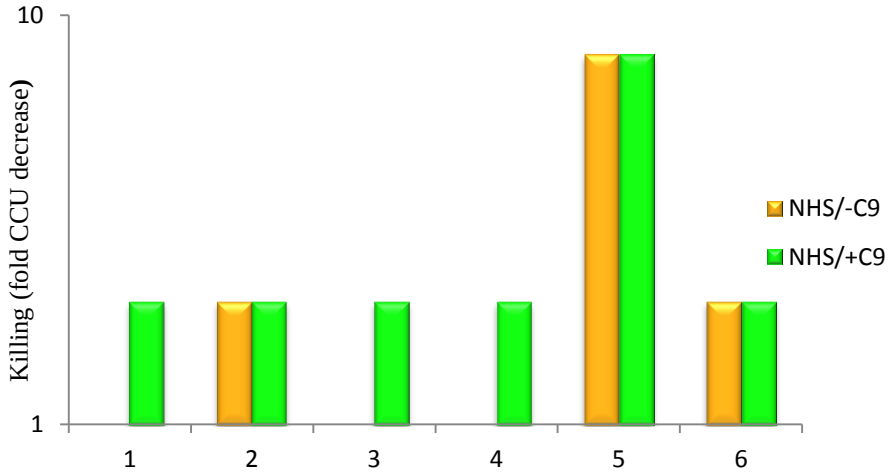


Figure 5.15 The ability of varying concentrations of adult and cord sera to kill (A) SV1 and (B) SV3. Red bars represent killing of adult sera and blue bars represent the killing of cord sera. Numbering of x-axis represents concentration of sera.

5.2.10 The supplementation of complement component C9 alone or combined with C6 and C8 did not change the bactericidal capacity of neonatal serum against *U parvum*

The bactericidal ability of neat 50% cord sera against SV1 and SV3 was not enhanced with serum supplementation with 5μg/ml of Quidel purified C9 (Figure 5.16). Since complement component C9 and to a lesser extent, C8 and C6 were the most deficient terminal components in neonatal serum; 50 μg/ml of each component was added to the selected cord serum. Serovar killing in response to incubation with cord sera was compared in parallel to the same sera supplemented with C6, 8 and 9. Figures 5.17 and 5.18 show that supplementation of cord serum with C6, C8 and C9 did not change its bactericidal effect on SV1, SV6 and SV14.

A SV1



B SV3

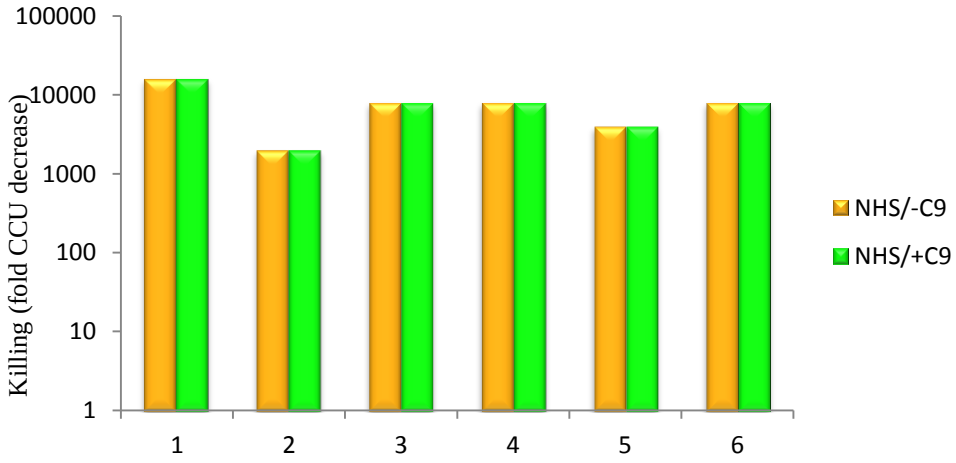


Figure 5.16 Effect of Supplemental C9 on the bactericidal activity of cord sera against (A) SV1, (B) SV3. Each number in the x axis indicates individual cord serum. Green bars represent serum supplemented with C9; Brown bars denote serum without C9 supplement.

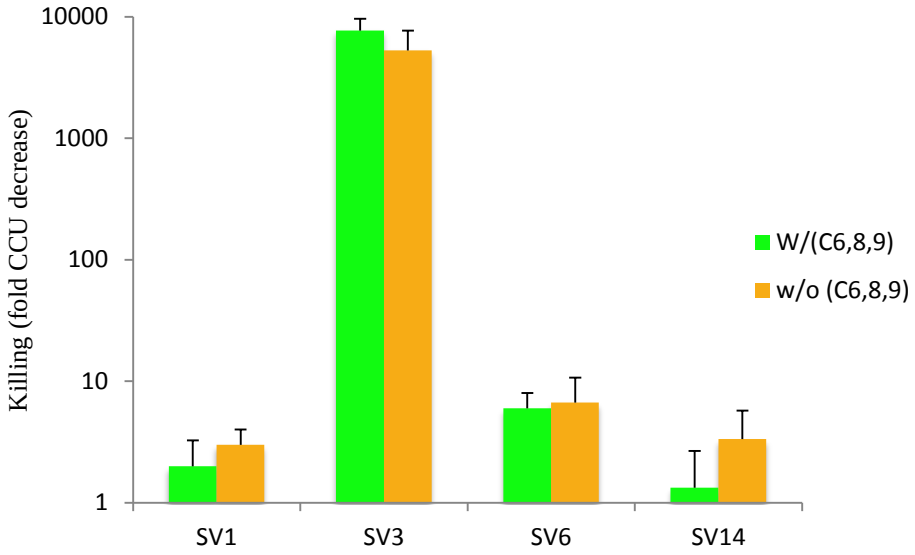


Figure 5.17 Effect of adding purified C6, C8 and C9 on the ability of cord sera to kill the four different serovars of U Parvum. Complement C6, C8 and C9 supplement did not enhance the Ureaplasmacidal capacity of cord serum. Green bars represent serum supplemented with C6, 8 and 9; Brown bars denote serum without supplement of C6-9.

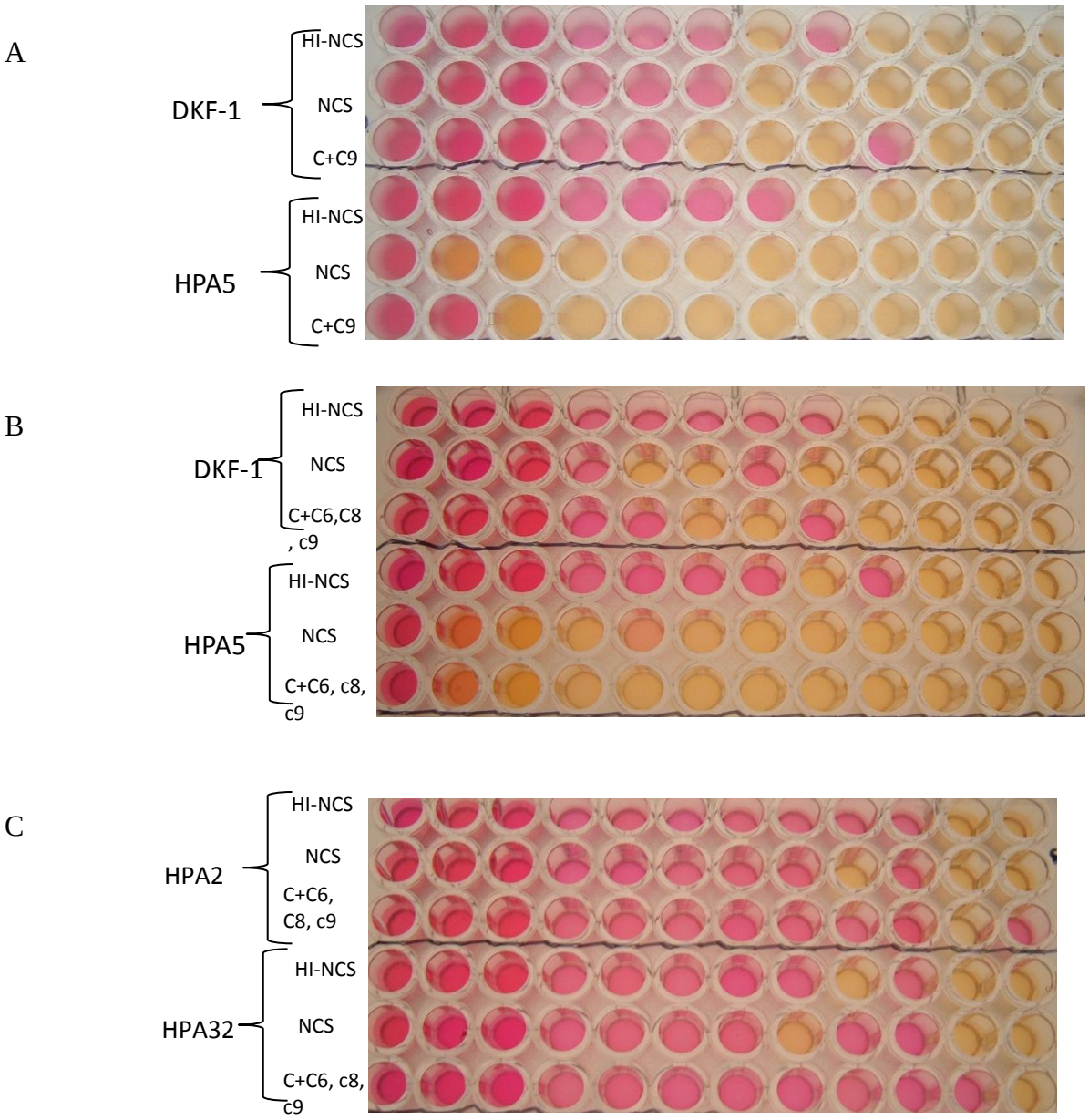


Figure 5.18 Complement killing of cord serum supplemented with C9 or combination of C6, C8 and C9. (A) Effect of adding C9 on the capacity of cord serum (NCS) to kill SV1 and SV3. (B and C) Ureaplasmocidal effect of cord sera supplemented with C6, C8 and C9 on the four representative serovars of U Parvum (B) DKF-1 (SV1), HPA5 (SV3), (C) HPA2 (SV6) and HPA32 (SV14). Killing was determined by calculating the fold decrease in CCU from NCS relative to the heat-inactivated normal cord serum (HI-NCS) control. (Yellow colour wells indicate no killing and pink colour indicates killing)

5.2.11 Detection of anti- *Ureaplasma* IgG responses within serum by Western blots

In order to detect the presence of any anti- *Ureaplasma* antibody in cord serum, Western blot analysis was performed using the 14 cord sera samples to detect reactivity against the four serovars representatives of *U. parvum* (figure 5.19). The blots from cord 1 and 2 show the two seronegative sera in this study. These membranes were subsequently probed with seropositive serum to rule out the possibility of false negative results. Blots from cord 3 and 4 had similar banding patterns of each respective serovars. DKF-1 lanes produced a dark band of just greater than 60 KDa; this band was conserved across all serovars blotted with these sera but with greater intensities. A second less intense band of just greater than 70KDa was also observed in most of the blotted sera for DKF1, showing the same pattern previously observed in adult serum by Beeton *et al* 2012. These bands do not correlate with a greater degree of SV1 killing. Serum from cord sample 14 revealed a conserved band from all serovars between 43 and 65 KDa, similar band was observed in sample 3, 4, 7 and 11.

An additional clear band about 41 kDa for HPA5 was also observed for most cord sera except in negative cord samples 1 and 2 along with cord 13 which showed the lowest killing capacity of SV3. Similarly, another band was seen for all serovars of about 84 KDa in most cord sera. HPA2 had a unique band with a slightly higher than 72KDa for cord 3 and 4. Cord 7, 12 and 14 produce a number of bands to each isolates with varying intensity. A final intense band of high molecular weight (around 130KDa) was seen in the lane containing

HPA32 of cord 3, 4, 6, 7, 8, 9 and 11sera. Due to using whole-cell lysates the bands present on the Western blot represented both internal (non-complement activating) and external (complement activating) antigens and individual band reactivity or intensity may not necessarily relate to magnitude of killing by serum.

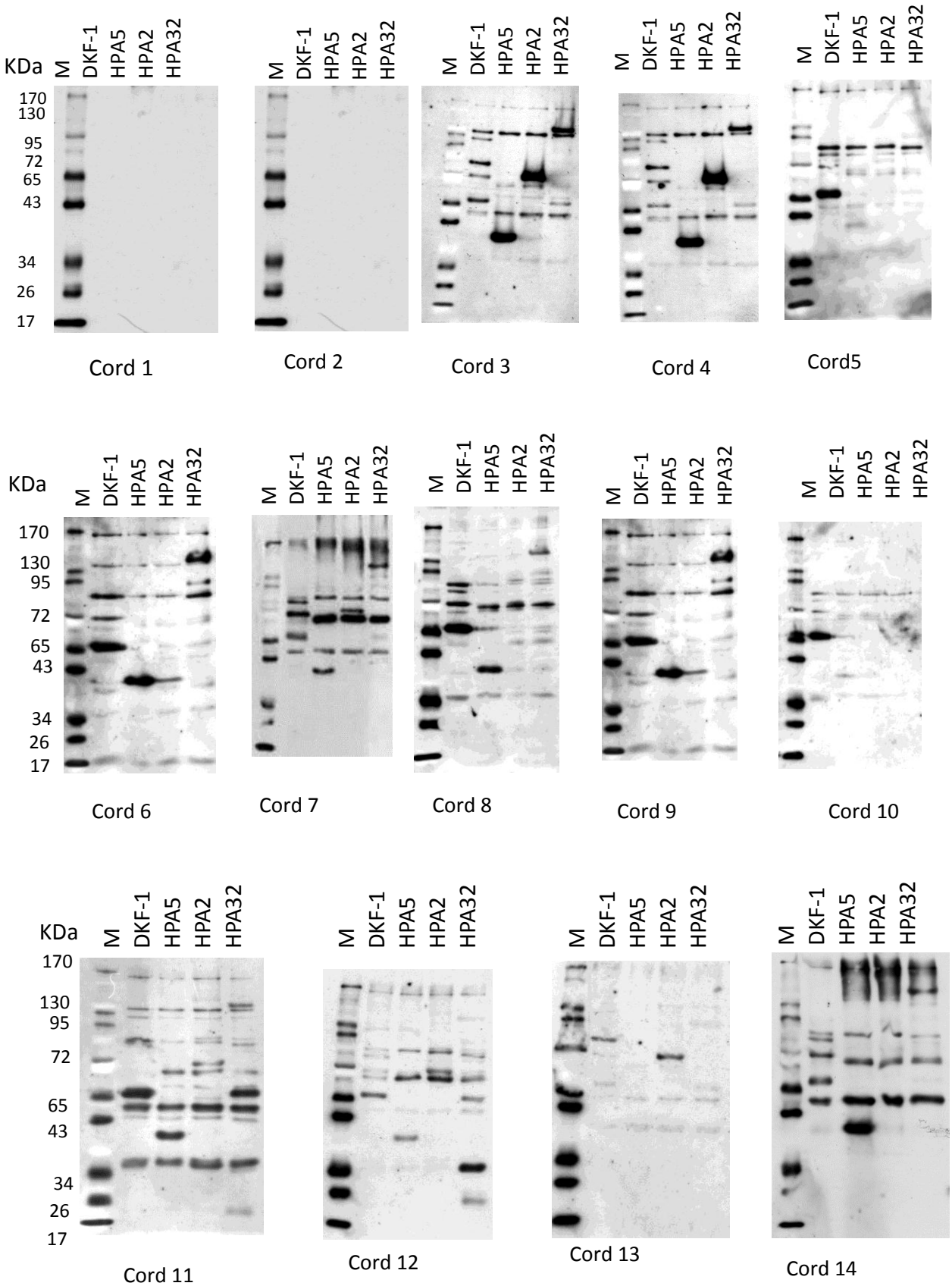


Figure 5.19 Immunoblots of SV1 isolate DKF-1, SV3 isolate HPA5, SV6 isolate HPA2 and SV14 isolate HPA32 probed with cord serum. Each blot image denotes individual cord serum.

5.3 Discussion

5.3.1 Terminal complement components levels (C6, C7, C8 and C9)

The concentrations of terminal complement components have been reported by a number of studies and they found that newborn serum contains low levels of these components compared to adults (Davis *et al*., 1979; Johnston *et al*., 1979; Drew and Arroyave, 1980 Stonntag *et al*., 1998b). The same findings were observed in our study where serum of normal full term newborns was low for components (C6, C8 and C9) of terminal complement system pathway compared to adult levels. One exception was C7 which was identical to adult level. Compared to adult levels, the average term neonatal levels of C6 was 12.8 mg/L (34.2% of adult level), C7 was 43 mg/L (101%), C8 was 14.1 mg/L (25.5%) and C9 was 2.9 mg/L (6.4%). In addition, the concentrations of these components were compared with international pooled adult standards and the percentages were 41.5, 94.3%, 30.4% and 8.3% for C6, C7, C8 and C9 respectively. Previous studies of terminal complement components levels in full term infants compared to adults ranged between 44-58 % for C6 levels, whereas its level in preterm neonates was 35-39 % and similarly low C8 level was demonstrated in term and preterm neonates with levels of 36-38% and 29% respectively. Reported C7 was 95-120% in term neonates in keeping with our results while adequate C7 level was demonstrated even in preterms as well at 73-75% (Adinolfi *et al*., 1981; Lassiter *et al*., 1992; Wolach *et al*., 1997).

One of our adult samples in this work had no detectable C7 when tested with Hycult mAb. This is consistent with the known polymorphism of C7 which was first detected based on charge difference by isoelectric focusing. C7 M/N polymorphism was another allotyping

based on reactivity to allospecific mAb. WU 4-15 recognizes all C7 molecules in homozygous $C7^{*}$ M individuals and 50% of C7 in heterozygotes but the reaction is very weak in $C7^{*}$ N homozygous individuals which is the allotype of this individuals. $C7^{*}$ M homozygous alleles is present in 99% of Caucasians (Würzner *et al*., 1992). Whereas the serum in our study that failed to react with WU 4-15 came from an individual from Tibet.

Levels of C9 were consistently low in previous studies but values varied considerably possibly due to different methodology. Higher percentage of C9 in cord relative to adult serum (50-55%) was reported by only one group of researchers using single radial immunodiffusion (RID) (Wolach *et al*., 1994; Wolach *et al*., 1997). Other groups reported cord C9 levels of 10-20% of adult level (Davies *et al*., 1979; Adinolfi *et al*., 1981; Høgåsen *et al*., 2000). Moreover, more recent study reported a median level that is 10.8% of adult levels and interestingly 13% of 72 newborns had C9 levels less than 2% of adult values (range of 0.6- 35.2 %) . Profound C9 deficiency in this study was correlated with gestational age and with ability of cord serum to form MAC (Høgåsen*et al*., 2000). Ueda *et al*. (1980) studied C9 in 2 groups of full term newborn on basis of their weight for gestational age. Both groups appropriate for gestational age (AGA) and small for gestational age (SGA) had C9 levels of 2.8 mg/ L which was 12% of maternal level. Maternal reference values are known to be higher than non- pregnant adults and in one study maternal C9 level was approximately double the amount of C9 in non-pregnant adult serum (Adinolfi *et al*., 1981).

Another difference between our present data and most of the previous reports in addition to method was the way of measurement and how levels were presented. Most previous reports expressed complement components levels solely as percentage rather than expressing the result in actual units like mg/ml.

Here in, amino acid hydrolysis was additionally used to accurately quantify affinity purified C6, C7, C8 and C9. The results of the present study are in accord with previous work demonstrating that terminal complement levels are 40-80% lower in infant than in the older child and adults (Ballow *et al*., 1974; Feinstein and Kaplan 1975; Adamkin *et al*., 1978).

5.3.2 Synthesis of terminal complements components

Complement in newborn are synthesised by the foetus since complement components are not transferred across the placenta (Ueda *et al*., 1980). The liver is the major site of synthesis of complement components (Ramadori *et al*., 1984) and C9 is known to be one of the acute phase proteins (Adinolfi and Lehner 1988). Evidence was also obtained from research on hepatoma cell lines which was able to synthesise terminal components C5, C6, C7 and C8 (Morris *et al*., 1982). Other tissues and cell types could produce C6 to C9 *in vitro* and extra hepatic synthesis of complement was reviewed by Morgan and Gasque (1997). Human umbilical vein endothelial cells have been shown capable of *in vitro* synthesis of all terminal complements C6-9 (Johnson and Hetland 1991; Langeggen *et al*., 2000., Langeggen *et al*., 2001). Similarly, peritoneal macrophages could synthesise complements C5 to C9 (Hetland and Bungum 1988).

Neutrophils were demonstrated to store large amounts of C6 and C7 that could be released upon stimulation. This store was not due to uptake from the serum nor synthesised in neutrophils but probably pre-synthesised during myelopoeisis (Høgåsen *et al*., 1995). Pulmonary synthesis of C5-9 was demonstrated by incorporation of radiolabelled amino acids in complement synthesised by type II pneumocytes and fibroblast cell lines derived from lung. Synthesised complement C5-C9 were visualised by autoradiography of double

immunodiffusion plates (Rothman *et al*., 1989). Platelets could also secrete C9 and C8 and to lesser extent C5, C6 and C7 when stimulated *in vitro* (Houle *et al*., 1989).

C7 level in cord is uniquely higher than levels of other terminal components. In addition, it is not an acute phase protein and therefore hepatic rule in C7 synthesis was re-evaluated. *In vivo* studies following orthotopic liver transplant supported the view that liver is the primary site of C6 and C8 synthesis, but not C7 (Hobart *et al*., 1977; Alper *et al*., 1980; Würzner *et al*., 1994). C7 was also detected in 2 siblings after renal transplant. Both of them had homozygous mutation in C7 gene leading to C7 deficiency, but transplant restored haemolytic activity and C7 was maintained at plasma level of 7-9% of normal values. C7 synthesis is believed to be in endothelial cells in the kidney (Rameix-Welti *et al*., 2007).

5.3.3 CH50

CH50 is considered the best single measurement of the functional integrity of the cascade of complement. Haemolytic activity is a useful indicator of complement component deficiencies since homozygous complement deficient individuals usually have abnormal CH50. In general, complement activity must be decreased by more than half to affect CH50 (Pettigrew *et al*., 2009).

The results of the present study show that haemolytic activity CH50 in cord sera was markedly lower than CH50 in adult sera. Fifty per cent haemolysis level was obtained with an average adult serum dilution of 1/22 compared to 1/12 for cord serum (p <0.05) with CH50 cord/adult ratio of 0.54 in keeping with previous studies where CH50 ratio compared to adult levels which was about 60% as shown in Table 5-1 with a range between 43% to 90% (Sawyer *et al*., 1971; Johnston *et al*., 1979; Strunk *et al*., 1979; Shapiro *et al*., 1981;

Notarangello *et al*., 1984; Ferriani *et al*., 1990; Sonntag *et al*., 1998b). Sawyer *et al* were the only team to report a level as high as 90% but there study included only 6 newborn and 4 of them had haemolytic disease of newborn which probably affected the result. The ratio dropped to 60% when compared to maternal CH50 (Sawyer *et al*., 1971). Another group of studies reported lower CH50 in newborn when compared with that of their mothers (range was 24-60%) due to higher CH50% in mothers than in non-pregnant adults (Kohler, 1968; Fireman *et al*., 1969; Adinolfi, 1970; Sawyer *et al*., 1971, Pedraz *et al*., 1980). High maternal CH50 could be related to higher levels of complement components, especially C9 which was reported to be double the level of non- pregnant adults (Adinolfi *et al*., 1981).

In the present work, quantitative analysis of terminal complement components showed that C9 was the most deficient component in cord sera (6.4% of adult level). When cord serum was supplemented with purified C9, CH50 results were restored to adult values despite the lower C6 and C8 levels (34.2 and 25.5 % respectively).

Haemolysis is due to production, deposition, and subsequent action of activated C5-C9 complex on the target cells. Since terminal complement components C6, C8 and C9 were demonstrated to be low in cord sera, it would be reasonable to expect that the observed defect in haemolytic activity of cord sera reflects their inadequate levels. In agreement with this, Adamkin *et al*., 1978 found that cord serum CH50 was more than 2SD below the mean CH50 of adult serum. Addition of purified C5 to C9 to cord serum to obtain final levels equivalent to these components level in normal adult serum only partially restored CH50. Maximal CH50% of cord sera was achieved by adding factor B and properdin along with components C3-C9. A study in newborn rats showed similarly low C9 levels and reduced haemolytic activity that was partially restored when exogenous human C9 was supplemented *in vivo* to newborn rats or added *in vitro* to their serum. Haemolytic activity matures gradually to 80%

in 3 weeks old rats. Moreover, haemolytic activity was better in unsupplemented sera taken from 1 week old rats than in sera from 2 days old supplemented with human C9 which may be explained by species specific affinity of native C5b-8 complex to bind more avidly to rat C9 than to exogenous human C9 (Lassiter *et al*., 1997). In addition, adding purified porcine C7 restored haemolytic activity of C7 depleted human serum in a dose dependent manner which denotes a high degree of functional and structural similarities of C7 in these species (Agah *et al*., 2000).

Fujita *et al*. (1992) in a case report of a 7 year old Japanese boy diagnosed with hereditary C9 deficiency showed that serum haemolytic activity of this boy was 30% of the normal CH50 level. In other reports of patients with hereditary deficiency of complement components, low haemolytic activity was restored when the deficient components were added to the serum. Würzner *et al* 1992 found that previously undetectable CH50 level in C6 deficient patient was restored when purified C6 supplement or normal human serum was added.

In a study from Korea, CH50 of C9 deficient individuals was approximately 30-40% of NHS (Kang *et al*., 2005). In the absence of C9, MAC consisting of C5b through C8 haemolyses erythrocytes, although its efficiency is lower than that of the C5b-9 MAC (Kang *et al*., 2005).

Table 5.1 Percentage of CH50 activity in term newborn relative to maternal CH50 ( ) and healthy adult volunteer serum

Study	Number of newborn studied	CH50 percentage	
Kohler, 1968	23	(60)	
Fireman *et al*., 1969	24	(53)	
Sawyer *et al*., 1971	6-11	(60), 90	(Compared with (Maternal) and adult serum.
Notarangelo *et al*., 1984	12	56	
Johnston *et al*., 1979	27		
Pedraz *et al*., 1980	58	(29)	
Drew and Arroyave 1980	11	52	
Strunk *et al*., 1979	30	81	
Shapiro *et al*., 1981	11 (AGA)	66	
	17 (SGA)	65	
Wolach *et al*., 1997	34	69	
Sonntag *et al*., 1998	125	43 median	
Present work	20	54	

5.3.4 Analysis of the complement killing assay

Humoral and cellular innate immunity are the first line of body defence against invading pathogens. The role of innate immunity is more crucial in newborn infants whose adaptive immune response is not functioning to its full capacity. The established predisposition of neonates to infection promotes extensive research on infants' response to pathogens. My

work assessed the complement mediated cytolytic activity of cord sera compared to adult sera against *Ureaplasma parvum* which was brought to attention lately as an important perinatal pathogen and potential suspect in pathogenesis of CLD.

Ureaplasma which is a commensal organism in the urogenital tract in many asymptomatic people have been reported to be the most commonly isolated organism from mothers especially in preterm labour (Cassel *et al*., 1986, Yoon *et al*., 1998). Abele-Horn *et al.* showed that from 170 mothers positive for *Ureaplasma* colonisation 10% experienced chorioamnionitis compared to none of the *Ureaplasma* negative group. Moreover, 35% of the *Ureaplasma* positive group had premature rupture of the membranes compared to 12% in the control group and 41% delivered prematurely compared to only 10% (Abele-Horn *et al*., 1997). Intrauterine infections and the ensuing inflammatory response results in the production of prostaglandins leading to uterine contractions, cervical dilations and membrane rupture and preterm delivery (Waites, 2005). A number of conditions in neonates have been associated with *Ureaplasma* infection following transmission from the mothers which include meningitis, systemic infection, pneumonia and CLD.

Bactericidal effect of newborn serum on *Ureaplasma parvum* serovars was studied in this chapter in the context of quantitative and functional investigation of neonatal complement system.

The killing assay described in this study was successfully used in our laboratory to identify the bactericidal activity of 12 sera from healthy adult volunteer against different strain of *U parvum* (Beeton *et al*., 2012). Here in, I used the same method to identify the bactericidal activity of 14 cord sera against the four serovars of *U parvum* compared to bactericidal effect of adult sera. The results in this study showed that all cord sera could kill *Ureaplasma* serovar (SV3) equivalently to adult serum even when samples from both groups do not have

specific anti-*Ureaplasma* IgG antibody when tested by immunoblotting. Two cord sera (14 %) killed SV1 by > 1000 fold compared to 5/12 adult (41 %). None of the cord sera could kill SV6 or SV14 in contrast to adult sera where 5/12 samples killed SV6 (41 %) and 4/12 killed SV 14 (33 %). Complement components C6, 8 and 9 were lower in cord than in adult sera as shown in section 5.2.5. However, adding these purified components to compensate for their deficiency in cord sera did not restore its bactericidal activity against SV6 or SV14 killing despite the presence of multiple anti-*Ureaplasma* IgG bands in the cord serum. Of note, adult samples capable of killing SV6 or 14 seem to be bactericidal for all serovars.

Serovars 1 and 6 were the most prevalent serovar isolated from a group of pregnant women who delivered prematurely (Fernández Molina *et al*., 2003; Knox *et al*., 2003; Yi *et al*., 2005). SV6 was the most common serovar identified in semen samples after a standard washing procedure used during assisted reproduction hence SV 6 is more adherent to host cells than other *Ureaplasma* serovars ((Knox *et al*., 2003). This finding is not completely understood but it could be potentially related to serovar pathogenicity and virulence.

Beeton *et al*., 2012 demonstrated that classical pathway is the predominant complement pathway involved in killing of all serovars. In addition, they speculated that killing of SV1, SV6 and SV14 was dependent upon the presence of IgG subclasses1, and 3 whereas killing of SV3 was C1q-dependent, but independent of any detectable anti-*ureaplasma* immunoglobulin.

Benstein *et al*., 2004 previously reported the ability of MBL from multiple species to bind SV3; however, they did not investigate complement activation or bactericidal activity. Minimal role of alternative and lectin pathways in *U parvum* serovar killing is supported by the negligible killing noted in the absence of Ca^{2+} and MBL, respectively. Furthermore, all

killing was abrogated by the addition of a function blocking anti-C1q monoclonal antibody, substantiating the classical pathway as responsible even for SV3 killing (Beeton *et al*., 2012). MBL levels have been reported significantly lower in preterm neonates but no difference was demonstrated between its level in full term neonates and adults (Lau *et al*., 1995). However, ureaplasmacidal effect was recently attributed to surfactant protein A, one of the collectins, which by opsonisation enhances ureaplasmacidal effect of macrophages (Okogbule-Wonodi *et al*., 2011). Moreover, the role of SP-A is illustrated by a study of SP-A knock- out mice with experimental *U parvum* pneumonia where delayed clearance of the infection as well as exaggerated inflammatory response was shown (Famuyide *et al*., 2009). SP-A binds equally to *U parvum* SV1, SV6 and *U urealyticum* serovars. We did not measure the level of SP-A in our study but it is known that SP-A is increased progressively with gestational age (Kaneko *et al*., 2001). However, SP-A does not activate complement and opsonisation for cellular ureaplasmacidal activity would not influence our cell-free killing assay. Other pathogen recognising receptors such as TLRs are engaged in recognising *Ureaplasma* and induction of inflammatory mediators release by macrophage and neutrophils. SV3 activates NF-K^B through TLR2 in cooperation with TLR1 and TLR6 (Shimizu *et al*., 2008).

Bacteriolysis probably plays an important role in intrauterine confinement of infection thus prevention of blood stream invasion by bacteria that are usually sensitive to the complement - mediated bactericidal activity in serum, such as serum killing capacity shown for *E coli* (Lassiter *et al*., 1992).

In normal serum, cytolysis may occur when complement proteins are assembled on the bacterial surface to form the membrane attack complex (C5-9). The process of bacteriolysis may be initiated after activation of the classical pathway or alternative pathway of the

complement. During activation of the classical pathway, C3b covalently binds to the bacterial surface, resulting in the formation of the C4b-2a-3b complex, which has the capacity to cleave C5 to C5a and C5b. Membrane bound C5b may then bind C6 and C7 to form a trimolecular complex, which may insert into the outer membrane surface of the bacteria (Hammer *et al*., 1975). C8 then binds to C5b-6-7 to cause leakage of ions (Kolb *et al*., 1972).The subsequent binding and polymerisation of several molecules of C9, a factor required for the efficient complement- mediated cytolysis, resulting in the formation of a large more stable membrane attack complex (Kolb *et al*., 1972; Joiner *et al*., 1985). Many studies found that the insertion of MAC into the cell membrane may result in sufficient injury to cause death of the bacteria (Joiner *et al*., 1985; Mackay and Danker 1990).

It is reported that there is an absolute requirement for all five components (C5b, 6, 7, 8 and C9) of this lytic complex for killing of a number of gram negative bacteria (Schreiber *et al*., 1979). Studies reported that poly C9 may be required to trigger a process of bacterial inner membrane dissociation (Kroll *et al*., 1983, Taylor and Kroll, 1984).

In adults with genetic deficiency of the proteins that constitute the MAC, investigators reported predisposition to recurrent Gram negative sepsis caused by some species like Neisseria (Würzner *et al*., 1992). The concentrations of C8 and C9 in the serum of human neonates have been reported to be 10-20 % of the concentration measured in serum from adult (Ballow *et al*., 1974) and the result of this chapter agreed with previous reports. The limited ability of neonate serum to eradicate bacteria may be due to insufficient concentrations of one or more of heat- stable complement proteins of the classical or the membrane attack pathway (C5-C9). However, supplementation of deficient components of

cord serum complement C6, C8 and C9 did not alter its Ureaplasmacidal capacity against the tested serovars.

This work on bactericidal capacity of cord serum resembles Lassiter's work on cord serum bactericidal effect against an important Gram negative neonatal pathogen, *E coli. In vitro* studies on cord serum showed that supplement of deficient C9 restored the killing capacity against *E coli* (Lassiter *et al*., 1997). The group did an experimental study on a rat model in which supplemental C9 enhanced the capacity of neonatal rats to kill pathogenic *E coli*. Survival of infected rats was positively correlated to the concentration of C9 and with bactericidal activity of the serum. Intra-peritoneal injection of C9 diminished the intensity of infection and enhanced survival of infected rats but protection was limited because of short half- life of exogenous C9 (Lassiter *et al*., 1997). However, in the present work I did not find any change in the degree of *Ureaplasma* killing capacity of cord serum supplemented with C9. Joiner *et al*. (1985) found that the precise immunological mechanism responsible for the diminished capacity of neonatal serum to kill *U parvum* is not clear yet.

Moreover, in a preliminary step, I added freshly purified cord neutrophils to *Ureaplasma* serovar culture to assess ureaplasmacidal effect of neutrophils on all serovars but no killing was observed. In a previous study, neutrophils isolated from healthy adults were able to phagocytose *Ureaplasma* in absence of antibody opsonisation. However, engulfed *ureaplasma* remained viable after phagocytosis which could be of pathogenetic importance. Adding complement enhanced the release of chemiluminescence and the role of complement is also supported by activation of C1q by *Ureaplasma*.

Killing of *Ureaplasma* serovars is modified not only by host factors but also by serovar variation in resistance to host immune response and on its inherent virulence factors. IgA protease, urease, phospholipase A and C are suggested *Ureaplasma* virulence factors (Glass

et al., 2000). Resistance to host defence and pathogenesis of chronic *Ureaplasma parvum* infection was also linked to difference in serovar ability to form protective biofilm (García-Castillo *et al*., 2008).

5.3.5 Serological status of the cord sera

To determine the serological status of the cord sera, Western Blots containing whole-cell lysates of *Ureaplasma* antigen were probed with 14 cord sera (Figure 5.19). Twelve samples out of the 14 examined sera showed some degree of reactivity, whereas, two were fully seronegative for anti- *Ureaplasma* IgG antibodies. To ensure the negative results were not falsely negative, the blots were subsequently probed with seropositive serum where expected banding was seen, therefore ruling out poor transfer. These seronegative sera failed to kill SV1, SV6 and SV14 but could kill SV3. A variety of different and conserved banding patterns of varying intensities was elicited by seropositive cord sera when blots were probed. High levels of killing were associated with the presence of multiple bands of higher binding intensity in cord samples 5, 6, 9 and14. An exception to this role was the absence of reaction to SV14 isolate HPA32 when exposed to cord 6, 7, 9 and 11 although high molecular weight band around 130KDa was seen. An interesting observation was the banding patterns produced by most cord sera against SV1 isolated DKF-1which was similar to that of adult sera described by Beeton *et al* 2012 who detected bands of 60 and 70 KDa. In a number of cases, such as with cord 14, bands were present but the degree of killing was minimal. This may represent an antibody response which was raised against internal antigens which would not be exposed in intact viable cells but manifested after cell lysis. Of course activation of complement can only occur following binding of the antibody to antigens present upon the surface of bacteria, and as western blot dose not distinguish between surface and internal antigens this could account for this finding.

5.4 Summary

This chapter has described an ELISA assay to measure the levels of terminal complement components and the function activity of the complement system in cord serum compared to adult. The key findings were that newborn serum contains low levels of C6, C8 and C9 with the exception of C7 which was identical to adult level. The haemolytic activity in cord sera was lower than CH50 in adult sera but adding C9 to cord sera restored its CH50 to adult level. Additionally this chapter has described an assay to quantify the bactericidal effects of cord and adult serum upon clinically important serovars of *Ureaplasma parvum.* The bactericidal capacity of neonatal serum compared to adult serum was impaired especially against SV1. SV3 was the most serum sensitive serovar whereas killing of the resistant serovars SV6 and 14 could not be induced by addition of the deficient components C6, C8 and C9.

Chapter Six

General Discussion

6.1 Overview

In this thesis I have sought to compare some aspects of the cellular (neutrophils) and non-cellular (complement) parts of innate immune system in newborns and adults. My research concentrated on the levels and functional activities of the triad of neutrophil proteases (PR3, Elastase and Cat G) released by primary granules, including exocytosis on certain neutrophil granule specific markers. I have sought to examine the differences in neutrophil surface expression of membrane proteinase 3 (mPR3), CD177, CD63, CD66b, CD35 and CD43 in cord and adult neutrophils. I have paid particular attention to the role of different stimulants in up-regulating the expression of these markers. Furthermore, intracellular levels of PR3, CD177, CD63, CD66b, CD43, CD35, SerpinB1, C5aR and C3aR were also examined in the resting neutrophils. Different neutrophil stimulants were used to determine whether any observed difference in release or surface expression of these neutrophil granule contents was due to variation in mobilisation, or related to inherent difference in total cellular amounts for adult and cord neutrophils. I have focused in detail on the functional and quantitative levels of terminal components C6, C7, C8 and C9 in neonates compared to adults. In addition, I have examined the role of complement activation pathways for the killing of *U parvum* in an in *vitro* system.

6.2 Cord blood neutrophil

Little is known about neutrophil function in neonates in relation to adult. Therefore, I studied the effect of maturity on human neutrophils degranulation among full term healthy neonates and adults. CD177 is a specific neutrophil antigen believed to be a high affinity receptor for mPR3 expression on neutrophil surface. CD177 is upregulated during bacterial infection and sepsis and plays a role in different diseases such as immune mediated neutropenia,

transfusion related acute lung injury and polycythemia rubra vera (PRV). Until recently little was known about the function of this receptor on neutrophil. I asked the question whether CD177 expression and other neutrophil markers were affected by maturity. Initially, I have confirmed the finding of previous studies that the adult neutrophil expression of CD177 was bimodal and CD177 expression showed inter-individual variation with the stability of the percentage of the positive CD177 expressing cells per individual even after the cells were activated with different stimuli (Halbwachs-Mercarelli *et al*., 1995; Witko-Sarsat *et al*., 1999; Schreiber *et al*., 2003; Wolff *et al*., 2003). I have demonstrated that CD177 expression was significantly higher in cord neutrophils compared to adult neutrophils and this observation has been reported only in one study before by Stroncek *et al*. (1998). PR3 is a main autoantigen in anti-neutrophil cytoplasmic antibody-associated vasculits and its surface presentation on neutrophils has pathogenic importance. PR3 expression on the surface of cord blood neutrophils has not been published before. The neutrophils with positive expression of both PR3 and CD177 varied considerably between individuals ranging from 0% to 100% but the mean percentages of CD177 and PR3 expression were higher in cord blood than in adult neutrophils. I have demonstrated that the expression of PR3 was very low and monomodal in resting neutrophils in all adult samples and also in some of cord samples. This pattern of expression changed to bimodal in response to different stimuli in agreement with a previous report (Hu *et al*., 2009), although their neutrophil stimulation methods were different and Hu *et al.* only examined adult cells. Herein, I detected mPR3 on the surface of neutrophils of two negative CD177 cord samples which suggests that CD177 was not the sole receptor for mPR3. However, expression of mPR3 in negative CD177 cord samples does not contradict the current understanding that CD177 remains the high affinity receptor for mPR3 expression. The mechanism leading to expression of PR3 on the surface of CD177-deficient neutrophils is not fully understood yet. In retrospect, I could have studied CD177 expression

on the neutrophils taken from the parents of the CD177 negative cord samples to see if it was inherited. Of note, despite expression of mPR3 on neutrophils from negative CD177 cord samples, it was monomodal even after stimulation. CD177 seems to be essential for bimodal expression of mPR3 on neutrophil surface. Further study of more CD177 negative samples is necessary to confirm this observation.

I demonstrated that CD177 and m PR3 expression was rapidly induced on the cell surface of neutrophils following exposure of both cord and adult samples to various stimulants. PR3 was found in primary granules, secondary granules as well as secretory vesicles and plasma membrane (Csernok *et al*., 1990; Halbwachs-Mecarelli *et al*., 1995; Witko-Sarsat *et al*., 1999), while CD177 was reported to be found in secondary granules and its expression was more or less of same intensity on myelocytes, metamyelocytes and segmented neutrophils, but was not found in promyelocytes. Transcription of the gene encoding the CD177 antigen is probably switched off at some myelopoietic stage in CD177 negative samples for those individuals that become bimodal as adults. However, those individuals that lack CD177 on all cells may have other mechanisms underlying the failure to express. Furthermore, the nature of CD177 and PR3 interaction are still unknown and importance of their role in host immunity is not fully understood. CD177 deficient populations have been described without any apparent underlying pathology.

In this study I confirmed the previous observation that term neonatal neutrophils expressed CD35 with an intensity that was similar to adult neutrophils. Shedding in response to stimulation was more pronounced in cord neutrophils. I observed that neutrophils from term neonates expressed significantly less CD35 on the plasma membrane in response to stimulation than neutrophils from adults. However, the percentage of CD35 expressing neutrophils was significantly lower in response to PMA and fMLP + cytochalasin B in adult cells, which could be related to the protease deficit found in neonatal neutrophils (MMP-9,

MMP-8 and elastase; unpublished results). However, only stimulation with F+C caused significant reduction in the percentage of CD35 expressing cord neutrophils, suggesting different methods of shedding may occur between these neutrophil groups.

All stimulants caused significant reduction in CD43 expression on adult neutrophils, particularly PMA. The same effect on CD43 expression was observed in cord blood neutrophils. The explanation for this could be that CD43 was enzymatically cleaved from the surface of activated neutrophils or the shedding could be mediated by neutrophil proteases.

In the present work, no significant difference in neutrophil CD63, CD66b, CD15 and CD16 expression was detected between cord blood and adult neutrophils.

Estimation of intracellular and surface expression of mPR3, CD177 and other markers was performed using flow cytometry; PR3 results were validated with more quantitative ELISA methods for lysed neutrophil PR3 and released supernatant PR3. Unstimulated cord neutrophils contained a higher PR3 content than adult cells, for both methodologies validating the significance of these result. Both methods showed that maximum PR3 release followed stimulation with fMLP + cytochalasin B even in CD177 negative cord samples.

6.2.1 Neutrophil proteases

Since significant function of neutrophil proteases is detected at the surface of activated neutrophils, measuring and quantifying membrane bound activities is of major interest for understanding their role during inflammation. I identified lower Cat G and elastase activity in neonatal neutrophils compared to adult neutrophils. However, similar proportion of each protease could be degranulated from the neutrophils following various pro-inflammatory stimuli for neonatal cells. This implies that lower intracellular stores translate into lower

releasable proteolytic capacity for neonates relative to adults. Neonates have a less mature immune system than adults that renders them more vulnerable to infection. Reduced protease content of neutrophils could be another factor that makes neonates less able to kill bacteria. Since my study examined Cat G and elastase content of a set number of neutrophils by functional assay of individual neutrophils, it was not possible to know whether the neutrophils themselves were smaller in cord blood neutrophils and contained the same density of granules as adults; nor if the neutrophils were the same size but contain smaller amount of proteases. In retrospect I could have put isolated neutrophils through a Coulter counter, a device that detects changes in electrical conductance cross a small aperture as fluid containing cells flow through it. This device measures cellular volume and would have enabled us to examine differences between adult and cord blood neutrophils.

Of note, Cat G functional activity was performed immediately after neutrophil separation. In 4 adult-cord blood neutrophil dyads, Cat G functional activity results were reproducible when the experiment was repeated next day (12 hours after the first experiment), indicating that storing cells and supernatants in these types of studies could have been batched for investigation without loss of activity.

All of cord blood samples in this work were obtained from full term newborn. Other studies have shown that differences in neutrophil function are greater in immature preterm neonatal cells, but as the ethics available for this study included only term newborns, cord samples from preterm neonates were not studied.

6.3 Chronic lung disease

Majority of spontaneous preterm delivery is associated with perinatal infection and premature neonates are particularly vulnerable to infection. A recent comprehensive meta-analysis that included 59 studies and 15,295 infants reported on association between chorioamnionitis and

development of CLD although it showed a significant publication bias leading to exaggeration of the magnitude of the association (Hartling *et al*., 2012).

Perinatal infection is implicated in the complex pathogenesis of CLD. Microbial infection of the lung in preterm ventilated infants leads to influx of inflammatory cells and release of pro-inflammatory cytokines. The role of *Ureaplasma* species in CLD pathogenesis was studied first in animal models. Response has varied from one study to another, possibly due to differing virulence of studied *Ureaplasma* serovars and host species differences. Moreover, humans are the specific host for *U parvum* and *U urealyticum* hence less prominent reaction in animal model is not surprising (Viscardi, 2012).

Neutrophil proteases and other proteolytic enzymes (MMP-8, MMP-9) degrade tissue matrix and the prolonged inflammation may impair the repair process in the critical window during lung development resulting in the characteristic abnormal lung growth found in new CLD. MMP-8 or neutrophil collagenase which has a major role in the proteolysis of collagen-I was elevated in BAL fluid from babies who eventually developed CLD (Sweet *el al*., 2004). Collagen-I is an important structural protein that provides the tensile strength in extracellular matrix (Davidson, 1990).

The action of these enzymes is regulated by different inhibitors and balance between proteases and their inhibitors is essential to prevent lung tissue damage. Neutrophil proteases have been linked to CLD in preterm newborn infants and the precursor acute RDS has been shown to be neutrophil-dominant pathology (Arnon *et al*., 1993). It is likely the persistence of neutrophils and their impact on enhancement and perpetuation of the ongoing inflammatory process allows for the development of the lung injury that characterises CLD (Kotecha *et al*., 2003).

I examined PR3 levels in BAL fluids from premature neonates, previously examined for MMP-9 and elastase levels (Davies *et al*., 2010). PR3 levels in serial BAL samples taken from this cohort were assessed using ELISA, although the complete set of samples was not available for analysis because they had been used up for the previous study by our laboratory. The data from this cohort study has shown that the peak in total PR3 levels was higher in infants who develop CLD compared to those babies with resolved RDS and higher than the ventilated term control group. No significant difference was detected between PR3 in term babies and resolved RDS patient samples. PR3 elevation in BAL fluid from preterm newborns who went on to develop CLD was episodic and coincided with peaks in elastase and MMP-9 levels in the same samples. This common episodic pattern was not fully explained but could be triggered by infection.

Previously more emphasis was put on the role of elastase in CLD but the contribution of PR3 seems to be underestimated in CLD. Elastase and PR3 are present in the same neutrophil granules so they should be released together when neutrophils are activated. In addition, PR3 was the most abundant of the neutrophil proteases with each adult neutrophil estimated to store 3pg of PR3, compared to 1.1 pg of elastase and 0.85 pg of CatG, respectively (Campbell *et al*., 2000). Furthermore, PR3 may be harder to regulate: affinity of AAT is much higher for elastase than for PR3 (Rao *et al*., 1991; Korkmaz *et al*., 2005) and it was predicted that 89% of AAT would bind elastase whereas only 11% would bind PR3 (Rao *et al*., 1991). In addition, PR3 is not inhibited by SLPI or antichymotrypsin while it is only minimally inhibited and transiently bound by elafin (Rao *et al*., 1991; Ying and Simon., 2001). In contrast to membrane bound elastase, mPR3 was resistant to inhibition by AAT (Korkmaz *et al*., 2005). The net result is that released PR3 which can degrade a wide range of lung matrix protein remains unopposed in the lung.

*Ureaplasm*a colonised neonates were more likely to develop CLD than *Ureaplasma* negative patients in a cohort of 230 preterm newborns (< 32 weeks) ventilated for 2-32 days (Viscardi, 2012). Updated strategies proposed to prevent CLD revolve around reducing the damage caused by proteases and on treating the inciting infection or colonisation. Protease inhibitors have been the focus of studies examining prevention of neutrophil protease harmful effects on developing lung. Multiple protease inhibitors may be a useful therapeutic intervention; however, further work is required to find inhibitors of choice with greater inhibitory effect and less susceptiblity to proteolytic degradation. Extensive work was done on neutrophil elastase interaction with inhibitors and the search was extended in the last years to find appropriate synthetic inhibitors that can inhibit the main proteases and improve the method of administration for optimal efficacy (Matsuzaki *et al*., 2005; Okayama *et al*., 2006).

Antibiotic treatment has also been of interest for prevention of pathogenesis of CLD. Earlier studies on erythromycin did not show any positive effect on prevention (Bührer *et al*., 2001; Baier *et al*., 2003). A newer generation of macrolide, azithromycin, which purportedly has anti-inflammatory properties, was shown to be capable of inhibiting production of pro-inflammatory cytokines by neutrophils and monocytes from tracheal aspirates of premature neonates (Aghai *et al*., 2007). Azithromycin, in a pilot randomised placebo-controlled double blind study, also reduced the use of steroids and duration of hospital stay and ventilation in the treated neonates. These effects were due to its anti-inflammatory effect and ability to alter the innate immune response to infection since *Ureaplasma* infected or colonised infants were excluded for some reason (Ballard *et al*., 2007). A more recent study by the same group has shown some benefit in the *Ureaplasma* colonised or infected subgroup of very low birth weight infant when treated with azithromycin where the odds ratio for CLD or death decreased to 0.026, 95% CI 0.001-0.618 (Ballard *et al*., 2011). A similar recent study on

Ureaplasma culture positive preterm newborns (birth weight 750-1250 g) concluded that clarithromycin (another related macrolide) prevented the development of CLD (Ozdemir *et al*., 2011). However, macrolide resistance has been noted in some *Ureaplasma* strains (particularly in the US and China), which should be regarded when considering prophylactic role of antibiotics as indiscriminate use of antibiotic could promote emergence of resistant bacteria. Moreover, other potential concerns are raised with macrolide antibiotics including hepatotoxicity and hearing impairment (Brown *et al*., 1997).

6.4 Complement system and *Ureaplasma*

Ureaplasma has recently been shown to be killed by adult human serum (Beeton *et al*., 2012). Therefore, an in vitro experiment was prepared to assess the role of membrane attack complex (MAC) mediated killing of the complement system using cord serum. The ureaplasmacidal capacity mediated by complement activity of cord serum was compared to adult serum against *Ureaplasma parvum*. Terminal complement components responsible for (C6-C9) forming the membrane attack complex (MAC) on the target cells, leading to pathogen cell lysis were compared. In chapter 5, I demonstrated that cord sera are deficient in C6, C8 and C9. A significant finding was that all cord sera could still kill *Ureaplasma parvum* serovar (SV3) equivalently to adult serum, irrespective of whether the serum contained specific anti-*Ureaplasma* IgG antibody when tested by immunoblotting. However, none of the cord sera could kill SV6 or SV14, unlike some adult sera. Some degree of killing was observed in SV1 although it was lower than the degree of killing noticed in adult serum. Beeton *et al.,* 2012 demonstrated that classical pathway was the predominant complement pathway involved in killing of all serovars. In addition, they speculated that killing of SV1,

SV6 and SV14 was dependent upon the presence of IgG subclasses 1 and 2 whereas killing of SV3 was dependent, in half of their samples on IgG3.

In normal serum, cytolysis may occur when complement proteins are assembled on the bacterial surface to form the membrane attack complex (C5-9). In this study I have confirmed previously published findings which showed that the concentrations of terminal complement components in newborn serum were significantly lower than adult level, with only one exception, C7, which was identical to the adult level. Although my results followed the trend of previous published work, I used more accurate methodology to obtain reference values compared with the older semi-quantitative methods previously used which are no longer used for clinical or research purposes (Davis *et al*., 1979; Johnston *et al.,* 1979; Drew and Arroyave, 1980; Sonntag *et al*., 1998b).

To optimise the sensitivity and specificity of my measurements, I tested panels of monoclonal antibodies for each component to be measured from commercial and non-commercial sources. Where necessary, sensitivity and range of assay were enhanced by immobilising monoclonal antibodies with rat anti-mouse IgG1 monoclonal antibody. In addition amino acid hydrolysis was used to accurately quantify affinity purified C6, C7, C8 and C9. The refined method was used in adult samples to evaluate the level of these components. Subsequently, analytical work was extended to test the terminal components levels in international pooled adult serum in order to provide a readily available revised reference levels for C6-C9.

Since the killing capacity of cord serum against *Ureaplasma* was lower than adult serum as shown in chapter 5, we postulated that this observation was related to the lower level of terminal complement components found in cord serum, compared to adult serum, particularly C9. However, adding purified C9 or adding a combination of C6, C8 and C9 to compensate

for their deficiency in cord sera did not restore its bactericidal activity against SV6 or SV14 killing, despite the presence of multiple anti-Ureaplasma IgG bands in the cord serum. Of note, adult serum samples capable of killing SV6 or 14 seem to be bactericidal for all serovars.

In a previous study, neutrophils isolated from healthy adults were able to phagocytose *Ureaplasma* in absence of antibody opsonisation. However, engulfed *ureaplasma* remained viable after phagocytosis which could be of pathogenetic importance (Taylor-Robinson *et al*., 1986). In a preliminary step, I added freshly purified cord neutrophils to *Ureaplasma* culture to assess ureaplasmacidal effect of neutrophils on all serovars but no killing was observed. Neutrophil killing of *Ureaplasma* needs to be addressed in future work using markers that can be detected by flow cytometry or similar methodology to detect the dynamics of neutrophil *Ureaplasma* phagocytosis and killing capacity.

Haemolytic activity is a useful indicator of complement component deficiency since homozygous complement deficient individuals usually have absent haemolytic functional activity (CH50). In general, complement component concentration must be decreased by more than half to affect CH50 (Pettigrew *et al.*, 2009). In this study I have confirmed the finding of previous work that cord serum has significantly lower complement haemolytic activity CH50 than adult serum (Shapiro *et al*., 1981; Notarangello *et al*., 1984; Ferriani *et al*., 1990; Sonntag *et al*., 1998b).

In agreement with previous reports (Davies *et al*., 1979; Adinolfi *et al*., 1981; Hoggasen *et al*., 2000) C9 was the most deficient component in cord sera (6.4% of adult level). Supplementation of cord serum with C9 alone was adequate to restore CH50 to adult values despite the lower C6 and C8 levels (34.2% and 25.5 % of adult values respectively).

The observed deficiency in neonatal complement function and the lower levels of multiple terminal complement components which decreased ability to form MAC required for effective bacteriolysis is likely to increase the risk of serious neonatal infection. Evidence of enhanced complement activation suggests a role in adverse pathological consequences in certain neonatal conditions; however, the exact mechanisms underlying activation are still undetermined. Further work on the neonatal complement system especially in premature infants is necessary for better management and therapy of these high risk infants.

6.5 Future Research

This thesis has raised a number of questions that need further investigation. Although PR3 is known to have the potential to promote inflammation and injure tissues, the biologic forms and the function of PR3 in neutrophils from healthy term and adults donors have received little attention. In this work I have shown that the expression of CD177 and PR3 was higher in term infants than adult. It would be interesting if further investigation included cell surface expression of PR3 and CD177 on neutrophils from premature infants.

Furthermore, neonates appear to be fairly uniform in CD177 expression at birth, relative to bimodal expression in adults; it would be useful to follow developing children to observe when the differentially expressing subsets emerge and the nature of the gene regulation that is responsible for this phenomenon. Functional studies should also focus on physiological role of CD177 expression on neutrophils and its interaction with PR3, relative to their antimicrobial function and ability to leave the vasculature in response to inflammation as well. Expression of CD177 in neutrophil haematopoietic stages could also be examined and linked with expression of PR3 in the developing lineage.

All our in vitro studies of neutrophil and complement were performed using cord blood from term infants collected following elective Caesarean section. I would like to assess the impact of decreasing gestational age on the ability of degranulation of neutrophils particularly the PR3 and CD177. In addition, study the ability of complement system in those preterm infants to kill *ureaplasma.* Further research on neutrophil proteases elastase, Cat G and PR3 studied herein would benefit from set groups in which neonates are divided according to gestational age, including extremely preterm infants between 23-28 weeks gestation in the presence of healthy adult group. This is of particular interest as the major site of haematopoesis changes from the liver to the bone marrow through this time period and could have a dramatic effect on the function and composition of neutrophils through these times

Working with preterm cord blood will be more challenging than term infants because significantly preterm infants are not often delivered by elective Caesarean section unless necessitated by foetal compromise or maternal ill health, so samples might not be easily obtained from this group. In addition, extremely preterm or severely compromised foetus frequently has a very small, thin umbilical cord which will yield only a very small blood sample therefore neutrophil separation might be difficult with the current methodology that need larger sample volumes.

Measurement of the exclusive functional activity of PR3 will require optimising a specific substrate for PR3 which is so far not available (PR3 substrates are readily cleaved by elastase). The results in this thesis showed that the expression of PR3 and its level in term infants was higher than that in adults. Study of the functional activity of this proteinase would be beneficial to see whether the high concentration reflects high functional activity or not

compared with adults. Serine protease triad content and activity should be studied also in preterm cord blood neutrophils.

Other categories of proteases and their inhibitors would also be of interest in the context of CLD include matrix metalloproteinases such as MMP-8 as well as cysteine proteases such as Cathepsin B, H, K, L and S which has not been studied in detail for premature and full term neonates compared to adults. Better understanding of the complex interaction between proteases and their inhibitors would facilitate effective prevention of proteolytic lung injury.

The role of PR3 in the pathogenesis of CLD in preterm infant has not been studied before and this would be an area of interest for further investigation. This could be investigated further using animal models where bacteria could be instilled into the lung to cause infection or by delivering protease directly to preterm animals lungs for brief period but otherwise giving optimal care including surfactant and antibiotic therapy and then to see if new CLD changes occur in the lung. The sheep model has been particularly well developed. However, only mice can be used in gene targeting to create PR3 knock-out animals to allow more definitive characterisation of the in vivo role of this enzyme in pathogenesis of CLD.

Having defined reference values of terminal complement components for term infants and normal healthy adults, the next step should be to evaluate the levels of these components in preterm neonates of different gestational age groups to allow accurate comparisons between studies and analysis using current most accurate methodology. However, preterm neonates can often have an underlying microbial trigger that could lead to activation and consumption of complement, which will have to be adequately controlled for in these studies.

As part of the terminal complement pathway, C5b should have been included in the investigation for the full complete terminal components profile in term and preterm newborns vs. adult serum.

This report on the Ureaplasmacidal capacity of cord sera against *U parvum* could be expanded more using term and preterm cord serum Ureaplasmacidal capacity on additional strains of clinical importance of these serovars which were not investigated here. Further work should also test Ureaplasmacidal capacity of cord sera on serovars of the other important species, *U. urealyticum* to determine any variation in their susceptibility to complement mediated killing.

In addition, further study on the mechanisms underlying variation in susceptibility to complement mediated killing should be undertaken. Possible mechanisms already exploited by many other pathogens, such as serum resistance through binding serum complement regulators to evade complement mediated killing, should be investigated. It would be of interest to explore the possibility of complement evasion by serovars, SV6 and SV 14 of *Ureaplasma* serovars to escape the ureaplasmacidal action of serum.

6.6 Final summary

In this thesis I sought to understand the effect of maturity on human neutrophils and complement function among neonate and adults. Neutrophils from term cord blood express more PR3 and CD177 on the cell surface than neutrophils from adult, but the amounts of surface CD66b, CD63,CD15, CD16, CD35 and CD43 expression were not significantly different even after stimulation.

I have shown that cord blood neutrophils deficient CD177are able to effectively express PR3 on their surface suggesting that CD177 was not the sole receptor for mPR3. Significant differences was demonstrated in the intracellular expression of both CD177 and PR3 in cord blood neutrophils compared to adult neutrophils, whereas, no obvious variations were detected in the expression of SerpinB1, C3aR, C5aR, CD66b, CD63, CD35 and CD43.

Neutrophils from cord blood contained less elastase and Cat G than neutrophils isolated from adult blood but a similar proportion of protease was released following proinflammatory stimulation.

Longitudinal analysis of BAL fluid samples from infants at risk of developing CLD demonstrated that PR3 concentration was significantly greater in infants who develop CLD compared to infants with resolved RDS. I have found significant relationships between episodic spikes of PR3 and MMP-9 in BAL fluid in both preterm infants at risk of developing CLD and in resolved RDS patients.

In the complement chapter, term neonates were found to have lower CH50 and lower levels of terminal complement component (except for C7) concentrations when compared to adult values.

Variation in sensitivity of *U. parvum* to bactericidal action of adult and cord serum showing that SV3 was the most sensitive serovars in both cord and adult serum while SV6 and 14 were resistant. The killing in adult serum was obviously more than cord serum in SV1. Substituting deficient complement component with purified proteins did not restore the bactericidal capacity of cord serum against SV6 and SV14 but supplementation of cord serum with purified C9 restored the CH50 to adult values.

Chapter Seven

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Appendix 1

Parents information leaflet for cord blood neutrophil study

INFORMATION SHEET Version 4 (17th February 2010) – HEALTHY ADULT CONTROL

Principle Investigators: Professor Sailesh Kotecha, Consultant Neonatologist
Contact Details: Neonatal Unit, 029 20 74 3374

Study Title

Developmental regulation of neonatal neutrophils and monocytes function.

Invitation

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

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

Thank you for reading this leaflet.

Part 1

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

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

1.2. **Why have I been chosen?**

We wish to obtain blood samples from three groups:

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

Because you are a healthy adult, we would like to obtain 20 – 30 mls (one tablespoonful is 10 ml) to use as controls for our experiments and compare the results with those from babies born on time or prematurely.

1.3. **Do I have to take part?**
It is up to you to decide whether or not you want to take part. If you do decide that you would like to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

1.4. **What will happen if I take part?**
We would like to obtain blood (20 – 30 ml) from your arm to use for our studies.

1.5. **What are the risks of taking part?**
Blood samples are routinely taken by our team. It usually results in a sharp pin prick on insertion of the needle.

1.6. **What are the benefits of taking part?**
We cannot promise that the study will help you but the information we get from this study may help us to improve the treatment of premature babies in the future.

1.7. **What if there is a problem?**
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

1.8. **Will my taking part be kept confidential?**
Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

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

Part 2

2.1. **What will happen if I don't want to carry on with the study?**
If you decide to withdraw from the study, we would like to use the data collected up to your withdrawal. Any stored blood samples that can be identified as yours will be destroyed if you wish.

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

2.3. **What will happen to the results of the study?**
We will publish the results in reputable medical journals and present the data at scientific and medical meetings. Your name and details will NOT be revealed at any stage. Please let us know if you would like a copy of the report.

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

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

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

2.5. **Will my taking part be kept confidential?**
All information which is collected about you during the course of the research will be kept strictly confidential. All data collected will be processed on a secure, password-protected computer at Cardiff University. Any information about you which leaves the hospital will have your name removed so that you cannot be recognised from it. We will assign a number to each subject and use this to label the samples obtained for the study.

2.6. **Who is paying for the study?**
The studies are being funded by the Wellcome Trust and Arriva Pharmaceuticals.

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

2.8. **Who can I contact for further information?**
You may contact Professor Sailesh Kotecha by telephoning 029 20 74 4187 or by email (***KotechaS@Cardiff.ac.uk***) to: ***Professor Sailesh Kotecha, Neonatal Unit, Heath Hospital, Heath Park, Cardiff CF14 4XN.***

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

Dr Salima Abdulla
Clinical Research Fellow

Professor Sailesh Kotecha
Consultant in Neonatal Medicine

CONSENT FORM – HEALTHY ADULT CONTROLS

Developmental regulation of neonatal neutrophils and monocytes function.

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

Please initial box

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

2. I understand that my participation is voluntary and that I am free to withdraw ☐ at any time.

3. I consent to my blood being used ☐

_____	_____	_____
Name	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient, 1 for researcher

Appendix 2

Abstracts and publications from this work

Poster - International Organisation for Mycoplasmology (IOM) July 2012 -Toulouse – France

Despite developmental complement deficiency, neonatal serum is bactericidal for Ureaplasma serovar 3, but complement component supplementation and addition of pooled IgG fails to induce killing of serovars 6 and 14.

Salima Abdulla, Sailesh Kotecha, O. Brad Spiller
Cardiff University, School of Medicine, Institute of Molecular and Experimental Medicine, Cardiff, CF14 4XN, U.K.

Compelemnt is a cornerstone of innate immunity, as well as modulating and enhancing humoral and cellular immunity. However, normal term neonatal serum is disadvantaged by a developmental insufficiency of lytic complement components, which is worsened by premature birth. Furthermore, normal neonatal serum contains only IgG transferred from the mother and low innate IgM levels. *Ureaplasma spp*. remains one of the most common infectious agents associated with premature labour and preterm newborns have higher rates of pulmonary *Ureaplasma* infection. We have previously characterised *Ureaplasma parvum* killing by adult serum: uraplasmacidal activity for *Ureaplasam serovars* (SV) 1, 6 and 14 required specific anti-ureaplasmal IgG and classical complement pathway activation, while SV3-killing was complement-independent, but IgG-independent. Here we extend these studies to neonatal serum.Complement function assay, *Ureaplasma* killing and new ELISA methods were used to compare serum from 20 adults and 20 uncomplicated term caesarean-delivered newborn umbilical cord serum. The ability of neonatal and adult serum to kill *Ureaplasma* SV3 were equivalent despite neonatal serum C6 and C8 levels being 25-30% and C9 levels only 6.6% of adult levels. Further, 7 of 14 neonatal sera could kill SV1, but none could kill SV6 or SV14. Reconstitution of the low complement components to adult concentrations corrected the functional neonatal serum deficit as measured by clinical assay, but did not increase the killing for SV1, 6 or 14. All but 2 of the neonatal sera contained anti-*Ureaplasma* antibodies reactive with all by immunoblot and addition of commercial purified pooled IgG with complement components to neonatal sera did not increase *Ureaplasma parvum* killing. However, complement mediated killing for SV3 could be removed by depletion of IgM with a specific immune-affinity column, suggesting that low levels of innate IgM in neonatal serum are sufficient to mediate ureaplasmacidal complement activation.

Poster - Postgraduate research day November 2011

PR3 expression and release by cord and adult neutrophils and its level in bronchoalveolar fluid.

Salima Abdulla, Sailesh Kotecha, Philip L Davies, O. Brad Spiller

Department of Child Health, Cardiff University, School of Medicine.

Background Proteinase 3 (PR3) is serine protease, ubiquitous in neutrophil granules and present on plasma membranes of CD177-positive neutrophils. PR3 in the inflamed lung may play a key role in host defence and airway remodelling.

Method Neutrophils isolated from ten paired cord and adult blood samples were stimulated with fMLP, Cytochalasin B (CytoB), lipopolysaccharide (LPS), IL-8 and phorbol ester (PMA). Flow cytometry was used to evaluate PR3 expression on unstimulated neutrophils and also in response to stimulants. PR3 ELISA assay was performed on supernatants and cellular fractions. PR3 level was also measured in bronchoalveolar lavage (BAL) samples obtained from ventilated preterm infants (<32 weeks gestation) and term controls.

Result unstimulated adult and cord neutrophils showed monomodal positive expression of membrane-bound PR3 (mPR3) whereas stimulation with fMLP, PMA and fMLP in combination with CytoB resulted in a biomodal pattern of mPR3 expression. Mean unstimulated cord neutrophil mPR3 expression (78.021±27.7) was significantly higher than that of unstimulated adult neutrophils (16.91±3.2; p = 0.0367). Following stimulation the mean mPR3 expression in both adult and cord cells was higher than on unstimulated cells. Cord neutrophils contain 6.25 ± 0.95 pg/cell of PR3 while adult neutrophil level was 3.71± 0.49 pg/cell. The proportion of PR3 released by cord and adult neutrophils was similar. Peak concentration of PR3 was significantly higher in BAL from infants who developed CLD (median 6.05 μg/ml) compared to preterm infants whose RDS resolved (3.44 μg/ml) and term control (0.74 μg/ml) (*P* 0.0096, 0.0076 respectively).

Poster - 13th European Meeting on Complement in Human Disease, Leiden, August 2011

Despite relative C9-deficiency, neonatal serum is bactericidal for Ureaplasma serovar 3, but addition of C6-C9 and specific IgG fails to induce killing of serovars 6 and 14.

Abdulla,S,, Hakobyan, S., Kotecha, S., Brad O, Spiller
Cardiff University, School of Medicine, Cardiff, UK,

Background: *Ureaplasma* remains one of the most common causes of premature labour and pulmonary *Ureaplasma* infection in preterm neonates. We previously demonstrated IgG1 and C1q dependent killing of *Ureaplasma serovars* (SV) 1, 6 and 14 for adult serum, but SV3 C1q-dependent killing was IgG-independent. Normal term neonatal serum has only maternal IgG, low non-specific IgM levels and a relative deficiency of C6, C8 and C9.

Results: Neonatal C6 and C8 levels were 25-30% of adult serum concentration and C9 levels were 6.6% of adult levels, however, ability of neonatal and adult serum to kill *Ureaplasma* SV3 were equivalent. Thirty percent of neonatal serum could kill SV1 and none could kill SV6 or SV14. Reconstitution of C6-C9 to adult levels corrected the CH50 deficit, but did not increase the killing for any of the serovars, despite measurable *anti-Ureaplasma* antibody presence in neonatal serum or addition of exogenous antibody. Killing results for SV3 suggest that low levels of immature IgM in neonatal serum are sufficient to mediate bactericidal activity.

Conclusion: Bactericidal deficit of neonatal serum has been described, and C9 relative deficiency is suspected to be a cause. Here we find *Ureaplasma* SV3 is unusually sensitive to neonatal serum, while killing of SV6 and 14 could not be induced by addition of purified C6, C8 and C9 with or without addition of anti-*Ureaplasma* antibodies. C1q-dependent killing of *Ureaplasma* SV3 is IgM-dependent, even in neonatal serum, and the target for IgM is likely carbohydrate that is serovar-specific

Poster - 13th European Meeting on Complement in Human Disease, Leiden, August 2011

Optimising measurement of terminal complement components in adult and newborn serum by ELISA by refining capture antibodies and purified standards.

Abdulla,S[1]., Hakobyan, S[1]., Sexton, P[2]., Kotecha, S[1]., Brad Spiller[1],O
1- Cardiff University, School of Medicine, Cardiff, UK, 2- Quidel Corporation, Santa Clara, CA, USA

Background: Accurate measurement of terminal complement components has been limited by using subjective methodology and use of poorly characterised standards.

Methods: State-of-the-art ELISAs were created for measuring C6, C7, C8, and C9, choosing the most sensitive monoclonal antibody from commercial and non-commercial sources. Where necessary, sensitivity and range of assay were enhanced by immobilising monoclonal antibodies with rat anti-mouse IgG1 monoclonal antibody. Amino acid hydrolysis was used to accurately quantify affinity purified C6, C7, C8 and C9. Confidence intervals for complement concentration were determined in 20 normal adults and 25 cord sera from uncomplicated caesarean-delivered term newborns.

Results: Adult C7 levels were 37.77 ± 2.89 mg/L, comparable to neonatal C6 levels of 38.83 ± 3.15 mg/L, while neonatal C6 (9.4 ± 1.00 mg/L) and C8 (12.5 ± 1.15 mg/L) levels were 25-30% of adult C6 (30.52 ± 1.55 mg/L) and C8 (50.7 ± 3.50 mg/L) levels ($p<0.001$). Neonatal C9 levels were very low (2.47 ± 0.60) compared to adult levels (37.31 ± 4.80 mg/L; $p<0.01$) and supplementation with purified C9 alone corrected the poor CH50 values for neonatal serum. All antibodies were able to detect their relative components in purified soluble C5b-9 complex to varying degrees; however, some anti-"neoepitope"C9 antibodies recognise purified C9 that was not denatured and retained function.

Conclusion: Optimised sandwich ELISA methods with definitively quantified ultrapure standards allows the determination of true confidence intervals for complement component concentration. These methods will be applied to the international standard serum pool to provide a readily available standard source.

Poster - South Wales and South West Microbiology Forum September 2010

Comparative Bactericidal deficit of neonatal and adult serum complement against *Ureaplasma parvum*

Salima A. Abdulla, Michael L. Beeton, Sailesh Kotecha, and O. Brad Spiller
Cardiff University, School of Medicine, Department of Child Health, Cardiff, CF14 4XN

Introduction: The complement system is essential for opsonisation and killing of pathogens. Infection remains a major cause of morbidity and mortality in newborns and their susceptibility is partly attributed to functional and quantitative deficits in innate immunity including the complement system. Little is known about the bacteriocidal role of complement in neonates against *Ureaplasma* (a major cause of premature birth and chronic lung disease).

Methods: The bactericidal capacity of adult compared to cord sera against 4 serovars of *Ureaplasma parvum* was determined. *Ureaplasma* survival (in colour changing units) was measured following incubation for one hour at 37 C with a 6.5- 50% titration of adult and cord sera as well as a heat inactivated matched control. ELISA assays were developed to measure C6-C9 terminal lytic complement components, and were supplemented where determined to be lower than adult levels. Degree of killing was determined by comparing fold decrease of *Ureaplasma* survival following incubation with heat inactivated versus normal sera.

Results: All cord sera could kill SV3 equivalently to adult serum. Six cord sera out of 15 (compared to 7/12 adult sera) killed SV1 and none of the cord sera could kill SV6 or SV14 (compared to 5/12 and 4/12 adult serum). Normal neonatal C6 levels were 37.7%, C8 levels were 33% and C9 levels were 8.7% of adult values; however, supplementation of cord sera to adult levels did not restore SV6 or SV14 killing. Immunoreactivity to SV6 and SV14 was as prevalent in cord sera as in adults by Western blot.

Conclusion: Cord sera had strong bactericidal activity for SV3, less killing effect upon SV1 and no bactericidal effect on both SV6 and SV14. Correcting complement components C6, C8 and C9 levels (or adding exogenous IgG) had no effect on bactericidal activity of cord sera.

Poster - I3-IRG Meeting 2010

Optimising measurement of terminal complement components in adult and newborn serum by ELISA by refining capture antibodies and purified standards.

Abdulla,S[1]., Hakobyan, S[1]., Sexton, P[2]., Kotecha, S[1]., Brad Spiller[1],O
1- Cardiff University, School of Medicine, Cardiff, UK, 2- Quidel Corporation, Santa Clara, CA, USA

Background: Accurate measurement of terminal complement components has been limited by using subjective methodology and use of poorly characterised standards.

Methods: State-of-the-art ELISAs were created for measuring C6, C7, C8, and C9, choosing the most sensitive monoclonal antibody from commercial and non-commercial sources. Where necessary, sensitivity and range of assay were enhanced by immobilising monoclonal antibodies with rat anti-mouse IgG1 monoclonal antibody. Amino acid hydrolysis was used to accurately quantify affinity purified C6, C7, C8 and C9. Confidence intervals for complement concentration were determined in 20 normal adults and 25 cord sera from uncomplicated caesarean-delivered term newborns.

Results: Adult C7 levels were 37.77 ± 2.89 mg/L, comparable to neonatal C6 levels of 38.83 ± 3.15 mg/L, while neonatal C6 (9.4 ± 1.00 mg/L) and C8 (12.5 ± 1.15 mg/L) levels were 25-30% of adult C6 (30.52 ± 1.55 mg/L) and C8 (50.7 ± 3.50 mg/L) levels ($p<0.001$). Neonatal C9 levels were very low (2.47 ± 0.60) compared to adult levels (37.31 ± 4.80 mg/L; $p<0.01$) and supplementation with purified C9 alone corrected the poor CH50 values for neonatal serum. All antibodies were able to detect their relative components in purified soluble C5b-9 complex to varying degrees; however, some anti-"neoepitope"C9 antibodies recognise purified C9 that was not denatured and retained function.

Conclusion: Optimised sandwich ELISA methods with definitively quantified ultrapure standards allows the determination of true confidence intervals for complement component concentration. These methods will be applied to the international standard serum pool to provide a readily available standard source.