The Role of Fas/FasL, Inflammatory Mediators and LPS-activated Macrophages in Human Neutrophil Apoptosis

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Contents

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Preface

The neutrophil is the first haemopoetic cell to arrive at the site of infection. In acute respiratory distress syndrome (ARDS), dense neutrophilic infiltrates are found in the lung in response to bacterial infection as well as generalised inflammatory stimuli, such as pancreatitis. At sites of infection, phagocytosis of bacteria by neutrophils enhances their subsequent apoptosis and clearance by macrophages however at inflammatory sites, the lifespan of the neutrophil is influenced by both pro- and anti-apoptotic factors in the inflammatory milieu. Furthermore subsequent macrophage phagocytosis of apoptotic neutrophils induces the macrophage to switch to an anti-inflammatory phenotype thereby hastening resolution of inflammation.

The Fas death receptor pathway is important in T lymphocyte apoptosis but its role in neutrophil apoptosis is controversial. We have shown that neutrophils express the Fas receptor (CD95) on their surface but there is no evidence of expression of its natural ligand (FasL). An agonistic anti-Fas monoclonal antibody (CH-11) accelerated neutrophil apoptosis under certain culture conditions. Lipopolysaccharide (LPS) originating from Gram-negative bacteria is often found at sites of inflammation. We have shown that LPS attenuated CH-11 - induced neutrophil apoptosis unless the Fas/FasL death receptor pathway was activated prior to the LPS signalling pathway. This LPS-mediated attenuation did not involve the p42/44 ERK, protein kinase C or phosphatidylinositol 3-kinase signalling pathway however the p38 MAPK and NF-κB pathway appeared to be partially involved. We have shown that neutrophils express the protein $cFLIP_s$ and that CH-11 and inflammatory mediators altered its expression.

Although macrophages are principally phagocytes, they are also important in determining the composition of the milieu at an inflammatory site. Macrophages have been shown to express FasL which can be shed and may contribute to the pools of sFasL found in the bronchoalveolar lavage fluid (BALF) in ARDS patients. We have shown that the conditioned supernatants from LPS-activated macrophages induced neutrophil apoptosis at early time points. The pro-apoptotic activity was mediated by TNF-α and was found in the fraction containing proteins with molecular

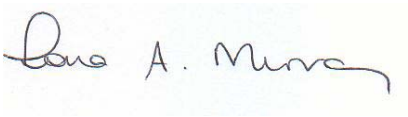
weights greater than 50kD. Macrophage phagocytosis of apoptotic neutrophils suppressed TNF-α production by LPS-activated macrophages and this was associated with loss of the pro-apoptotic activity.

In summary, our data suggest that Fas/FasL fratricide does not appear to be involved in spontaneous neutrophil apoptosis. However LPS attenuates Fas-induced apoptosis unless the Fas/FasL death receptor pathway is activated prior to LPS signalling pathways. The signalling pathways involved in this attenuation are not clear but may involve cellular FLIP. Furthermore, activated macrophages secrete inflammatory mediators and at early time points, TNF-α appears to be the most important in inducing neutrophil apoptosis.

Author's declaration

The experimental design of the work presented in this thesis was by me and by my supervisors, Dr Simon Brown and Professor Adriano Rossi. All experimental work was performed by me.

I declare that this thesis has been composed by me and is a record of the work performed by me. This thesis has not previously been submitted for a higher degree.



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Abstracts arising from this work

Fas-mediated apoptosis in the human neutrophil is attenuated by lipopolysaccharide via a NF-κB dependent mechanism. **Scottish Thoracic Society**, November 2003 (oral presentation).

Lipopolysaccharide attenuates Fas-mediated apoptosis in the human neutrophil via a NF-κB dependent mechanism. **American Thoracic Society**, May 2003 (poster presentation).

Pro-inflammatory mediators attenuate Fas-mediated death of the neutrophil *in vitro*. **British Thoracic Society**, December 2001 (oral presentation).

Ligation of Fas (CD95, Apo-1) on human neutrophils induces apoptosis which is partially mediated by p38 MAPK. **Medical Research Society – Third Prize**, November 2001 (poster presentation).

TNF-α mediates neutrophil apoptosis by lipopolysaccharide-activated macrophages. **American Thoracic Society**, May 2007

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Abbreviations

$15dPGJ_2$	15-deoxy-Δ^{12}, Δ^{14}-PGJ_2
ALI	Acute lung injury
Apaf-1	Apoptotic protease-activating factor 1
ARDS	Acute Respiratory Distress Syndrome
AS	Autologous serum
ATP	Adenosine 5'-triphosphate
BALF	Bronchoalveolar lavage fluid
BD	Becton Dickinson
BPI	Bactericidal/permeability increasing protein
CD	Cluster of differentiation (antigen)
CD11/CD18	β_2 integrin
CD62E	E-Selectin
CD62L	L-Selectin
CD62P	P-Selectin
cDNA	Complementary DNA
cFLIP	Cellular FLICE - like inhibitory protein
CM	Conditioned media
CTL	Cytotoxic T lymphocytes

DAG	Diacylglycerol
DD	Death domain
DED	Death-effector domain
DEPC	Diethylpyrocarbonate
DISC	Death-inducing signalling complex
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal related kinase
FACS	Fluorescent activated cell sorting
FADD	Fas-associated protein with death domain
FasL	Fas ligand
FBS/Fbs	Foetal bovine serum
FITC	Fluorescein Isothiocyanate
fMLP	fMet-Leu-Phe
G-CSF	Granulocyte- colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HDM	Human peripheral blood-derived macrophages
HDMC	Human peripheral blood-derived mononuclear cells

HRP	Horseradish -peroxidase
ICAD	Inhibitory caspase-activated DNase
ICAM-1	Intercellular adhesion molecule type 1
ICE	Interleukin-1β- converting enzyme
IFN-γ	Interferon-γ
IL-1	Interleukin-1
IL-1β	Interleukin-1β
IL-10	Interleukin-10
IL-6	Interleukin-6
IL-8	Interleukin-8
IMDM	Iscove's modified Dulbecco's medium
IPTG	Isopropyl β-D-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
LB	Luria-Bertani
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
mAb	Monoclonal antibody
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase

MMP-9	Matrix metalloproteinase-9
MPO	Myeloperoxidase
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NO	Nitric oxide
PAF	Platelet activating factor
PAK2	p21-activated protein kinase 2
PBS/Pbs	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PE	Phycoerythrin
PECAM-1, CD31	Platelet-endothelial cell adhesion molecule-1
PI 3	Phosphatidylinositol 3
PKC	Protein kinase C
PMA	Phorbol 12-myrisate 13-acetate
PMN	Polymorphonuclear leukocyte
PMSF	Phenylmethylsulfonyl fluoride
PPAR-γ	Perioxisome proliferating activating receptor-γ
PRP	Platelet-rich plasma

PSGL-1	P-selectin glycoprotein ligand-1
RA	Rheumatoid arthritis
rhFasL	Recombinant FasL
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sFasL	Soluble Fas ligand
SHP-1	Src homology domain 2 (SH-2)-containing tyrosine phosphatase-1
siRNA	Small interfering RNA
sLe^x, CD15s	sialyl-$Lewis^x$
SLPI	Secretory leukocyte protease inhibitor
TBE	Tris-borate buffer
TBS	Tris buffered saline
TBST	Tris buffered saline containing 0.1% (v/v) Tween 20
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor

TNF-α	Tumour necrosis factor-α
TNFR	Tumour necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand

Chapter 1

Introduction

1.1 The Cells

Macrophages reside in the lung quiescent but armed to deal with invading organisms while neutrophils marginate in the lung vasculature, quiescent but ready to mobilise to sites of infection or inflammation. The role of these cells, their response to inflammatory mediators and their role in the resolution of inflammation will be explored.

1.2 The Lung

The vertebrate lung is essential to life; physiologically, gas exchange is its main function however immunologically, its role in innate immunity is also important. The lung's innate immune system is tightly regulated. It has to remain quiescent to allow daily exposure to ubiquitous non-virulent organisms while at the same time being able to mount immune responses. Furthermore, it must prevent over-zealous immune or inflammatory responses which result in tissue damage.

Acute Respiratory Distress Syndrome (ARDS) is an example of lung inflammation secondary to both direct and indirect lung injury. While pneumonia and aspiration of gastric contents are the commonest causes of direct lung injury, sepsis, severe trauma with shock and multiple transfusions are the commonest causes of indirect lung injury. Overall sepsis is the leading cause of ARDS accounting for up to 40% of the total cases (Ware and Matthay, 2000). Patients with ARDS, in the early stages, are profoundly hypoxaemic and refractory to oxygen therapy. This reflects disruption of the blood-air barrier caused by the diffuse alveolar damage (figure 1.1). In this acute or exudative phase, neutrophils, macrophages, erythrocytes, hyaline membranes and protein-rich oedema are found in the alveolar spaces. This is largely secondary to

widespread injury to the endothelium and alveolar epithelium. This inflammatory process can either resolve or progress to a fibrotic phase. In the fibrotic phase, histologically there is evidence of fibrosis, an acute and chronic inflammatory cell infiltrate and only partial resolution of the pulmonary oedema.

In ARDS and acute lung injury (ALI), which is a less severe form of ARDS, there is debate in the scientific community regarding the pathogenic role of the neutrophil. It has been argued that they are merely, a stereotypical part of the inflammatory response. However, on balance, the evidence suggests that the neutrophil plays a central role. Although neutropenic patients develop ARDS (Laufe *et al.*, 1986), their pulmonary function worsens during recovery from the neutropenia (Azoulay *et al.*, 2002). Furthermore, in animal models, depletion of neutrophils prior to the pathogenic stimulus, reduces the extent of the lung injury (Heflin and Brigham, 1981; Abraham *et al.*, 2000).

Neutrophils may exacerbate lung injury in several ways; secondary necrosis of neutrophils, due to delayed neutrophil clearance, results in release of their toxic granules and thereby exacerbation of the tissue injury and secondly, activated neutrophils in the inflamed tissue secrete pro-inflammatory mediators. Bronchoalveolar lavage fluid (BALF), from patients with ARDS, delays the apoptosis of peripheral blood neutrophils *in vitro*. Blocking antibodies have shown that most of this anti-apoptotic activity is due to the cytokines granulocyte - colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Matute-Bello *et al.*, 1997). However BALF also contains other cytokines, including interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) which are the main pro-inflammatory cytokines secreted by an activated neutrophil (Park *et al.*, 2001).

In animal models, lung injury can be induced by several methods including intratracheal administration of endotoxin. Anti-inflammatory strategies targeting the neutrophil, for example anti-oxidant therapies, inhibit the development of ALI after endotoxin administration by reducing neutrophil infiltration and secretion of pro-

inflammatory cytokines. In turn, this is associated with a reduction in oedema formation and extent of lung injury (Blackwell *et al.*, 1996; Liu *et al.*, 1997).

In health, apoptotic neutrophils are only occasionally seen in peripheral blood or bronchoalveolar lavage fluid due to their rapid phagocytosis by macrophages. Neutrophil clearance appears to be important in resolution of ALI *in vivo*. In the resolution phase of an oleic acid model of ALI in rats, apoptotic neutrophils were seen and were cleared, by phagocytosis, by alveolar macrophages (Hussain *et al.*, 1998).

In the intensive care setting, it has been observed that ventilator-associated pneumonia is more common in patients with ARDS compared to those without ARDS (Dreyfuss and Ricard, 2005). There is evidence to suggest that the systemic inflammatory response is also immunosuppressive and may therefore increase susceptibility to bacterial and fungal infections (Munford and Pugin, 2001). This is supported by the observation that in an animal model of endotoxin-induced ALI, the alveolar macrophages retrieved in BALF from ALI animals exhibited poorer bacterial phagocytic activity than those retrieved from control animals (Jacobs *et al.*, 1986).

1.3 The Neutrophil – a professional phagocyte

The principal role of the neutrophil in the cellular orchestra of the innate immune system is that of a professional phagocyte, the importance of which was first realised by Elie Metchnikoff in the 1880's. The neutrophil phagocytoses invading bacteria and in so doing accelerates its own apoptosis (Watson *et al.*, 1996).

1.3.1 Neutrophil Maturation

The neutrophil starts life in the bone marrow as a pluripotent cell of the myeloid lineage and over a two week period passes through six different stages of differentiation and maturation: myeloblast, promyeloblast, myelocyte, metamyelocyte, non-segmented (band) neutrophil and segmented neutrophil. The segmented neutrophil is the mature, terminally differentiated, cell that exits the bone

marrow. It contains cytoplasmic granules and a lobulated chromatin-dense nucleus with no nucleolus and is therefore also known as a polymorphonuclear leukocyte (PMN). It remains in the circulation, in a quiescent state, for 4 to 10 hours before marginating and entering tissues where it survives for 1 to 2 days (Breton-Gorius and Reyes, 1976).

1.3.2 Neutrophil Recruitment

In quiescent states, there are two pools of neutrophils, a circulating pool and a marginated tissue pool. Margination occurs in narrow, mainly pulmonary capillaries and in response to a vasodilating stimulus such as exercise the neutrophils re-enter the circulating pool resulting in a relative neutrophilia. An inflammatory stimulus will also induce a leucocytosis by stimulating the bone marrow to produce more polymorphonuclear cells (Witko-Sarsat *et al.*, 2000).

In response to bacterial products and pro-inflammatory cytokines, neutrophils sequester in the lungs by inducing a conformational shape change such that the neutrophil becomes trapped even in vasodilated capillaries. One hypothesis is that, in response to G-protein-coupled seven-transmembrane ("serpentine") receptor ligation, soluble G-actin assembles into an outer ring of F-actin filaments (Worthen *et al.*, 1989; Hogg and Walker, 1995) which reduces neutrophil deformability. Mature circulating neutrophils constitutively express L-selectin (CD62L) and it also appears to be important in pulmonary sequestration; in L-selectin deficient mice, there was only a transient neutropenia in response to infused complement fragments compared to the circulating neutropenia observed in wild type animals (Doyle *et al.*, 1997). Furthermore, an L-selectin blocking monoclonal antibody reduced alveolar capillary neutrophil sequestration in a rabbit endotoxin model of ALI (Kuebler *et al.*, 2000). L-selectin signalling may also contribute to the change in neutrophil deformability. In a recent study, L-selectin cross-linking induced actin assembly and increased neutrophil retention in an *in vitro* model of the microvascular capillary bed (Simon *et al.*, 1999).

At sites of inflammation, inflammatory mediators, including histamine, thrombin and oxygen radicals, induce the endothelial expression of P-selectin (CD62P) which

interacts primarily with P-selectin glycoprotein ligand-1 (PSGL-1) present on neutrophils (Moore *et al.*, 1995; Hidari *et al.*, 1997). Later, locally produced TNF-α and interleukin-1 (IL-1) stimulate the endothelium to synthesize and express E-selectin (CD62E) (Bevilacqua and Nelson, 1993; Cronstein and Weissmann, 1993). The endothelial selectins bind to their ligands on the neutrophil which include sialyl-Lewis^x (sLe^x, CD15s) and PSGL-1, forming a tight but rapidly dissociating bond, which along with the shear stress of blood flow gives rise to the intermittent tethering motion of neutrophil rolling (Bevilacqua and Nelson, 1993; Albelda *et al.*, 1994). In addition, inflammatory stimuli, including interleukin-8 (IL-8), fMet-Leu-Phe (fMLP, a bacterial cell wall-derived tripeptide) and complement factor C5, activate the neutrophil thereby inducing the surface expression of CD11/CD18 (β_2 integrin). At the same time, activation induces a conformational shape change of the ligand CD11/CD18 which then has greater avidity for its receptor, intercellular adhesion molecule type 1 (ICAM-1) (Williams and Solomkin, 1999). ICAM-1 expression, on post-capillary venules and also capillary endothelial cells in the lung, is upregulated by the inflammatory stimuli. This stable bond between β_2-integrins and ICAM-1 allows neutrophil adhesion to the endothelium.

However the individual role of adhesion molecules in neutrophil recruitment appears to depend upon the stimulus. In murine streptococcal pneumonia, neutrophil emigration is CD18-independent while in murine pneumonia secondary to *Escherichia coli* and *Pseudomonas aeroginosa*, it is CD-18 dependent (Mizgerd *et al.*, 1999; Doerschuk *et al.*, 2000). Furthermore in murine streptococcal pneumonia neutrophil emigration was also independent of E-, P- and L-selectin (Mizgerd *et al.*, 1996). In humans, the importance of adhesion molecules in neutrophil recruitment is apparent in patients who have leucocyte adhesion deficiency-I (LAD-I) due to a congenital deficiency of CD18. These patients die prematurely (often before the age of 10) from recurrent skin and mucosal infections due to a paucity of tissue neutrophils while at the same time having a relative blood leucocytosis (Anderson *et al.*, 1985; Lekstrom-Himes and Gallin, 2000). It has also become apparent that neutrophils can respond heterogeneously. Neutrophils released from bone marrow in response to acute streptococcal pneumonia express a higher concentration of L-

selectin and migrate more slowly into the inflammatory site than circulating neutrophils (Lawrence *et al.*, 1996).

1.3.3 Neutrophil Priming

Neutrophils can be "primed" such that subsequent stimulation provokes a larger response than a non-primed cell. Priming can result in biochemical changes within the cell (Walker and Ward, 1992) including intracellular generation of cytokines or lipid mediators, for example, leukotriene B_4 (LTB_4) and platelet activating factor (PAF) (Doerfler *et al.*, 1989) so that the cells have increased "ammunition" when activated. Inflammatory mediators including lipopolysaccharide (LPS) and fMLP, prime neutrophils to induce a shape change as a result of modifications to their intracellular actin (Worthen *et al.*, 1989). This is a key step in making neutrophils less deformable. However various processes involved in sequestration of neutrophils (Kitagawa *et al.*, 1997) can themselves lead to priming including crosslinking of neutrophil adhesion receptors (Waddell *et al.*, 1994; Liles *et al.*, 1995).

Neutrophils have many different types of receptor on their surface. Serpentine receptors are G-protein-linked seven transmembrane receptors. There are also single transmembrane domain receptors that require immobilization or crosslinking such as immunoglobulin (Fc) receptors and integrins and then there are single transmembrane receptors which bind growth-related cytokines such as TNF-α or GM-CSF. Triggering of the former two receptor pathways often has a dual effect; priming at low concentrations of the ligand and activation at higher concentrations. However, triggering of the single transmembrane receptor pathway for growth-related cytokines tends to result in priming alone (Hallett and Lloyds, 1995). A two stage biological process however prevents inappropriate activation of neutrophils in the bloodstream.

1.3.4 Transendothelial Migration

In the lung, transendothelial neutrophil migration from the capillary bed, in response to inflammatory stimuli, occurs mainly by penetrating interendothelial junctions or at bicellular or tricellular corners of endothelial cells. However a transcellular route has also been described (Burns *et al.*, 2003). Under the influence of a chemotactic

gradient, neutrophils penetrate the endothelial layer (diapedesis) and migrate through the interstitium. Diapedesis occurs predominantly between endothelial cells and both *in vitro* and *in vivo* data suggest that platelet-endothelial cell adhesion molecule 1 (PECAM-1, CD31) is important in this step. CD31 is a member of the immunoglobulin superfamily and has now been linked to many biological functions (Jackson, 2003). Its role in migration involves a homotypic interaction between CD31 on the neutrophil and CD31 on the endothelial cell resulting in transient remodelling of the junction (Muller *et al.*, 1993). The interaction of CD31 on endothelial cells with CD31 on transmigrating neutrophils also leads to upregulation of α6 integrins and recently both α6 integrins and neutrophil elastase have been shown to be important in neutrophil migration through the perivascular basement membrane (Wang *et al.*, 2005).

1.3.5 Phagocytosis

Microorganisms can either be recognized directly by pattern recognition receptors on the neutrophil surface or more commonly the microorganisms are first opsonized by IgG antibodies or complement and then recognized by neutrophil $F_c\gamma$ receptors and complement receptors (CR1, CR3) respectively. Recognition of the microorganism and binding to the neutrophil surface stimulates phagocytosis.

This involves a complex series of morphological and biochemical processes which differ depending whether phagocytosis is antibody-mediated or complement-mediated. The antibody-coated microorganism is initially engulfed by an invaginating plasma membrane to form a phagocytic vesicle. Thereafter, the contents of neutrophil granules, usually in combination with toxic substances generated during the respiratory burst, kill the engulfed bacteria. The importance of the respiratory burst can be seen in patients with chronic granulomatous disease who have an absence of or abnormality in, a component of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system. The neutrophils of these patients are able to phagocytose bacteria but they are unable to kill their involuntary guests. Patients thus die prematurely from bacterial infections, particularly with catalase

positive bacteria such as *Staphylococcus aureus* that can intrinsically break down hydrogen peroxide (Winkelstein *et al.*, 2000).

More recently, it has been observed that mice deficient in two neutrophil granule proteases (cathepsin G and elastase) were more susceptible to fungal and bacterial infections. Similarly *in vitro*, neutrophils treated with protease inhibitors were unable to kill ingested *Candida albicans* and *Staphylococcal aureus* Furthermore, the mice lacking cathepsin G and elastase succumbed to infection with these organisms despite their neutrophils having a fully functional respiratory burst. This challenged the accepted dogma that hypochlorous acid is the main microbicidal product in the phacocytic vesicle. Reactive oxygen species (ROS), generated by the respiratory burst and released into the endocytic vacuole, create a highly negatively-charged environment. The accompanying release of the neutrophil acidic granules is insufficient for neutralisation and it is therefore accompanied by an influx of positively charged potassium ions in a pH dependent manner. This increased ionic strength favours the release of the aforementioned cationic proteases from their resting state, bound to anionic proteoglycan matrix. Furthermore, the resulting alkaline pH of the endocytic vacuole is optimal for the function of these proteases which in their activated state, contribute to microbial killing (Reeves *et al.*, 2002).

1.3.6 The Respiratory Burst

Neutrophil activation results in a large increase in the concentration of cytosolic free calcium (Ca^{2+}) that triggers NADPH oxidase to form from its membranous and cytosolic components. This complex enzyme is composed of at least five members that are dissociated and thus inactive in quiescent neutrophils. Upon activation, the cytosolic components translocate to the plasma membrane to assemble the active oxidase. Activation of this oxidase requires a large increase in cellular oxygen consumption termed the respiratory burst (Clark, 1999). During this process, oxygen is reduced by NADPH oxidase to the univalent superoxide anion. This is converted by superoxide dismutase to hydrogen peroxide. Although hydrogen peroxide is itself toxic, it can interact with myeloperoxidase (MPO) found in neutrophil azurophil granules to form hypochlorous acid that is metabolised to hypochlorite and chlorine.

Hypochlorous acid is 100 to 1000 times more effective then hydrogen peroxide. Hydrogen peroxide, catalysed by iron (Fe^{2+}) also decomposes to the hydroxyl radical that is another powerful and important microbicidal reactive oxygen intermediate (ROI) but its importance in neutrophils is controversial (Rosen and Klebanoff, 1979; Ward *et al.*, 1983).

It is well documented that murine neutrophils generate nitric oxide (NO) in response to cytokines (Nathan and Hibbs, 1991) however it is controversial whether resting or activated human neutrophils produce reactive nitrogen species (Yan *et al.*, 1994; Padgett and Pruett, 1995; Evans *et al.*, 1996; Wheeler *et al.*, 1997). The differences in studies may be due to experimental conditions and technical difficulties in measuring NO *in vitro*.

1.3.7 Neutrophil Granules

Neutrophils contain three different types of granules and also secretory vesicles. Their content and function is shown in table 1.1. The granules are formed sequentially during neutrophil maturation in the bone marrow with primary granules being formed first at the myeloblast stage, secondary at the myelocyte stage, tertiary at the band cell stage and secretory vesicles at the segmented stage, of development (Borregaard and Cowland, 1997).

The ease with which these granules are subsequently mobilized in the mature cell is in the reverse order of their development. Secretory vesicles are mobilized in response to inflammatory mediators to induce surface expression of receptors, for example the β_2 integrins, which as discussed above are important in neutrophil adhesion to endothelium. Thereafter the tertiary granules are mobilized; gelatinase is thought to be important in neutrophil migration as its substrates include collagen IV (found in basement membranes) and collagen V (an important constituent of extracellular matrix). The primary granules can be further subdivided into those that contain defensins and those that do not. The latter granules develop first during neutrophil bone marrow maturation and subsequently they are mobilized last. It is likely that there is minimal extracellular release of the defensin-poor granules under

normal physiological conditions as they principally discharge their toxic contents into the phagosome (Sengelov *et al.*, 1995).

Two rare diseases with granule deficiencies illustrate their importance to normal host immunity. Patients with Chediak-Higashi disease suffer from recurrent bacterial infections as a result of mutations in their Lyst gene that encodes a cytoplasmic protein important in lysosomal trafficking; their neutrophils contain giant granules that result from primary and secondary granule fusion. Recurrent bacterial infections are also seen in patients suffering from specific granule deficiency; their neutrophils lack secondary granules and defensins (Lekstrom-Himes and Gallin, 2000).

Table 1.1 Neutrophil Granules

	Primary Granules	Secondary Granules	Tertiary Granules	Secretory Vesicles
Synonyms	Azurophilic, non-specific, basophilic	Specific, eosinophilic, acidophilic	C-Particles, Secretory vesicles	
Lysosomal acid hydrolases	β-Glucuronidase, acid-phosphatase, cathepsin B, cathepsin D			
Neutral serine proteases	Elastase, Cathepsin G, Proteinase 3	Plasminogen activator		
Neutral metalloproteinases		Collagenase	Gelatinase	
Microbicidal factors	Myeloperoxidase, lysozyme, defensins, cationic proteins, BPI	Lysozyme, cytochrome b	NADPH oxidase	
Adhesion molecules/ chemotactic factor receptors		Receptors for laminin, fibrinogen, vitronectin, fMLP, TNF, C3		CD11b, CD16
Miscellaneous		Vitamin B12-binding protein, lactoferrin		

1.3.8 Neutrophils and Host Defense

Bactericidal/permeability increasing protein (BPI) is a 55kDa protein, found in the primary granules, that has both LPS neutralizing activity (by binding to the Lipid A moiety of LPS) (Weersink *et al.*, 1993) and bactericidal activity for specifically Gram-negative bacteria (Elsbach and Weiss, 1993). BPI is bactericidal, as the name suggests, by enhancing bacterial membrane permeability and stimulating hydrolyses of membrane phospholipids by phospholipase (Elsbach, 1998). Furthermore BPI enhances Gram-negative bacteria phagocytosis (via neutrophil complement receptors) by accelerating their opsonisation by complement fragments (Nishimura *et al.*, 2001). Recombinant BPI has been used with some success, in Phase III clinical trials, in children with meningococcal septicaemia. There were fewer amputations in the treatment group and the functional status of those in the treatment group was higher following the illness (Levin *et al.*, 2000).

Defensins are 3- to 5-kDa cationic peptides of which there are four α-defensins in the primary granules of human neutrophils. They kill microorganisms by increasing their membrane permeability and act synergistically with BPI against Gram-negative bacteria. Defensins may also be involved in the transition from innate to adaptive immunity. Neutrophil α- and β-defensins are chemotactic for T cells and immature dendritic cells (Ganz *et al.*, 1990). Furthermore antigen-specific responses are enhanced by human α-defensins.

Cathepsin G, in addition to its direct antimicrobial effect, has also been shown to have chemotactic activity for monocytes (MacIvor *et al.*, 1999). Furthermore, lysozyme not only cleaves peptidoglycans that form bacterial cell walls, it can also kill bacteria non-enzymatically (Ganz *et al.*, 1986). Lactoferrin from secondary granules sequesters iron essential for microbial respiration. Secretory leukocyte protease inhibitor (SLPI) via its N-terminal domain has some antimicrobial activity *in vitro* against both Gram-negative and Gram-positive bacteria (Sallenave, 2002). The C-terminal domain is an inhibitor of neutrophil elastase and may also be involved in regulating intracellular responses to LPS (Williams *et al.*, 2006).

1.4 Apoptosis

Kerr, Wyllie and Currie in 1972 coined the term "apoptosis" to describe a distinctive type of cell death, programmed cell death. The features of an apoptotic body are "marked condensation of both nucleus and cytoplasm, nuclear fragmentation and separation of protuberances that form on the cell surface to produce many membrane-bounded, compact but otherwise well-preserved cell remnants of greatly varying size" (Kerr *et al.*, 1972). The term apoptosis itself originates from the Greek word for falling leaf.

Apoptosis is important both developmentally and in maintenance of normal tissue structure and function. For example, disruption of normal apoptotic pathways is an important mechanism by which cells transform and acquire malignant potential. Furthermore, in the immune system, the mode of cell death induced by cytotoxic T lymphocytes is apoptotic. The apoptotic mode of cell death is important as it allows senescent and potentially toxic cells to be cleared without inciting an inflammatory or immune response. In contrast necrosis, classically illustrated in the form of red blood cells being placed in hypotonic medium, such as water, involves disruption of their cellular membranes and release of haemoglobin. Loss of cellular membrane integrity allows release of potentially toxic intracellular contents, as described earlier in terms of the neutrophil (Haslett, 1992). As with most scientific principles, necrosis and apoptosis are at two ends of the spectrum and there is probably a continuum with less definite modes of cell death in between.

Mammalian cells have two main routes to apoptosis: the internal and the external pathway. The external pathway is mediated upstream by death receptors while the internal pathway is mitochondria dependent. Downstream the pathways converge on a group of proteins, "the final common pathway". Some of these key proteins have been identified. The DNA ladder nuclease (caspase-activated DNase or CAD) forms an inactive complex with an inhibitory subunit known as ICAD (Nagata, 2000). Caspase-3 cleaves ICAD resulting in the release and activation of CAD (Liu *et al.*, 1997; Enari *et al.*, 1998; Sakahira *et al.*, 1998). Caspase-mediated cleavage of the nuclear lamins are responsible for nuclear shrinkage and budding (Rao *et al.*, 1996;

Buendia *et al.*, 1999) while cleavage of cytoskeletal proteins, fodrin and gelsolin, results in loss of cell shape (Kothakota *et al.*, 1997). Membrane blebbing is a function of p21-activated protein kinase 2 (PAK2), and it is activated by caspase cleavage between the negative regulatory subunit and the catalytic subunit (Rudel and Bokoch, 1997).

1.4.1 Caspases

Caspases, a group of cysteine proteases, are central to the neutrophil's apoptotic machinery. These proteins are phylogenetically conserved having CED homologues in the worm *Caenorrhabis elegans*. Their catalytic centre has a highly conserved pentameric sequence, which includes a cysteine residue. They cleave substrates after aspartic acid residues and the four residues amino-terminal to the cleavage site, confer substrate specificity (Earnshaw *et al.*, 1999). Caspases involved in apoptosis can be divided into two groups, initiator and effector caspases. Initiator caspases include caspase-2, -8, -9 and -10; their autoactivation is tightly regulated. Effector caspases include caspase-3, -6 and -7; they are activated by initiator caspases. However some caspases, including caspase-1 or interleukin-1β- converting enzyme (ICE), are involved in both apoptotic and inflammatory pathways (Rowe *et al.*, 2002). Caspases, including caspase –1; –3; –4 ; –8 and –10 are present in neutrophils (Yamashita *et al.*, 1999).

1.4.2 The "internal" apoptotic pathway

Mitochondria, the powerhouse of the cell, are important in apoptosis. Furthermore cytochrome c, the electron carrier, is central to this pathway's machinery. It is released into the cytosol, in response to cellular damage and stress, where it interacts with apoptotic protease-activating factor 1 (Apaf-1) to form the "apoptosome" (Li *et al.*, 1997). This induces oligomerization of Apaf-1 and the complex is then able to recruit and subsequently activate caspase 9. Triggers include radiation and cytotoxic drugs.

Neutrophils are known to have structures which resemble mitochondria (Maianski *et al.*, 2002). Recent research has confirmed that although human neutrophils have

only small amounts of cytochrome c, the Apaf-1 dependent pathway is important in their apoptosis (Murphy *et al.*, 2003).

Cytochrome c release from the mitochondria is regulated by the Bcl-2 family of proteins (Reed *et al.*, 1996). Although there are conflicting results in the literature, it is now generally agreed that human neutrophils constitutively express pro-apoptotic proteins, Bax, Bid, Bak and Bad but do not express the anti-apoptotic protein Bcl-2 (Akgul *et al.*, 2001).

1.4.3 Death Receptors and the "external" apoptotic pathway

Death receptors are a subset of the tumour necrosis factor receptor (TNFR) family characterized by an intracellular "death domain". The receptors are type-1 membrane proteins while their ligands are type-2 membrane proteins. The extracellular C termini of the type-2 membrane proteins must homotrimerise to form the active ligand. The ligands can exert their function in either a membrane-bound form or can be proteolytically cleaved to a soluble form. Binding of the trimerised ligand to its receptor triggers an intracellular signalling cascade.

1.4.4 The Fas/FasL death receptor pathway

In 1989, a monoclonal antibody, anti-Fas antibody, was first described. It had been raised against a human surface antigen using human diploid fibroblast FS-7 cells as the immunogen (Yonehara *et al.*, 1989). Subsequently, the Fas antigen (CD95) was found to be ubiquitously expressed in human cells. In the same year, the monoclonal antibody Apo-1 was also described, although its antigen was initially found on only activated or malignant lymphocytes (Trauth *et al.*, 1989). Yonehara *et al* (1989) demonstrated that anti-Fas antibody, when combined with interferon-γ, killed cells expressing the Fas antigen.

The nucleotide sequence of the Fas antigen was discovered using a complementary DNA (cDNA) library; the sequence molecularly coded for a 319 amino acid polypeptide (Itoh *et al.*, 1991). Its extracellular domain showed homology to human tumour necrosis factor receptors. When murine cells were transformed with human Fas antigen cDNA and treated with anti-Fas antibody, they underwent apoptosis. In

1993, the ligand (FasL) was identified in a cytotoxic T lymphocyte hybridoma which killed Fas^+ but not Fas^- cells (Suda *et al.*, 1993).

Mutants of the MRL mouse strain which develop lymphadenopathy and splenomegaly have been described (Andrews *et al.*, 1978); an autosomal recessive mutation on mouse chromosome 19 was found in the mutant referred to as *lpr* (Watanabe *et al.*, 1991) while an autosomal recessive mutation on mouse chromosome 1 was found in the mutant referred to as *gld* (Ramsdell *et al.*, 1994; Takahashi *et al.*, 1994; Hahne *et al.*, 1995). It is now known that the *lpr* mutation truncates transcription of the gene encoding Fas while the *gld* point mutation, near the C terminus of the coding region for FasL, abolishes the ability of FasL to bind to Fas (Nagata and Suda, 1995). A human lymphoproliferative syndrome has also been described associated with mutations in the Fas gene on human chromosome 10 (Bettinardi *et al.*, 1997).

The Fas/FasL interaction is primarily important in T lymphocyte development and homeostasis. Positive and negative selection in the thymus, as part of T cell development, appears to be normal in the *lpr*/*gld* mice however clonal deletion of autoreactive T cells in the periphery appears to be aberrant. Furthermore, cytotoxic T lymphocytes (CTL) appear to use both the perforin/granzyme and Fas/FasL pathways to mediate their cytotoxic effect (Krammer, 2000).

It has also been suggested that the expression of FasL in the eye and Sertoli cells of the testis confers immune privilege by killing invading immune cells (Bellgrau *et al.*, 1995; Griffith *et al.*, 1995). Transplant immunologists were obviously excited by this discovery and subsequently myoblasts expressing FasL were shown to "protect" transplanted pancreatic islet cells (Kang *et al.*, 1997). Furthermore, FasL expression on colonic tumour cells induced cell death in a human T-cell leukaemia cell line (Seino *et al.*, 1997). Several studies then emerged which refuted the established paradigm that FasL is important in immune privilege. When pancreatic islet cells expressing FasL were transplanted, the FasL expressing cells rather than protecting the islet cells from rejection, resulted in more rapid rejection due to infiltrating neutrophils (Kang *et al.*, 1997). Membrane-bound FasL (40kda) is cleaved by matrix

metalloproteases to a 26-29kDa soluble form (sFasL) (Kayagaki *et al.*, 1995). It has been shown that while human sFasL is chemotactic for mouse and human neutrophils, it does not induce their apoptosis (Ottonello *et al.*, 1999). The generation of sFasL reduces expression of the active membrane-bound form and as sFasL can bind to Fas without inducing apoptosis, it is also a natural antagonist (Schneider *et al.*, 1998). Thus, it was proposed that sFasL, cleaved from the transplanted islet cells, induced neutrophil chemotaxis. These neutrophils, in turn, were primarily responsible for the islet cell destruction. It has also been suggested that FasL-tumour expressing cell lines indirectly augment neutrophil infiltration by inducing secretion of interleukin-1β (Il-1β) from neutrophils or by activating resident macrophages to secrete pro-inflammatory cytokines (Muzio *et al.*, 1996). FasL-tumour expressing cell lines have also been shown to induce cytotoxic activity in neutrophils (Kang *et al.*, 1998). However the eye and testis may be protected from this neutrophilic infiltration due to the presence of transforming growth factor-β (TGF-β) in their cellular microenvironment (Chen *et al.*, 1998).

Neutrophils have been shown to express Fas and anti-Fas antibody accelerates their apoptosis (Iwai *et al.*, 1994). Initially they were shown to express FasL and release sFasL which was able to induce death in activated Jurkat cells (Liles *et al.*, 1996). Furthermore blocking the Fas receptor, partially suppressed spontaneous neutrophil apoptosis suggesting that Fas/FasL may mediate neutrophil apoptosis by both an autocrine and paracrine pathway (Liles *et al.*, 1996). Fas-mediated induction of neutrophil apoptosis might therefore accelerate their clearance from sites of inflammation. At such sites cytokines and inflammatory mediators including GM-CSF, LPS and complement factor C5a are known to prolong neutrophil survival (Haslett, 1997; Ward *et al.*, 1999).

1.5 The Macrophage

Macrophages, large mononuclear phagocytes found in tissues, were first described by Elie Metchnikoff over 100 years ago (Karnovsky, 1981). They originate in the bone marrow, move into the bloodstream as monocytes and finally into the tissues where they differentiate into macrophages (Territo and Cline, 1975). Macrophages

function in the innate immune system primarily as phagocytes, phagocytosing not only airborne particulates that reach alveoli but also microorganisms.

The macrophage has signalling pattern recognition receptors that respond to pathogen-associated molecular patterns e.g. mammalian Toll-like receptors (TLRs) that recognise bacterial lipopolysaccharide (LPS), viral double-stranded RNA, unmethylated CpG dinucleotides (common in bacterial DNA), mannans of yeast, glycolipids of mycobacteria, lipoproteins of bacteria and parasites and lipoteichoic acids of Gram-positive bacteria. LPS is initially bound by lipopolysaccharide-binding protein (LBP) and this complex binds to the membrane-anchored protein, CD14. CD14 has no cytoplasmic domain and requires TLR4 for transduction of the LPS signal. TLR4 activates nuclear factor-κB (NF-κB) and stimulates the expression of chemokines and cytokines, important in mediating inflammatory responses, including, interleukin-8 (IL-8), leukotriene B_4 (LTB_4), interleukin-1 and tumour necrosis factor-α (TNF-α) (Palsson-McDermott and O'Neill, 2004). Endocytic pattern recognition receptors are also found on the surface of macrophages and include the macrophage mannose receptor and the macrophage scavenger receptor. These receptors enhance phagocytosis of microorganisms by the macrophage. Phagocytosed bacterial antigens are then processed intracellularly and presented by these professional antigen-presenting cells to the adaptive immune system. However macrophages also phagocytose apoptotic neutrophils and are therefore important in the resolution of inflammation (diagram 3.1).

1.5.1 Alveolar macrophages

Macrophages become highly specialized and specifically adapt to microenvironments. In the lung, macrophages are exposed to inhaled microbes, toxic substances and fine particulate matter. They must remain in a quiescent state in response to harmless antigens but be able to become activated in response to infectious microbes. Alveolar macrophages, in their quiescent state, do not produce inflammatory cytokines and downregulate expression of the phagocytic receptor CD11b such that they display poor phagocytic activity (Holt, 1978). Their importance in maintaining a tonic state was elegantly demonstrated when alveolar

macrophages were eliminated *in vivo* using clodronate-filled liposomes, this led to overt inflammatory reactions in response to harmless particulate and soluble antigens (Thepen *et al.*, 1989). Furthermore, when alveolar macrophages were mixed with dendritic cells *in vitro*, they suppressed T-cell activation through release of prostaglandins, interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (Holt, 1993).

In animal models of ALI, it has been shown that the rapid recruitment of neutrophils and later recruitment of blood monocytes to the lung is dependent on resident alveolar macrophages (Maus *et al.*, 2002). In response to intratracheal monocyte chemoattractant, the recruitment of alveolar monocytes was largely dependent on the β_2 integrin CD11b/CD18 and CD54 as well as the β_1 integrin CD49d while the co-administration of intratracheal monocyte chemoattractant and LPS resulted in amplified monocyte recruitment dependent additionally on CD11a and CD106 (Maus *et al.*, 2002).

The lung microenvironment itself determines the macrophage phenotype. The breakthrough in our understanding of this process came when it was shown that $\alpha_v\beta_6$ integrin-deficient mice had activated alveolar macrophages due to a lack of TGF-β activation (Morris *et al.*, 2003). The $\alpha_v\beta_6$ integrin can activate latent TGF-β by binding to the latency-associated peptide, an N-terminal inactivating fragment of TGF-β. This knowledge has recently been expanded. Alveolar macrophages, in the quiescent state closely adhere to alveolar epithelial cells which induces expression of the $\alpha_v\beta_6$ integrin on the epithelial cells. This leads to local activation of TGF-β which in turn binds to its receptor expressed on macrophages, inducing phosphorylation of SMAD-2 and -3 thereby suppressing macrophage phagocytosis and cytokine production. When alveolar macrophages are exposed to infectious agents such as LPS, signalling through their Toll-like receptors leads to a rapid loss of contact with the alveolar epithelial cells, downregulation of $\alpha_v\beta_6$ integrin expression on the epithelial cells, loss of activation of TGF-β and priming of the macrophage to secrete pro-inflammatory cytokines (Takabayshi *et al.*, 2006).

During the inflammatory process, as discussed earlier, monocytes are recruited to the alveolar space and these cells have an inflammatory phenotype (Warmington *et al.*, 1999). It takes a few days before these cells acquire the suppressive phenotype of alveolar macrophages (Bilyk and Holt, 1995). Recently the mechanism by which macrophages switch back to their suppressive phenotype after exposure to infectious agents was further elucidated; a few days after exposure to the infectious agent, lymphocytes secreting interferon-γ (IFN-γ) stimulated the production of matrix metalloproteinase-9 (MMP-9) by the alveolar macrophages. MMP-9 can activate latent TGF-β and by the mechanism described above, this switches the macrophage back to its anti-inflammatory phenotype (Takabayshi *et al.*, 2006).

An elegant proteomic study has shown differential protein expression between human alveolar macrophages and their precursor blood monocytes which reflect their different phenotypes (Wu *et al.*, 2005).

1.6 The Macrophage, the Neutrophil and Inflammation

At a cellular level, the processes involved in the resolution of inflammation have been further elucidated. Neutrophils produce prostaglandins and leukotrienes from arachadonic acid (diagram 4.1.). During neutrophil activation, the NF-κB signalling pathway induces transcription of genes encoding enzymes which switch the production of arachidonic acid metabolites to lipoxins (Levy *et al.*, 2001). Lipoxins are known to slow the entry of neutrophils to inflamed sites, reduce vascular permeability and stimulate macrophage phagocytosis of apoptotic neutrophils (Serhan *et al.*, 1995; Takano *et al.*, 1998; Godson *et al.*, 2000). Recently other groups of anti-inflammatory lipid mediators have been identified, including resolvins and protectins, which are metabolites of omega-3 polyunsaturated fatty acids (Serhan *et al.*, 2002; Hong *et al.*, 2003; Marcheselli *et al.*, 2003).

The ageing inflammatory neutrophil, as discussed previously, usually dies by apoptosis. The macrophage recognizes several surface receptors on the apoptotic cell, including phosphatidylserine, the integrin $\alpha_v\beta_3$ and CD36, resulting in phagocytosis (Savill *et al.*, 1989; Hart *et al.*, 1996; Fadok *et al.*, 1998). In so doing,

the macrophage itself, switches to an anti-inflammatory phenotype (Fadok *et al.*, 1998). Macrophages are themselves cleared from inflamed sites by the draining lymphatics (Bellingan *et al.*, 1996).

The Fas-FasL pathway may also be important in the interaction between macrophages and neutrophils at inflamed sites. Macrophages express FasL and sFasL has been identified in the conditioned supernatants of activated macrophages (Brown & Savill, 1999). The conditioned supernatants containing sFasL accelerated neutrophil apoptosis but other factors were also present which contributed to the observed induction of neutrophil apoptosis (Brown & Savill, 1999). The nature of these factors will be explored further.

1.7 Aims of this project

Neutrophilic inflammation in the alveoli is the hallmark of acute lung injury (ALI)/adult respiratory distress syndrome (ARDS). In early ARDS, bronchoalveolar lavage fluid (BALF) delays neutrophil apoptosis but this is largely due to granulocyte- colony stimulating factor (G-CSF) and granulocyte/macrophage - colony stimulating factor (GM-CSF) (Matute-Bello *et al.*, 1997). However in late ARDS, our group showed that BALF accelerated neutrophil apoptosis (Donnelly & Haslett, unpublished observation). BALF from patients with ARDS has also been shown to contain soluble FasL (sFasL) (Matute-Bello *et al.*, 1999). The Fas/FasL pathway is important in cellular apoptosis and it is therefore proposed that Fas-mediated induction of neutrophil apoptosis will accelerate resolution of inflammation by apoptotic clearance of neutrophils from the lung and their subsequent macrophage phagocytosis. The role of this death pathway in regulating neutrophil longevity as well as the effect of "the inflammatory milieu" will be explored. As sepsis/endoxaemia is the leading cause of ALI/ARDS in humans, the role of the LPS-activated macrophage in control of neutrophil longevity and the importance of the Fas/FasL pathway in this regard will be determined. Thus, the role of Fas/FasL, inflammatory mediators and LPS-stimulated macrophages on human neutrophil apoptosis *in vitro* will be investigated.

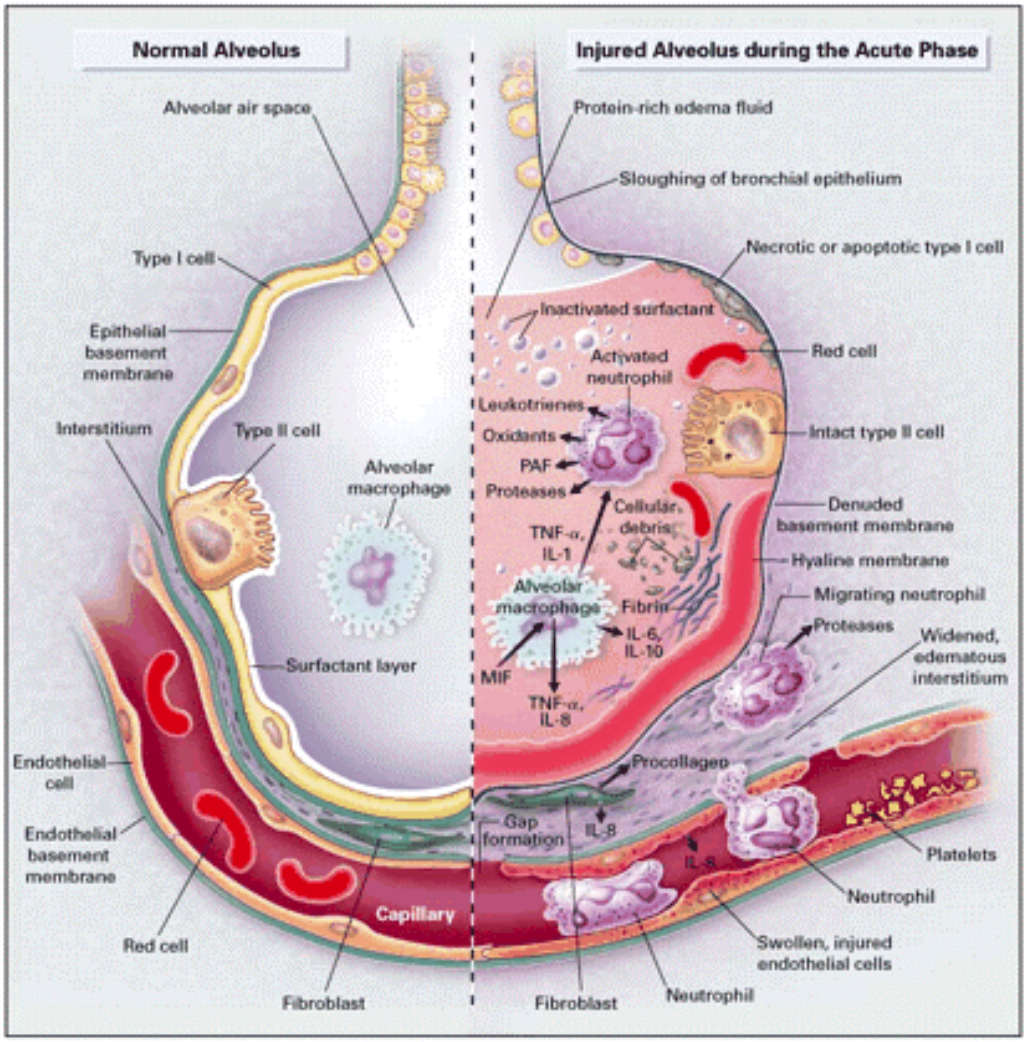


Figure 1.1. The alveolus in normal l.ung and ARDS.

In ARDS (right-hand side) there is damage to the bronchial and alveolar epithelium and pulmonary endothelium. A hyaline membrane has formed next to the denuded membrane. As a result of the leaky blood-air barrier, there is a protein-rich exudate in the alveolus. Neutrophils have adhered to the damaged endothelium and activated neutrophils have transmigrated into the alveolus where they are producing inflammatory mediators. The alveolar macrophage has been activated by macrophage inhibitory factor (MIF) to secrete cytokines (TNF-α, IL-1, IL-6, IL-8 and IL-10) that are important in regulating the inflammatory response.

(Adapted from Ware and Matthay, 2001)

Chapter 2

Material and Methods

2.1 Materials

2.1.1 Biochemicals

Unless otherwise stated, all chemicals were obtained from Sigma, Poole, UK. All tissue culture reagents were purchased from Life Technologies, Paisley, UK.

2.1.2 Antibodies and Pharmacological Inhibitors

The antibodies used in this study are detailed in table 2.1. The pharmacological inhibitors used in this study are detailed in table 2.2.

2.2 Cell Biology Techniques

2.2.1 Neutrophil and monocyte isolation from human peripheral blood

Human polymorphonuclear leukocytes and mononuclear leukocytes were isolated from peripheral human blood (80-320ml) taken from healthy donors as previously described by Haslett *et al.*, (1985). This technique was carried out under sterile conditions using endotoxin-free reagents and tissue culture plastics to prevent inappropriate cell activation. Unless otherwise stated it was carried out at room temperature. The blood was citrated (0.38%) (Phoenix pharmaceuticals) to prevent clotting, mixed by gentle inversion and centrifuged (Sanyo Mistral 3000i centrifuge) for 20 minutes at 220G with zero brake. Centrifugation separates the blood into a lower dense cellular layer, the packed cell volume (PCV), which constitutes approximately 45% of the circulating blood volume and contains the red blood cells, above which there is the buffy coat, rich in leukocytes, and the supernatant which is platelet-rich plasma (PRP). The supernatant was carefully removed and 10ml of PRP was added to glass bottles with 220µl 1M calcium chloride. At 37°C, the calcium activates the platelets to form a platelet plug and thus "autologous serum" was

obtained. Dextran sedimentation of the packed cell volume (6ml of 6% dextran and 19ml 0.9% (w/v) saline were added to the PCV), separated the residual red cells and leukocytes. After 30 minutes at room temperature, the upper leukocyte rich layer was removed and washed at 220G for 6 minutes.

The neutrophils/eosinophils and mononuclear cells were separated by centrifugation through discontinuous isotonic Percoll gradients (55/68/81%) at 700g for 20 minutes with zero brake. The mononuclear cells were isolated from the 55/68% interface and washed twice in phosphate buffered saline (PBS) without cations before being resuspended at $4x10^6$/ml in Iscove's modified Dulbecco's medium (IMDM) (GIBCO) containing 20IU/ml penicillin/streptomycin. Monocytes were enriched by selective adherence to tissue culture plastic, a method previously demonstrated by Ackerman and Douglas (1978). Non-adherent lymphocytes were removed by aspiration and the wells washed twice with Hank's balanced salt solution (HBSS). Over a period of 5 days, when the adherent monocytes were cultured in IMDM containing 10% autologous serum, they differentiated into mature monocyte-derived macrophages (figure 2.1b). As autologous serum contains variable cytokines, the adherent monocytes were also differentiated in serum-free media with 250U/ml recombinant human GM-CSF (R&D Systems) (Vincent *et al.*, 1992). The neutrophils were isolated from the 68/81% interface and washed twice in phosphate buffered saline (PBS) without cations. The purity of the neutrophils was greater than 97% as assessed by forward/side scatter analysis on a EPICS Coulter flow cytometer (purity of granulocyte population) and by morphology (figure 2.1a). Cytospins of harvested cells were stained with Diff Quik and a minimum of 500 cells per slide were counted.

2.2.2 Assessment of apoptosis and necrosis

Apoptosis was assessed quantitatively by assessment of morphology and the differential binding of Annexin V to apoptotic cells. Cytospins were prepared in duplicate or triplicate for each experimental condition; 120µl of cells centrifuged at 300G onto glass slides, fixed in methanol and stained with Diff-Quik (Gamidor, Abingdon, UK). Cells were counted using oil immersion microscopy (x100

objective lens) to determine the proportion of cells with distinctive apoptotic morphology i.e. cell shrinkage, nuclear condensation (figure 2.2a). A minimum of 500 cells/slide were counted and the results were expressed as the mean ± SEM.

Annexin V binds to phosphaditylserine exposed on the surface of apoptotic cells. Fluorescein isothiocyanate (FITC)-labelled recombinant human Annexin V (Bender Medsystems, Vienna, Austria) was diluted (1:200) in binding buffer and 180µl was added to 20µl of cells ($5x10^6$/ml) (in duplicate or triplicate) and incubated at 4°C for 10 minutes before flow cytometric analysis on EPICS XL2 (figure 2.2b). This is a previously validated method of assessment of apoptosis (Majewska *et al.*, 2000; Vermes *et al.*, 2000). In the assessment of apoptosis using Annexin V, we used cells which had not undergone apoptosis as a negative control however other groups have used an EDTA control to indicate the calcium-dependent specific binding of Annexin V (Boas *et al.*, 1998; Cederholm *et al.*, 2005). Necrosis was determined by assessing the integrity of cells (at least 500) by virtue of their exclusion of the vital dye trypan blue.

Apoptotic rates assessed by morphology and Annexin V binding were highly correlated ($n=12$, $r = 0.93$, $p<0.0001$, data not shown).

2.2.3 Cell Culture

All cell culture was carried out with aseptic technique within a tissue culture hood (Envair Class II) and cultures were maintained in an incubator at 37°C/5% CO_2. Heat-inactivated serum was prepared by heating serum at 56°C for 60 minutes.

2.2.3.1 A549 cells

A549 cells are derived from a human lung carcinoma (ECACC ref. 86012804). Media for use with the A549 cells was Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2mM glutamine and 20IU/ml penicillin/streptomycin. The cells were adherent and cultures were split (1:6) on reaching 70-90% confluency (approximately every 3 days) using trypsin (0.05% w/v)/ethylenediaminetetraacetic acid (EDTA) (0.02% w/v). The

clones stably transfected with FasL were a kind gift from Dr Shirley O'Deas (MRC Centre for Inflammation).

2.2.3.2 Jurkat E6.1 cells

Jurkat cells are a human leukaemic T cell lymphoblastic cell line (ECACC ref. 88042803). Media for the Jurkat T cell line was RPMI 1640 supplemented with 10% heat-inactivated FBS, 2mM glutamine and 20IU/ml penicillin/streptomycin. The cells grew in suspension and cultures were maintained between 3-9 x 10^5/ml.

2.2.3.3 K562/KFL9 cells

The K562 cell line (ECACC ref. 89121407) was established from a pleural effusion of a patient with chronic myelogenous leukaemia in terminal blast crisis. Media for the K562/KFL9 cell line was RPMI 1640 supplemented with 10% heat-inactivated FBS, 2mM glutamine and 20IU/ml penicillin/streptomycin. The cells grew in suspension and cultures were maintained between 1-10 x 10^5/ml. K562 cells stably transfected with FasL (KFL9) were a kind gift from Dr D. Kaplan (Case Western Reserve University, Cleveland, USA) to Dr Simon Brown (MRC Centre for Inflammation).

2.2.3.4 THP-1 cells

The THP-1 cell line (ECACC ref. 88081201) was established from the peripheral blood of a patient with acute monocytic leukaemia. Media for the THP-1 cell line was RPMI 1640 supplemented with 10% heat-inactivated FBS, 2mM glutamine and 20IU/ml penicillin/streptomycin. The cells grew in suspension and cultures were maintained between 2-9 x 10^5/ml.

2.2.4 The effect of inflammatory mediators on constitutive and Fas-induced neutrophil apoptosis and investigation of their signalling pathways using pharmacological inhibitors

Freshly isolated neutrophils (5x10^6/ml) were initially cultured in IMDM with 10% autologous serum (AS) or 10% FBS or phosphate buffered saline (PBS). The neutrophils were incubated in 96 flat-bottomed plastic flexi-well plates (Costar) with activating anti-Fas antibody (CH-11) (Upstate Cell Signalling Solutions, USA) and

recombinant FasL (SUPERFasL) (Alexis Biochemicals Corporation, USA) at varying concentrations for 3 hours and apoptosis assessed by morphology (n=2) and Annexin V binding (n=2). Jurkat cells were used as a positive control as Fas stimulation is known to accelerate their apoptosis. The neutrophils were also pre-incubated for 1 hour in a shaking water bath with the poly-caspase inhibitor zVAD (100μM) (Calbiochem, UK) the agonistic anti-Fas antibody, ZB4 (500ng/ml) (Upstate Cell Signalling Solutions, USA) and the neutralising anti-FasL antibody NOK-1 (500ng/ml) (BD Biosciences, USA) prior to being plated out in 96 flat-bottomed plastic (Costar) flexi-well plates with CH-11 (500ng/ml) for 3 hours. Apoptosis was assessed by morphology (n=2) and Annexin V binding (n=2).

To assess the effect of inflammatory mediators, the freshly isolated neutrophils ($5x10^6$/ml) in PBS and 10% FBS were pre-incubated for 1 hour in a shaking water bath with LPS (0.1μg/ml) (*E.coli* 0127:B8, Sigma), fMLP (10nM) (Calbiochem) and GM-CSF (50U/ml) (R&D Systems) prior to being plated out in 96 flat-bottomed plastic flexi-well plates (Costar) with CH-11 (500ng/ml). Apoptosis was assessed by morphology (n=2) and Annexin V binding (n=2) after a further 3 hours incubation.

The different intracellular signalling pathways were assessed by pre-incubating the freshly isolated neutrophils for 1 hour in a shaking water bath with various pharmacological inhibitors prior to being plated out in 96 flat-bottomed flexi-well plates with CH-11 (500ng/ml) and apoptosis assessed as above.

2.2.5 THP-1, transfer of conditioned media and its effect on human neutrophil apoptosis

THP-1 cells ($1x10^6$/ml), in media as above, were activated with phorbol 12-myrisate 13-acetate (PMA) (25ng/ml) and incubated on fibronectin-coated plates overnight. The cells were then washed and incubated in fresh media for a further 24 hours. The differentiated THP-1 cells were then stimulated with LPS (1μg/ml) for varying time periods before being co-incubated with fresh neutrophils ($2x10^6$/ml) for 4 hours or their conditioned media being transferred to fresh neutrophils ($2x10^6$/ml) and incubated for 4 hours.

2.2.6 Human monocyte-derived macrophages, transfer of conditioned media and its effect on human neutrophil apoptosis

The human monocyte-derived macrophages were differentiated for 5 days in either IMDM and 10% autologous serum or serum-free media supplemented with 250U/ml recombinant human GM-CSF (R&D Systems) before stimulation with 1μg/ml LPS (*E.coli* 0127:B8, Sigma) for the appropriate time. The serum-free macrophages also required purified human lipopolysaccharide binding protein (found constitutively in serum) (Hycult Biotechnology, MA, USA).

The conditioned supernatants were centrifuged at 300G for 5 minutes and then ultra centrifuged at 100 000G for 10 minutes before transfer to freshly isolated neutrophils at $2x10^6$/ml and incubated for the appropriate time. In some experiments, the conditioned media was further fractionated through Centricon 50kD filters according to the manufacturer's instructions. The freshly isolated neutrophils ($5x10^6$/ml) were also pre-incubated with an anti-TNF-α antibody (3μg/ml) (R&D Systems, USA) and antagonistic anti-Fas antibody (ZB4, 500ng/ml) (Upstate Cell Signalling Solutions, USA) for 1 hour in a shaking water bath before incubation with the conditioned media. In addition, the conditioned media was itself heat-inactivated at 56°C for 1 hour.

In the experiments requiring apoptotic neutrophils, on day 4 of macrophage differentiation, fresh neutrophils ($5x10^6$/ml) were incubated overnight in 50ml tissue culture flasks and apoptosis assessed by morphology. Macrophages were co-incubated with the apoptotic neutrophils for 4 hours at 37°C. For cytokine analysis and two-dimensional gel electrophoresis, the conditioned supernatant was stored at -70°C.

2.3 Biochemical assays and methods

2.3.1 Receptor surface expression assessed using flow cytometry

Flow buffer was prepared by adding 0.1% (w/v) azide and 0.2% (w/v) bovine serum albumin to 500ml phosphate buffered saline without cations. Cells, 100μl ($5x10^5$/ml), were plated onto 96 well round-bottomed plastic flexi-well plates

(Costar) and centrifuged (200G) for 2 minutes at 4°C. All subsequent steps were carried out at 4°C. The supernatant was discarded and the cells resuspended by gentle vortexing. Rabbit immunoglobulin, 10µl (1:20), was added for 5 minutes to block immunoglobulin receptors. The plates were again centrifuged, the supernatant discarded and cells resuspended by gentle vortexing. The primary antibody was appropriately diluted in flow buffer and added to the resuspended cells for 30 minutes before washing off the non-bound antibody (twice). The secondary FITC/ phycoerythrin (PE)-conjugated antibody was appropriately diluted and added to the resuspended cells for 30 minutes before washing off the non-bound antibody (twice). The cells were then resuspended in 200µl flow buffer and binding was assessed by flow cytometry.

2.3.2 Preparation of cell lysates for Western blotting

Lysates were prepared at 4°C. Cells in suspension were pelleted by centrifugation and the supernatant discarded. To minimize problems with proteolysis, lysates were prepared by methods normally used for electrophoretic mobility shift assay (EMSA) preparation (Ward *et al.*, 1999). The cell pellet was resuspended in 100µl lysis buffer (buffer A (10mM Tris-HCL, pH 7.8, 1.5mM EDTA, 10mM KCL), 0.5mM dithiothreitol, 1µg/ml aprotinin, leupeptin and pepstatin A, 1µM 4-(2-aminoethyl) benzenesulphonyl fluoride, 1mM sodium orthovanadate, 0.5mM benzamidine and 2mM levamisole) and placed on ice for 10 minutes. Following the addition of 0.1 volumes of 10% Nonidet P-40 (w/v), the cells were vortexed briefly and centrifuged at 12000G for 2 minutes at 4°C. The supernatants were transferred to clean Eppendorfs and the nuclear pellet discarded. The protein content of the supernatants was estimated as below and the appropriate volume of 6x sample buffer (0.375M Tris-HCL, pH 6.8, 12% sodium dodecyl sulphate (SDS), 15% β-mercaptoethanol, 40% glycerol and 0.03% bromophenol blue) was added to the supernatants prior to heating at 95°C for 5 minutes. Lysates were stored overnight at 4°C prior to further usage.

2.3.3 Protein estimation

The protein concentration of lysates was estimated using the Pierce protein assay system which is based on the Bradford Coomassie brilliant blue dye binding system. A range of bovine serum albumin standards was used to construct a standard curve using spectrophotometry and protein samples were diluted to fall within the linear range of this curve. Protein concentrations of all samples were routinely measured in duplicate.

2.3.4 Polyacrylamide gel electrophoresis

A Bio-Rad mini trans-blot module (Protean II) was used. In order to maximize resolution of the protein of interest, the appropriate percentage sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separating gel was used. The appropriate quantity of 30% acrylamide/bis-acrylamide was added to the separating buffer (e.g. 20ml of 9% separating gel solution required 8.82ml of deionised water, 6ml of 30% acrylamide/bis-acrylamide , 5ml of 1.5M Tris base pH 8.8, 0.08ml of 20% SDS, 0.08ml of 10% ammonium persulphate and 20ul TEMED). A 3% stacking gel solution was also prepared (for a final volume of 8ml, 5.51ml of deionised water, 0.8ml of 30% acrylamide/bis-acrylamide, 1ml of 0.5M Tris base, pH 6.8, 0.08ml of 10% SDS, 0.6ml of 12% ammonium persulphate and 8µl of TEMED were required) (Laemmli, 1970). Standardised amounts of protein for each sample were loaded and the proteins resolved by electrophoresis at 150V. (The electrophoresis buffer was 250mM Tris/1.92M glycine (pH 8.3) and 0.1% SDS)

2.3.5 Immunoblotting

Proteins resolved by polyacrylamide gel electrophoresis were transferred to nitrocellulose membranes at 80V for 1 hour. (The transfer buffer was 250mM Tris/1.92M glycine (pH 8.3) and 20% methanol). Transfer efficiency and equality of loading were assessed by staining membranes with Ponceau S solution for 3 minutes followed by washing with distilled water. Ponceau S was removed by washing with Tris buffered saline (TBS) (20mM Tris-HCl, pH 7.6, 136mM sodium chloride) containing 0.1% (v/v) Tween 20 (TBST).

The nitrocellulose membranes were then blocked in TBST with 5% (w/v) non-fat powdered milk (Marvel) for a minimum of 1 hour at room temperature. Blots were incubated with primary antibodies at the recommended concentration in TBST or TBS with 1% (w/v) bovine serum albumen, overnight at 4°C. Three washes of 10 minutes each using TBST were then carried out prior to incubation with the appropriate horseradish peroxidase conjugated secondary antibody for 1 hour in TBST at room temperature. Blots were washed with three washes of 10 minutes each using TBST prior to developing. Protein bands were visualised by enhanced chemoluminescence (Amersham) and autoradiography.

2.3.6 Re-probing with anti - β-actin antibody

The immunoblots were stripped in buffer (62.5mM Tris base (pH 6.7), 0.075% β-mercaptoethanol and 2% SDS) for 1 hour at 50°C. Three washes of 10 minutes each using TBST were then carried out prior to blocking in TBST with 5% (w/v) non-fat powdered milk (Marvel) for a minimum of 1 hour at room temperature. The blots were then incubated with 1:50 anti - β-actin antibody for 1 hour at room temperature. Three further washes of 10 minutes each using TBST were then carried out prior to incubation with 1:2000 horseradish peroxidase conjugated goat anti-mouse antibody for 1 hour in TBST at room temperature. The blots were then washed and developed as above.

2.3.7 Two-dimensional gel electrophoresis

ExcelGel SDS gradient 8-18, ExcelGel buffer strips (Pharmacia Biotech), a Multiphor II electrophoresis unit and a MultiTemp II thermostatic circulator were used in these experiments which were carried out in duplicate. The samples were dissolved in reducing SDS sample buffer and denatured as previously described. Light paraffin oil (1ml) was used as insulating fluid and placed on the thermostatic circulator at 15°C before the gel was placed on the cooling plate so that the polarity of the gel corresponded to that of the plate. The cathodic and anodic buffer strips were applied to their respective sides of the gel. The applicator strip was placed 5mm from the cathodic buffer strip and 20ul of sample applied.

Electrophoresis was commenced at 300V, 2mA for 1 minute and gradually increased over the next 30 minutes to 3500V which was maintained for approximately 2 hours until the Bromophenol Blue front had reached the anode. The gel was immediately fixed (40% ethanol, 10% acetic acid) for 30 minutes and incubated overnight at room temperature (250ml of incubation solution contained 75ml ethanol, 17g sodium acetate·$3H_2O$, 1.3ml glutaraldehyde (25% w/v) 0.5g sodium thiosulphate and distilled water). The gel was washed for 5 minutes, three times, in distilled water. The gel was stained for 40 minutes in silver solution (250ml contained 0.25g silver nitrate, 50µl formaldehyde and distilled water). The protein bands were developed in developing solution (250ml contained 6.25g sodium carbonate, 25µl formaldehyde and distilled water) for approximately 15 minutes before stopping the reaction (with 3.65g EDTA-Na_2·2 H_2O in 250ml distilled water for 10 minutes. The gel was washed for 5 minutes, three times, in distilled water and preserved in 10% glycerol.

2.3.8 Quantitative analysis of inflammatory cytokines in cell supernatants using a flow cytometric bead array

The bead array was purchased from Becton Dickinson (BD) and measured IL-8, IL-1β, interleukin-6 (IL-6), interleukin-10 (IL-10) and TNF-α. Initially the cytokine standards were reconstituted and diluted appropriately. The standard curve covered concentrations from 20-5000pg/ml. The capture beads for each cytokine are in individual bottles and were pooled at the start of the experiment. To each assay tube, 50µl of mixed capture beads, 50µl of PE detection reagent and 50µl of either a standard or test sample were added. The samples were then incubated at room temperature, protected from light, for 3 hours. The samples were then washed with 1ml wash buffer and centrifuged at 200G for 5 minutes. The supernatants were carefully removed and discarded and the cytokine beads resuspended in 300µl of wash buffer before being analysed on the BD flow cytometer.

2.4 Molecular Biology

2.4.1 RNA isolation

Cells ($5x10^6$/ml) were pelleted and resuspended in 1ml Tri reagent (Sigma). Chloroform (0.2ml) was added, the samples shaken vigorously for 15 seconds and allowed to stand for 5 minutes at room temperature. The samples were then centrifuged at 12000G for 15 minutes at 4°C which separated the mixture into three phases; a red organic phase containing protein, interphase containing DNA and colourless upper phase containing RNA. The aqueous phase was transferred to a fresh RNase-treated tube to which 0.5ml of isopropanol was added and allowed to stand for 5 minutes at room temperature. The samples were then centrifuged at 12000G for 10 minutes at 4°C. The RNA pellet was washed with 1ml 75% ethanol and centrifuged at 7500G for 5 minutes at 4°C. The RNA pellet was air dried and 50µl formamide water added. The quantity of RNA was measured on a spectrophotometer.

2.4.2 First strand cDNA synthesis

First strand cDNA synthesis was performed using a Superscript first strand synthesis system (Life Technologies). RNA (0.5µg) was incubated with 1µl RNase inhibitor, 1µl oligo dT (0.5µg/ml), and diethylpyrocarbonate (DEPC)-treated water (added to a final volume of 11 µl) at 70°C for 10 minutes. Reaction mixture containing 10x reverse transcription buffer (2 µl), 0.1M DTT (2µl) and 25mM magnesium chloride (4 µl) were added and incubated at 42°C for 2 minutes. Superscript II (1 µl) was added and gently mixed before the reverse transcription reaction was allowed to proceed at 42°C for 60 minutes before termination by heating to 70°C for 10 minutes. The cDNA was stored at -20°C until required.

2.4.3 Purification of plasmid DNA

The cDNA of the Fas receptor (GenBank: NM_000043) in the pCMV-SPORT6 plasmid (GenBank: BC012479, ATCC: MGC-21432) was purchased from LGC Promochem, UK. The freeze-dried colonies were initially grown in 1ml LB (Luria-Bertani) with 50µg/ml ampicillin for 5 hours at 37°C with continuous shaking before

being added to 4ml LB with 50µg/ml ampicillin and grown overnight at 37°C with continuous shaking.

DNA was purified using the QIAprep Miniprep system (Qiagen, Crawley). The cells in 2ml bacterial culture were pelleted, the supernatant discarded and the cells resuspended in buffer P1 (250µl) containing RNase A. Buffer P2 (250µl) was then added to lyse the bacteria under alkaline conditions and gently inverted 4-6 times to mix. Lastly buffer N3 (350µl) was added to neutralise and adjust to high-salt binding conditions in one step. The tube was gently inverted to mix and then centrifuged at 13 000rpm for 10 minutes and the supernatant carefully transferred to the Qiaprep column. The column was centrifuged at 13 000rpm for 1 minute and the flow-through discarded. The column was then washed with 0.75ml buffer PE and centrifuged for 1 minute. The flow-through was again discarded before centrifuging for an additional 1 minute. The DNA was eluted by adding 50µl buffer EB (10mM Tris·Cl, pH 8.5) and standing for 1 minute before centrifuging for 1 minute. A Qiagen Plasmid Maxi kit was used for the purification of larger quantities of the plasmid DNA. 100µl of the bacterial broth was grown in 100ml volumes overnight. The cells were then pelleted by centrifugation at 6000G for 15 minutes at 4°C and resuspended in 4ml buffer P1. Buffer P2 (4ml) was added to lyse the bacteria , gently mixed and incubated at room temperature for 5 minutes. Chilled buffer P3 was added (4ml), gently mixed, incubated for 15 minutes at 4°C and centrifuged at 20000G for 30 minutes at 4°C. The supernatant was removed and re- centrifuged at 20000G for 30 minutes at 4°C. A Qiagen-tip 100 was equilibrated by applying 4ml buffer QBT and allowing the column to empty by gravity. The supernatant was applied to the column and allowed to enter the resin by gravity flow. The Qiagen-tip was washed twice with 10ml buffer QC. The DNA was then eluted with 5ml buffer QF and the DNA precipitated by adding 3.5ml isopropanol and centrifuging at 15000G for 30 minutes at 4°C. The DNA pellet was washed with 2ml 70% ethanol, centrifuged at 15000G for 10 minutes, allowed to air dry and dissolved in 10mM Tris·Cl, pH 8.5. The DNA was quantified and assessed using spectrophotometry and agarose gel electrophoresis.

2.4.4 Polymerase chain reaction

Mastermix (for FasL cytoplasmic tail) (total volume 50µl) containing 10x polymerase chain reaction (PCR) buffer (5µl) 50mM magnesium chloride (1.5µl), dNTP mix (final concentration 125µM of each dNTP) and forward and reverse primers (final concentration of each 500nM) was prepared and added to each 0.5ml Eppendorf tube. For FasL, the forward primer was 5'-GGA TTG GGC CTG GGG ATG TTT CA-3' and the reverse primer 5'-TAA TAC GAC TCA CTA TAG GGT TGT GGC TCA GGG GCA GGT TGT TG-3'giving a product of 343kB. For human GAPDH the forward primer was 5'-TGC CTC CTG CAC CAC CAA CTG C-3' and the reverse primer 5'-AAT GCC AGC CCC AGC GTC AAA G-3' giving a product of 456kB. For the Fas cytoplasmic tail, the primers are shown in diagram 4.5b. Template cDNA (10ng/50µl) or distilled water as control was added prior to the addition of 1.25 units of the enzyme *Taq* DNA polymerase (GIBCO). The following thermal cycling profiles were used. For FasL and GAPDH, samples were denatured at 94°C for 3 minutes, then 35 cycles of: denaturation at 94°C for 45 seconds, primer annealing at 63°C for 30 seconds and DNA extension at 72°C for 1.5 minutes. A final extension at 72°C was performed for 10 minutes. For the Fas cytoplasmic tail, samples were denatured at 94°C for 2.5 minutes, then 25 cycles of: denaturation at 94°C for 30 seconds, primer annealing at 51°C for 30 seconds and DNA extension at 72°C for 40 seconds. A final extension at 72°C was performed for 5 minutes. Oligonucleotide primers were synthesized by MWG-Biotech, Ebersberg, Germany.

2.4.5 Agarose gel electrophoresis of DNA

DNA samples were mixed with 6x blue loading buffer and run on 1% agarose gels with Tris-borate electrophoresis (TBE) buffer and ethidium bromide (2µl in 50ml) at 100V for 30 minutes. The bands were visualised on an ultraviolet transilluminator and images recorded on Polaroid film.

2.4.6 Gel extraction and purification of DNA

DNA extraction from agarose was performed using a QIAquick Gel Extraction kit (Qiagen, Crawley, UK). The DNA fragment was excised from the agarose gel using a clean scapel blade and placed in an Eppendorf tube. The fragment was then

incubated at 50°C for 10 minutes in buffer QG to dissolve the agarose. Isopropanol (100µl) was added and each sample placed in a spin column. DNA was applied to the membrane by centrifugation for 1 minute at 13000rpm in a microfuge. The flow-through was discarded and the membrane washed with 750µl of buffer PE before further centrifugation for 1 minute at 13000rpm. The flow-through was again discarded and the column centrifuged to remove all traces of wash buffer. The column was then placed in a clean Eppendorf tube and the bound DNA eluted by the addition of 50µl buffer EB followed by centrifugation at 13000rpm for 1 minute.

2.4.7 Subcloning of DNA

The Pinpoint^{TM} Xa-1 T-Vector system (Promega, Madison, WI, USA) was used to subclone the DNA product into the Pinpoint^{TM} Xa vector. The Pinpoint^{TM} Xa vector contains a biotin purification tag coding region upstream of the multiple cloning region (figure 4.4a): this encodes a peptide which becomes biotinylated in *E. coli* allowing the protein of interest to be purified.

2.4.7.1 Ligation of DNA into vector

The Pinpoint^{TM} Xa vector system exploits the property of *Taq* DNA polymerase to add a single deoxyadenosine to the 3' end of the PCR product. The linearised Pinpoint^{TM} Xa vector has complimentary 3' overhanging deoxythymidine residues thus improving the efficiency of ligation of the PCR product into the plasmid. Promega supplied control DNA (368bp) which encodes the *lacZ* α-peptide. When cloned into the vector a fusion protein is created between the biotinylation segment and the α-peptide. For ligation, 7µl of fresh PCR product or 3µl of the control DNA or distilled water (negative control) were mixed with 1µl of vector, 1µl of 10x T_4 DNA ligase buffer and 1µl of T_4 DNA ligase and incubated at 15°C overnight.

2.4.7.2 Transformation of competent *Escherichia coli*

High efficiency JM109 competent cells (Promega) were used. Aliquots of competent cells (50µl) were gently mixed with 3µl of plasmid DNA from the ligation step and incubated on ice for 30 minutes. Cells were then heat shocked at 42°C for 50 seconds followed by incubation on ice for 2 minutes. Following the addition of

500µl SOC medium (Invitrogen), the cells were gently mixed (225rpm) for 1 hour at 37°C. The cells were pelleted at 2500G for 3 minutes, the supernatant removed and the cells resuspended in 100µl SOC medium and spread onto prewarmed LB (Luria-Bertani) agar (Life Technologies) selective plates containing 50µg/ml ampicillin. Plates were then incubated overnight at 37°C. Eight colonies from the PCR insert plate were picked and incubated in 5ml LB with 50µg/ml ampicillin and grown overnight at 37°C with shaking. The DNA was purified using the QIAprep Miniprep system (Qiagen, Crawley) as above.

2.4.7.3 Restriction enzyme digest

The purified plasmid DNA was digested in a 10µl volume containing 1µl 10x reaction buffer and 0.5µl enzyme (Promega) for 2 hours at 37°C. The products of digestion were resolved on agarose gels and visualised using ultraviolet light as previously described.

2.4.7.4 DNA sequencing

The plasmid containing the insert in the correct orientation was identified using restriction enzyme digest. A sample containing 2µg of the purified plasmid DNA was made up to 5µl with distilled water before 0.5µl 3M sodium acetate pH 5.2 (check) and 12.5µl 100% ethanol were added. Samples were left for 1 hour at -20°C. The precipitated DNA was spun down at 15000rpm at 4°C for 10 minutes and washed with 500µl 70% ethanol. The pellet was air dried for 1 hour. Using SP6 primers, DNA encoding the "insert" was sequenced commercially by MWG Biotech. The cloned sequence was verified using DNA Strider 1.3f8 with the Fas receptor sequence in the NCBI nucleotide database (NM_000043).

2.4.8 Protein Purification

The biotinylated fusion product produced in *E.Coli* (JM109) was purified using the SoftLink™ Soft Release Avidin Resin (Promega, Madison, USA). This resin allows elution of the protein under non-denaturing conditions. *E. coli* produce a single

endogenous biotinylated protein, biotin carboxyl carrier protein (BCCP), (22.5kDa) that, in its native conformation, does not bind to avidin. This renders the affinity purification highly specific for the recombinant fusion protein. The resin is monomeric avidin and the biotinylated fusion protein is eluted from the resin in a non-denaturing 5mM biotin solution.

2.4.8.1 Large scale culture and induction

A freshly isolated bacterial colony (clone 4) was incubated in 5ml of LB (Luria-Bertani), containing biotin (2µM final concentration) and ampicillin (50µg/ml), overnight at 37°C with shaking. The 5ml culture was then transferred to 500ml LB (Lubia-Bertani), containing biotin (2µM final concentration) and ampicillin (50µg/ml) and incubated for 2 hours at 37°C with shaking. Protein expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) (100µM final concentration) and incubating for a further 5 hours at 37°C with shaking.

2.4.8.2 Bacterial cell lysis

The cells were pelleted at 4000G for 10 minutes and the supernatant discarded. The cells were weighed and resuspended in 10 volumes (ml/gram cell paste) of cell lysis buffer (50mM Tris-HCl (pH 7.5), 50mM NaCl, and 5% glycerol) containing phenylmethylsulfonyl fluoride (PMSF) (final concentration 1mM) at 4°C.

2.4.8.3 Lysozyme/Detergent method

Lysozyme (final concentration of 1mg/ml) was added and the solution continuously stirred for 20 minutes at 4°C. Triton X (final concentration 0.1%) was added and stirring continued for five minutes at 4°C. DNase I (200U) was added to reduce the viscosity of the solution and stirring continued for an additional 10 minutes. The crude lysate was centrifuged at 10000G for 15 minutes at 4°C to remove cellular debris.

2.4.8.4 Preparation and regeneration of SoftLink™ Resin

At room temperature, the column was equilibrated with 0.1M $NaPO_4$ (pH 7.0). The non-reversible binding sites on the fresh SoftLink™ Resin were preabsorbed by washing with 5mM biotin in 100mM sodium phosphate buffer (pH 7.2) at a flow rate of 6ml biotin/hour/ml of SoftLink™ Resin until two column volumes had passed through. The flow was stopped for 15 minutes to allow biotin binding. The column was then regenerated by washing the column with 8 column volumes of 10% acetic acid, then 8 column volumes of 100mM $NaPO_4$ (pH 7.0). The pH of the eluate was monitored until it reached pH 6.8. The flow was then stopped for 30 minutes to allow the avidin to refold and finally the column was re-equilibrated with cell lysis buffer.

2.4.8.5 Resin column protein purification

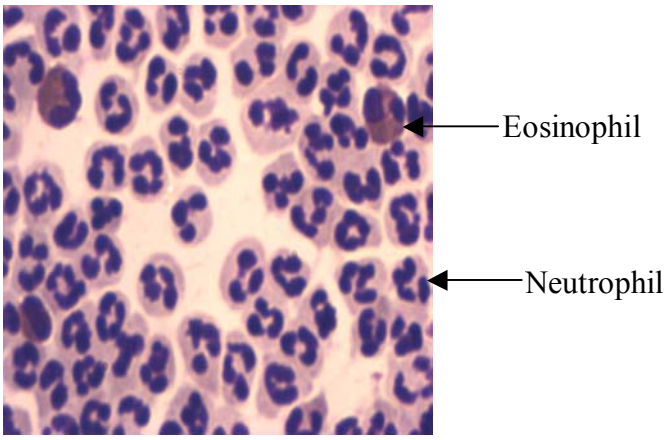
The supernatant (cell extract) was applied to the prepared resin column (6ml/hour) at 4°C. The column was then washed with five column volumes of cell lysis buffer and to elute the protein, a stabilizing buffer containing 5mM biotin was added. Initially a 0.5ml fraction was collected and the flow stopped for 15 min to increase the concentration of protein in the eluate before restarting collection of 0.5ml fractions. The fractions were tested and protein concentration quantified by monitoring the absorbance at A280. Proteins from each fraction were then run on a 12% SDS gel, transferred to a nitrocellulose membrane and detected using HRP-Streptavidin (1:2500).

2.5 Statistics

All values are presented as mean $\pm$ SEM. The data were evaluated statistically as shown in the table below. P values < 0.05 were considered to be statistically significant.

Figure	Page	Test	Post-test
3.1(c)	58	ANOVA	Student-Neuman-Keuls
3.2(b)	59	ANOVA	Student-Neuman-Keuls
3.3(b)	60	ANOVA	Student-Neuman-Keuls
4.1(a)	81	ANOVA	Student-Neuman-Keuls
4.1(b)	81	ANOVA	Student-Neuman-Keuls
4.2(a)	82	ANOVA	Student-Neuman-Keuls
4.2(b)	82	ANOVA	Student-Neuman-Keuls
4.3(a)	83	ANOVA	Student-Neuman-Keuls
4.3(b)	83	ANOVA	Student-Neuman-Keuls
4.3(c)	83	ANOVA	Student-Neuman-Keuls
4.4(b)	84	ANOVA	Student-Neuman-Keuls
4.5(a)	85	ANOVA	Student-Neuman-Keuls
5.1(b)	103	ANOVA	Student-Neuman-Keuls
5.2(a)	104	ANOVA	Student-Neuman-Keuls
5.4(b)	106	ANOVA	Student-Neuman-Keuls

a)



b)

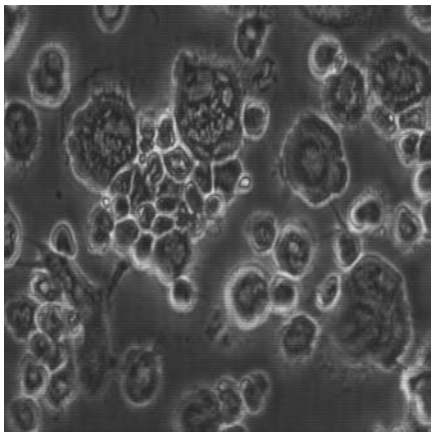
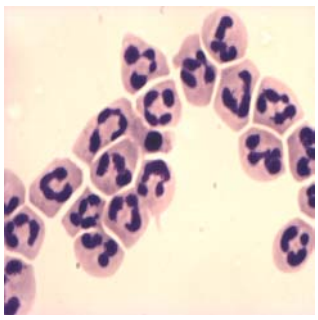
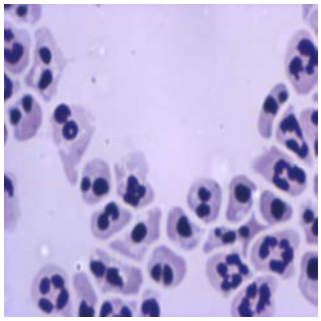


Figure. 2.1. Granulocytes and macrophages (a) Freshly isolated granulocytes (light microscopy under oil immersion, x100 objective,) (b) Human peripheral blood mononuclear cell-derived macrophages (day 5) cultured in IMDM with 10% autologous serum (phase contrast microscopy, x 40 objective)

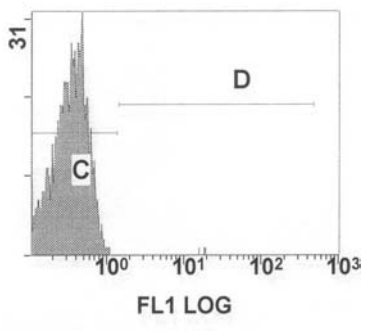
ai)



aii)



bi)



bii)

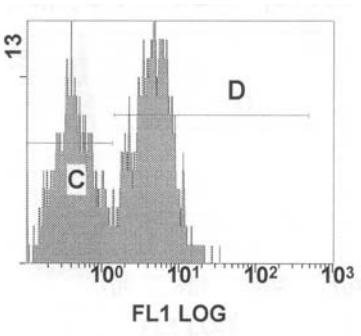


Figure 2.2 Fresh and apoptotic neutrophils (a) Morphology (i) Fresh neutrophils (ii) Apoptotic neutrophils (light microscopy under oil immersion x100 objective) (b) Apoptosis assessed by flow cytometry with apoptotic cells showing Annexin V binding (i) Fresh neutrophils (ii) Apoptotic neutrophils

Table 2.1. Antibodies used to assess receptor surface expression

Hybridoma clone (antigen)	Primary antibody	Secondary antibody	FITC/PE-conjugated antibody	Control antibody
CH-11 (Fas)	1:25	FITC-goat anti-mouse Ig $F(ab)_2$ 1:40		IgM (1:25)
ZB4 (Fas)	1:40	FITC-goat anti-mouse Ig $F(ab)_2$ 1:40		IgG_1 (1:40)
Alf2.1a (FasL)			1:50	FITC-conjugated IgG_1 (1:50)
3G8 (CD16)	1:50	FITC-goat anti-mouse Ig $F(ab)_2$ 1:40		IgG_1 (1:50)
My4 (CD14)	1:50	FITC-goat anti-mouse Ig $F(ab)_2$ 1:40		IgG2b (1:50)
10.1 (CD64)	1:50	FITC-goat anti-mouse Ig $F(ab)_2$ 1:40		IgG1 (1:50)
HK14 (HLA-DR)	1:50	FITC-goat anti-mouse Ig $F(ab)_2$ 1:40		IgG2b (1:50)

Table 2.2 Antibodies used in immunoblotting

Protein	Source	Primary Antibody	Secondary Antibody
Rabbit anti-human IκB-α	Cell Signalling	1:500	1:2500 HRP donkey anti-rabbit
Mouse anti-human FasL (Clone 33)	Transduction		1:2000 HRP goat anti-mouse
Rabbit anti-human FLIP	R & D Systems	1:1000	1:2000 HRP goat anti-rabbit
Mouse anti-human caspase-8	BD Pharmingen	1:125	1:2500 HRP goat anti-mouse

Table 2.3 Antibodies

Immunogen	Antibody	Application	Manufacturer
Human Fas (Apo-1, CD95)	Mouse anti-human Fas, clone CH11	Activating	Upstate
Human Fas (Apo-1, CD95)	Mouse anti-human Fas, clone ZB4	Antagonistic	Upstate
Human FasL (CD95L)	Mouse anti-human FasL, clone NOK-1	Inhibitory	BD Biosciences
Human FasL (CD95L)	Mouse anti-human FasL, clone 33	WB	BD Transduction
Soluble human FasL	Mouse anti-human FasL/FITC, clone Alf-2.1a	FACS	Ancell
Human Caspase-8	Mouse anti-human caspase-8, clone 4-1-20	WB	BD Pharmingen
IκB-α	Rabbit anti- IκB-α	WB	Cell Signalling Technology
Human/mouse FLIP	Rabbit anti-human/mouse FLIP	WB	R&D Systems
Human TNF-α	Mouse anti-human TNF-α	Inhibitory	R&D Systems
Human CD16	Mouse anti-human CD16	FACS	Beckman Coulter
Human CD14	Mouse anti-human CD14	FACS	Beckman Coulter
Human CD64	Mouse anti-human CD64	FACS	Ancell
Human HLA-DR	Mouse anti-human HLA-DR	FACS	Sigma

FACS: Fluorescent activated cell sorting

WB: Western Blotting

Table 2.4 Pharmacological Inhibitors

Enzyme(s)	**Pharmacological Inhibitor**	**Manufacturer**
Poly-caspase inhibitor	z-Val-Ala-DL-Asp-fluoromethylketone (zVAD)	Calbiochem
p38 MAPK	SB 203580	Calbiochem
p42/44 ERK	PD 98059	Cell Signaling
Phosphatidylinositol 3-kinase (PI 3-kinase)	LY 294002	Calbiochem
Protein kinase C	Ro-31-8220	Calbiochem
Protein kinase Cδ	Rottlerin	Calbiochem
Protein kinase Cα	Go 6976	Calbiochem
NF-κB	15-deoxy-$\Delta^{12,14}$-prostaglandinJ_2	Biomol

Table 2.5 Recombinant proteins

Recombinant protein	**Manufacturer**
FLAG-tagged soluble human FasL (SUPERFas Ligand)	Alexis Corporation
Human GM-CSF	R&D Systems
Human TNF-α	R&D Systems

2.5 Manufacturer

Alexis Biochemicals Corporation, San Diego, CA, USA

Ancell Corporation, North Bayport, MN, USA

Bachem, Bubendorf, Switzerland

BD Biosciences, San Jose, CA, USA

Beckman Coulter, Fullerton, CA, USA

Biomol, Plymouth Meeting, PA, USA

Calbiochem, Nottingham, UK

Cell Signaling Technology, Beverly, MA, USA

R&D Systems, Minneapolis, MN, USA

Upstate Cell Signaling Solutions, Lake Placid, NY, USA

Chapter 3

Neutrophil Apoptosis and Fas/FasL

3.1 Introduction

During apoptosis, the neutrophil undergoes cell shrinkage, chromatin condensation, membrane blebbing and loses the multilobed shape of its nucleus. The apoptotic bodies are then "eaten" by phagocytes such as macrophages (Savill *et al.*, 1989). If aged neutrophils are not effectively phagocytosed, they release their potentially injurious contents into the surrounding inflamed tissue which may potentiate tissue injury (Mecklenburgh *et al.*, 1999). Furthermore, when macrophages ingest apoptotic neutrophils, they switch to an "anti-inflammatory" phenotype (Fadok *et al.*, 1998). The discovery of this mode of cell clearance explained how cells are effectively removed without inciting an inflammatory response (diagram 3.1.).

At a molecular level, the apoptotic neutrophil displays different surface receptors. Immunoglobulin superfamily members including CD31 (PECAM-1), CD50 (ICAM-3), CD63 and CD87 (urokinase plasminogen activator receptor) are downregulated. There is also reduced expression of receptors involved in the inflammatory process including CD32 (FcγRII), CD35 (CR1), and CD88 (C5a receptor). CD16 (FcγRIII), CD15 (Le^x) and CD120b (TNF-α receptor) are shed from the cell surface while the phosphatidylserine receptor becomes externally exposed and apoptotic neutrophils are therefore able to bind fluorescently-labelled- Annexin V (Dransfield *et al.*, 1994; Homburg *et al.*, 1995; Hart *et al.*, 2000). At a functional level, apoptotic neutrophils *in vitro* are less responsive to external stimuli including fMLP. Other cellular functions including chemotaxis, adhesion, phagocytosis, enzyme secretion and generation of a respiratory burst are downregulated (Whyte *et al.*, 1993; Dransfield *et al.*, 1995). The DNA of apoptotic neutrophils also undergoes cleavage to form a characteristic nucleosomal ladder (Wyllie, 1980).

Neutrophils are short-lived and the majority undergo spontaneous apoptosis in less than 24 hours (Colotta *et al.*, 1992; Squier *et al.*, 1995). They have been shown to express Fas and anti-Fas antibody accelerated their apoptosis (Iwai *et al.*, 1994). Neutrophils also express FasL and release sFasL which can induce death in activated Jurkat cells (Liles *et al.*, 1996). Furthermore blocking the Fas receptor, partially suppressed spontaneous neutrophil apoptosis suggesting that the Fas/FasL may mediate neutrophil apoptosis by both an autocrine and paracrine pathway (Liles *et al.*, 1996). Therefore, this Fas-mediated fratricide in spontaneous neutrophil apoptosis will be further explored.

3.2 Results

3.2.1 Fas is functionally expressed on the surface of human neutrophils

The death receptor Fas is expressed in various tissues. We demonstrated that it was expressed on neutrophils using indirect immunofluorescent labelling with the mouse anti-human Fas IgG monoclonal Ab (mAb), ZB4 (figure 3.1a). This has previously been shown with the FITC-conjugated anti-human Fas mAb, UB2 (Liles *et al.*, 1996; Renshaw *et al.*, 2000). The activating anti-Fas antibody, CH-11, induces apoptosis in Fas-expressing cells. It also induced apoptosis in neutrophils at an optimum concentration of 500ng/ml (figure 3.1b). Neutrophils were short-lived, *in vitro*, with 70% of neutrophils undergoing apoptosis by 20 hours (figure 3.1c). The levels of spontaneous and Fas-induced neutrophil apoptosis were highest at 3 hours in a serum-free system, (Pbs, 34.9 $\pm$ 3.7%; CH-11, 64.5 $\pm$ 7.4%, n=3, $p<0.01$) however by 20 hours, cells cultured in serum-free media were necrotic as assessed by their inability to exclude trypan blue. In the presence of serum, the spontaneous rates of apoptosis were not affected by the type of serum however the induction of apoptosis by CH-11 was significantly greater at both 3 hours (As.CH-11, 26.1 $\pm$ 4.4%; Fbs.CH-11, 57.1 $\pm$ 6.8%, n=3, $p<0.001$) and 20 hours (As.CH-11, 69.1 $\pm$ 4.8%; Fbs.CH-11, 89.2 $\pm$ 2.6%, n=3, $p<0.05$) when the cells were cultured in the presence of foetal bovine serum (Fbs).

3.2.2 Anti-Fas antibodies

There are several anti-Fas antibodies (Komada *et al.*, 1999). The prototype agonistic anti-Fas antibody (CH-11) is an IgM antibody while the antagonistic antibody (ZB4) is an IgG antibody. Both CH-11 and ZB4 bound to the Fas receptor (figure 3.2a) but the binding of CH-11 was not saturated at concentrations (500ng/ml) which maximally induced neutrophil apoptosis (figure 3.1b). CH-11 and ZB4 have been shown to bind to the same epitope on the Fas protein however ZB4 has a much greater affinity (Komada *et al.*, 1999). It has been suggested that serial triggering of the Fas receptor, due to the IgM monoclonal antibody having a weak affinity, may explain the agonistic properties of CH-11 while the antagonistic IgG antibody has a strong affinity (Komada *et al.*, 1999). ZB4 (500ng/ml) had no effect on spontaneous

neutrophil apoptosis (control, 4.1 ± 0.8%; ZB4, 4.8 ± 1.0%) and when neutrophils were pre-incubated with ZB4 for 1 hour, it completely inhibited CH-11-induced apoptosis (CH-11, 30.5 ± 3.1%; ZB4.CH-11, 4.7 ± 1.1%, n=3, $p<0.001$) (figure 3.3b). This suggests that Fas/FasL is not an important mechanism of spontaneous neutrophil apoptosis and that CH-11 mediated its effect through the Fas death receptor alone. Caspases are known to be important mediators downstream of death receptors. The poly-caspase inhibitor, zVAD (control, 4.1 ± 0.8%; zVAD 3.8 ± 0.4%) had no effect on spontaneous neutrophil apoptosis but completely inhibited CH-11-induced apoptosis (CH-11, 30.5 ± 3.1%; zVAD, 3.7 ± 0.5%, n=3, $p<0.001$). This suggests that caspases are important in the Fas/FasL death receptor pathway in neutrophils. Contrary to our observations, there is also data suggesting that spontaneous neutrophil apoptosis is caspase dependent (Pongracz *et al.*, 1999).

3.2.3 FasL and Neutrophil Apoptosis

Human FasL is physiologically active in its membrane-bound form. When it is cleaved by metalloproteases to a soluble form, sFasL, its pro-apoptotic activity is downregulated (Schneider *et al.*, 1998; Tanaka *et al.*, 1998). Furthermore only multimerized or membrane-bound FasL induced apoptosis of murine granulocytes (Villunger *et al.*, 2000). SUPERFasLigand (Alexis) is genetically engineered human soluble recombinant FasL with a FLAG-tag and is biologically active (Thilenius *et al.*, 1997). Preliminary results showed that SUPERFasLigand (rhFasL) induced apoptosis in both Jurkat cells (human leukaemic T lymphocyte cell line) and human neutrophils however Jurkat cells were more sensitive (figure 3.3a). The agonistic antibody CH-11 (38.1% ± 4.2%) induced significantly more neutrophil apoptosis than rhFasL (8.6% ± 2.3 %, n=3, $p<0.001$). The anti-human FasL monoclonal antibody, NOK-1, has been shown to neutralize the cytotoxic activity of FasL (Kayagaki *et al.*, 1995; Orlinick *et al.*, 1997; Oyaizu *et al.*, 1997; Villunger *et al.*, 1997; Walker *et al.*, 1997). Pre-incubation with the anti-FasL antibody, NOK-1 and the anti-Fas antibody, ZB4 inhibited the cytotoxic effect of rhFasL (figure 3.3b). However, the effect of the agonistic anti-Fas antibody CH-11 (38.1 ± 4.2%) was also significantly inhibited by NOK-1 (27.6 ± 7.2%, n=3, $p<0.05$) and ZB4 (5.7 ± 1.4%, n=3, $p<0.001$) (figure 3.3b). Furthermore NOK-1 had no effect on spontaneous

neutrophil apoptosis. This suggests that NOK-1 partially inhibits CH-11 – induced neutrophil apoptosis although the mechanism is not known. Both NOK-1 and ZB4 had no effect on spontaneous neutrophil apoptosis which suggests that the Fas/FasL pathway in neutrophils is unlikely to mediate suicide or fratricide.

3.2.4 FasL expression on the neutrophil

The seminal paper in this field suggested that neutrophils express Fas and FasL (Liles *et al.*, 1996). Initially, to assess cell surface FasL by flow cytometry and whole cell FasL by Western blotting, a gift of FasL stably transfected A549 (lung adenocarcinoma cell line) cells were used as a positive control while Jurkat cells (human T-cell leukaemia cell line) were used as negative control (Smith *et al.*, 1998). Using an anti-human FasL monoclonal antibody, clone 33 (Transduction), FasL was shown to be present by Western blotting in Jurkat cells, control A549 cells and two stably transfected A549 cell lines (PC3 and PC4) (figure 3.4a). As the negative control Jurkat cell line, the control A549 cells and the transfectants all expressed FasL, we compared messenger ribonucleic acid (mRNA) levels of FasL in these cells to those of the myeloid leukaemia cell line, K562 and its FasL stable transfectant, KFL9. It has been shown that the K562 cell line does not express surface FasL while the stable transfectant KFL9 does express FasL (Renshaw *et al.*, 2000). Using RT-PCR, we showed that neither the A549, PC3, PC4 nor K562 cell line expressed mRNA for FasL whilst the KFL9 cell line did express mRNA for FasL (figure 3.4b). Thus PC3 and PC4 were not stably transfected with FasL and the anti-FasL monoclonal antibody, clone 33 appears to bind non-specifically to a 37kDa protein.

Monocytes have mRNA for FasL but only express it on the surface when activated (Lu *et al.*, 2002). Using the same primers as above, we showed using RT-PCR, that fresh neutrophils and K562 cells do not express mRNA for FasL whilst mononuclear cells and KFL9 cells do express mRNA for FasL (figure 3.5a). Furthermore, using a FITC-conjugated anti-human FasL monoclonal antibody, Alf-2.1a (Ancell), KFL9 cells expressed FasL on their surface but neither fresh neutrophils, mononuclear cells nor K562 cells expressed FasL on their surface (figure 3.5b).

3.3 Discussion

Neutrophil death by apoptosis is important in resolution of inflammation. If a neutrophil is allowed to die by primary or secondary necrosis, it will release its pro-inflammatory granules and induce or exacerbate tissue injury.

Human neutrophils undergo spontaneous apoptosis *in vitro*. The mechanisms controlling constitutive neutrophil lifespan are not clear. Akgul *et al*, (2000) proposed that control of the transcription and translation of short-lived, anti-apoptotic proteins, A1 and Mcl -1 may be important in determining neutrophil lifespan. Neutrophils are destined to die as they constitutively express the pro-apoptotic proteins Bax, Bid, Bak and Bad which have a long half-life and therefore the relative levels of the opposing anti-apoptotic proteins, A1 and Mcl-1 may be the key in determining the neutrophils' fate. It has been suggested that the Fas/FasL pathway may be important in constitutive neutrophil apoptosis since the antagonistic anti-Fas monoclonal antibody, ZB4 delayed spontaneous neutrophil apoptosis *in vitro* (Liles *et al.*, 1996). However, we have shown that when neutrophils were cultured in phosphate buffered saline, ZB4 had no effect on constitutive neutrophil apoptosis. In addition, the neutralizing anti-FasL antibody (NOK-1) had no effect on constitutive neutrophil apoptosis. This confirmed that the Fas/FasL pathway is not important in spontaneous neutrophil apoptosis (Brown and Savill, 1999; Renshaw *et al.*, 2000). Furthermore, both fresh and inflammatory neutrophils from Fas/FasL-deficient mice undergo constitutive apoptosis at the same rates as neutrophils from wild-type mice (Fecho *et al.*, 1998; Villunger *et al.*, 2000). Although spontaneous neutrophil apoptosis was independent of the Fas death receptor, other groups have shown that caspase 8 is important (Khwaja and Tatton, 1999; Daigle *et al.*, 2002). Recently it was shown that as neutrophils age, reactive oxygen species accumulate and via acid sphingomyelinase trigger lipid raft clustering of the death-inducing signalling complex components, including caspase 8, thus initiating apoptosis (Scheel-Toellner *et al.*, 2004).

Death receptors belong to the tumour necrosis receptor family. Currently seven receptors, namely TNF-R1, Fas, TRAMP/DR3, TNF-related apoptosis-inducing

ligand- receptors 1,-2, -3 (TRAIL-R1/ TRAIL-R2/TRAIL-R3) and decoy receptor 6 (DR6) have been identified that signal through an intracellular "death domain". TNF-α, induces neutrophil apoptosis *in vitro* at early time points (up to 8 hours) and survival at later time points (Murray *et al.*, 1997). Neutrophils have also been shown to be susceptible to "anti-Fas - mediated cell death" (Iwai *et al.*, 1994). We have shown that the activating anti-Fas antibody (CH-11) optimally activated neutrophil apoptosis *in vitro* at 500ng/ml and had a greater effect at earlier time points. The anti-Fas antibody loses its potency over time hence the variability between experiments. Within experiments, variability in anti-Fas antibody potency was minimized by repeating individual experiments in as short a period as possible.

Autologous serum attenuated the effect of CH-11 at 3 hours while completely inhibiting its effect at 20 hours. When peripheral blood neutrophils from post-surgery patients were treated with CH-11 *in vitro*, autologous plasma significantly attenuated the effect of CH-11 (Iwase *et al.*, 2006). Some cytokines have been shown to delay neutrophil apoptosis (Ward *et al.*, 1999) and the attenuation of the effect of CH-11 by autologous serum may be due to the presence of cytokines in the serum. However, in the post-surgical patients, neutralization of either granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-6 (IL-6) or interleukin-8 (IL-8) had no effect on the attenuation of CH-11-induced apoptosis mediated by plasma (Iwase *et al.*, 2006). The mechanism by which serum attenuates Fas-mediated neutrophil apoptosis requires further elucidation. Interestingly, sera from patients with infection, has been shown to induce neutrophil apoptosis *in vitro*. This was partially attenuated by anti-FasL antibody. Infection was associated with increased monocyte expression of FasL and increased serum levels of FasL (Nwakoby *et al.*, 2001). The Fas-triggered neutrophil apoptosis was caspase-dependent unlike TNF-α - induced neutrophil apoptosis which also has a caspase-independent death pathway (Maianski *et al.*, 2003). Recently two more death receptors, TRAIL-R2 and TRAIL-R3 were found to be expressed on neutrophils and anti-TRAIL antibodies have been shown to induce neutrophil apoptosis (Renshaw *et al.*, 2003).

The Fas/FasL pathway is important in peripheral deletion of activated T cells. Fresh cytotoxic T cells do not express FasL but upon T receptor ligation, the Fas ligand gene on chromosome 1 is transcribed and translated (Suda *et al.*, 1995; Tanaka *et al.*, 1995; Vignaux *et al.*, 1995). Jurkat cells (human leukaemic T lymphocyte cell line) have been shown to constitutively express FasL using the non-specific polyclonal anti-FasL (N20) antibody (Santa Cruz) (Martinez-Lorenzo *et al.*, 1996) however using Alf1.2 and NOK-1 they do not express FasL (Smith *et al.*, 1998). We have also shown that Jurkat cells do not express mRNA for FasL. However upon activation, cytotoxic T cells, which initially store newly synthesized FasL in their lytic granules, direct their lytic granules to the area of T cell surface in tight contact with the target cell by a process of "polarized degranulation" (Bossi and Griffiths, 1999). Furthermore, microvesicles expressing bioactive FasL and APO2 ligand/TNF-related apoptosis-inducing ligand (TRAIL) are released from Jurkat cells upon mitogenic stimulation (Martinez-Lorenzo *et al.*, 1999). Macrophages, upon phagocytosis of opsonized zymosan, also release bioactive FasL in microvesicles (Brown and Savill, 1999).

Soluble FasL (sFasL) is released from the membrane and it can block the apoptosis induced by the intact membranous form (Kayagaki *et al.*, 1995; Suda *et al.*, 1995; Tanaka *et al.*, 1995). While multimerized FasL induced Jurkat cell apoptosis, sFasL had minimal effects even at very high concentrations (Schneider *et al.*, 1998). Furthermore, only multimerized sFasL has been shown to induce apoptosis of murine bone-marrow derived granulocytes (Villunger *et al.*, 2000). In these studies, SUPERFasLigand (rhFasL) (Alexis Biochemicals) was used and while it induced human neutrophil apoptosis, Jurkat cells were more sensitive. The neutrophil apoptosis induced by rhFasL could be neutralized by the anti-FasL antibody, NOK-1 or apoptosis inhibited by pre-incubation with the anti-Fas antibody ZB4. We also found that, in human neutrophils, the agonistic anti-Fas antibody (CH-11) was a more potent inducer of apoptosis than rhFasL.

We have shown that human neutrophils express Fas. This is widely accepted (Liles *et al.*, 1996; Renshaw *et al.*, 2000). However the expression of FasL by human neutrophils and its role in mediating autocrine and paracrine cell death is

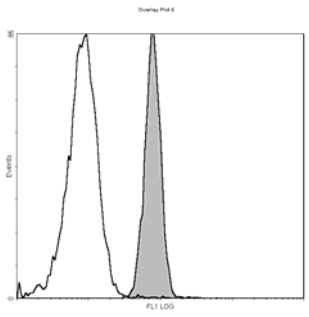
controversial. Liles *et al* (1996), in their seminal paper, showed that neutrophils express FasL on their surface using a Fas-immunoglobulin (Fas-Ig) fusion protein and indirect immunofluorescence. They also showed FasL protein expression by Western blotting using the anti-FasL monoclonal antibody, clone 33 (Transduction). Genetically-engineered antibodies are becoming more important as "tools" for scientists. However badly engineered tools can give misleading results; using a polyclonal rabbit immunoglobulin raised against a C-terminal peptide from FasL (Santa-Cruz), neutrophils express FasL (Mincheff *et al.*, 1998) however this antibody has been shown to bind non-specifically, when used in flow cytometry to detect surface expression of FasL (Smith *et al.*, 1998). Furthermore there have been conflicting results regarding FasL expression in thyrocytes; Stokes *et al* (1998) showed that when the anti-FasL monoclonal antibody, clone 33 (Transduction), is used in Western blotting, it binds non-specifically to FasL. We have also shown that this mAb, clone 33, bound to a 37kDa protein in Jurkat cells and A549 cells, both of which are known to lack FasL expression (Smith *et al.*, 1998). A second anti-FasL monoclonal antibody, G247-4 (Transduction) has been used in Western blotting to show the upregulation of FasL expression in activated cytotoxic T cells (Bossi and Griffiths, 1999) however when we used this antibody, we were unable to block non-specific protein binding. The anti-FasL monoclonal antibodies, NOK-1 and Alf-2.1a bind specifically to FasL and have been used in flow cytometry to detect surface expression of FasL (Smith *et al.*, 1998; Renshaw *et al.*, 2000). We have shown, using Alf-2.1a, that neutrophils do not express surface FasL.

In view of these conflicting results, we also looked at the mRNA expression of FasL using the reverse transcription polymerase chain reaction (RT-PCR). We showed that A549 cell lines, that were thought to be "stably-transfected" with FasL, did not in fact express mRNA for FasL. Furthermore, neutrophils did not express mRNA for FasL but KFL9 cells and mononuclear cells did. Previously, Brown & Savill (1999) have suggested that neutrophils do not express FasL, by Western blotting and RT-PCR. However they used the monoclonal antibody (clone 33) whose specificity for FasL has been challenged (Stokes *et al*, 1998) but they showed that clone 33 cross-reacts with β-actin (Brown and Savill, 1999). Subsequently Renshaw *et al,* (2000),

found that both fresh and ageing neutrophils, as well as activated neutrophils, do not express FasL using RT-PCR. They also failed to show surface FasL expression on neutrophils using the monoclonal antibody, Alf-2.1. Subsequently three studies, to date, have been published showing FasL expression on neutrophils (Jaber *et al.*, 2001; Kim *et al.*, 2001; Hu *et al.*, 2005). Contaminating mononuclear cells which have mRNA for FasL may account for the neutrophil expression of FasL seen by Jaber *et al* (2001) and Hu *et al* (2005). Although, Kim *et al*, (2001) used the non-specific monoclonal antibody (clone 33), they also showed mRNA expression of FasL in highly purified neutrophil preparations. The reason for this disparity is not clear.

We have confirmed earlier findings that neutrophils do not have mRNA for FasL and do not express FasL on their surface. Monoclonal antibodies with a high specificity for FasL were used in these experiments. As a consequence, the Fas/FasL pathway does not have a role in mediating constitutive neutrophil apoptosis. However triggering of the Fas death receptor pathway by anti-Fas monoclonal antibodies or multimerized recombinant FasL does accelerate neutrophil apoptosis but their effect is attenuated by factors in serum. Furthermore, Fas-mediated induction of neutrophil apoptosis is caspase-dependent.

a)



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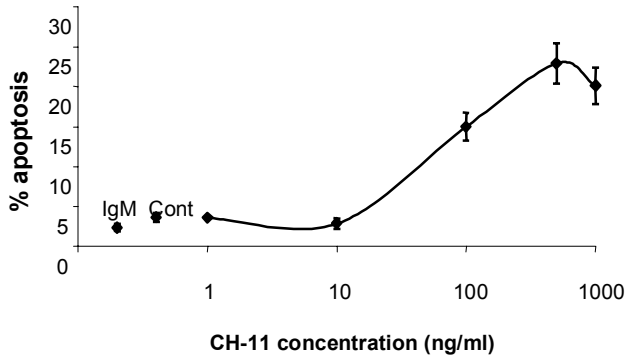

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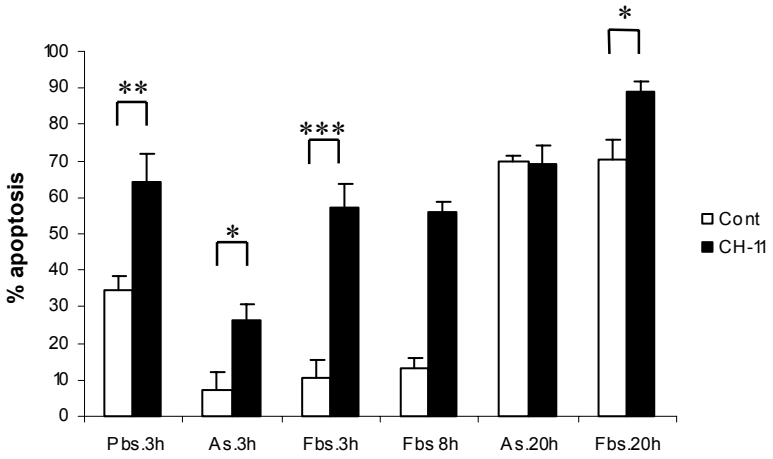
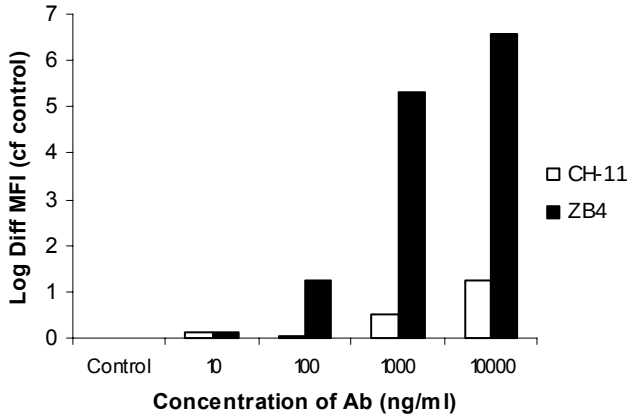


Figure 3.1. Fas and the neutrophil

(a) Expression of Fas on neutrophil surface assessed by flow cytometry. Isotype control antibody is shown as an outline curve. Specific binding of Fas is shown as a shaded curve. Freshly isolated human neutrophils were labelled with anti-Fas antibody ZB4 (1:40) and detected with FITC-conjugated goat anti-mouse immunoglobulin (1:40). (b) PMN (5 x 10^6/ml) were cultured in phosphate buffered saline (Pbs) with 10% foetal bovine serum (Fbs) with a control IgM antibody and varying concentrations of the agonistic anti-Fas antibody, CH-11. Apoptosis was assesses at 3 hours by Annexin V binding. (c) PMNs (5 x 10^6/ml) were cultured in Pbs with 10% autologous serum (As), 10% Fbs and CH-11 (500ng/ml). Apoptosis was assessed at 3, 8 and 20 hours by Annexin V. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

a)



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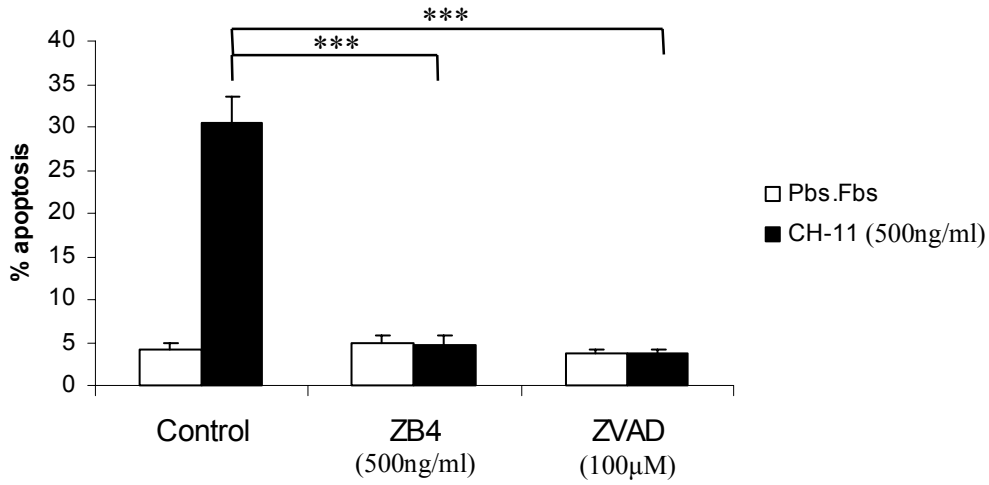
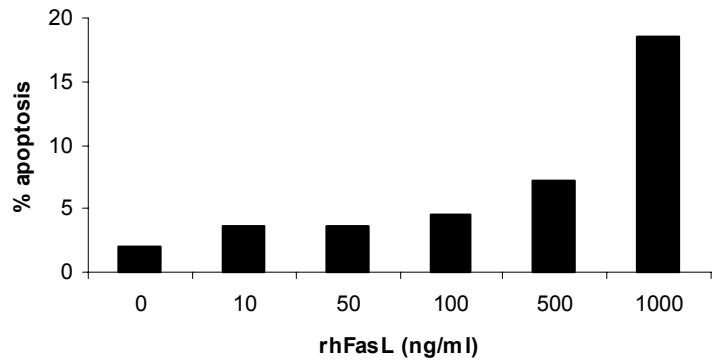


Figure 3.2. Agonistic and antagonistic anti-Fas antibodies

(a) Binding of CH-11 and ZB4 to Fas on neutrophils as assessed by flow cytometry. Freshly isolated human neutrophils were labelled with anti-Fas antibody (agonistic IgM antibody, CH-11 and antagonistic IgG antibody, ZB4) and detected with FITC-conjugated goat anti-mouse immunoglobulin (1:40) (n=1). (b) Neutrophils (5 x 10^6/ml) in Pbs, 10% Fbs were pre-incubated for 1 hour in a shaking water bath with either Pbs and 10% Fbs, ZB4 (500ng/ml) or zVAD (100μM). They were then incubated for a further 3 hours in flexi-well plates with or without CH-11 (500ng/ml) and apoptosis was assessed by Annexin V (n=3). ***$p<0.001$.

ai)



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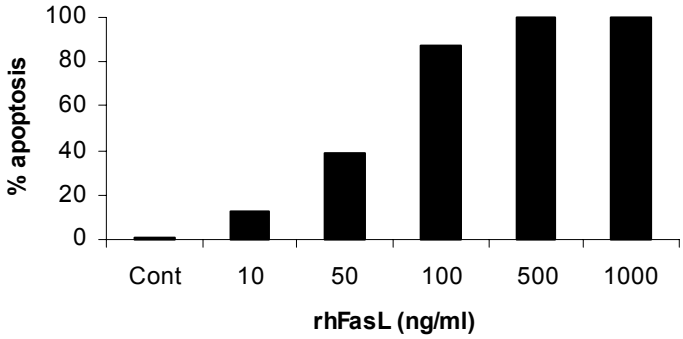

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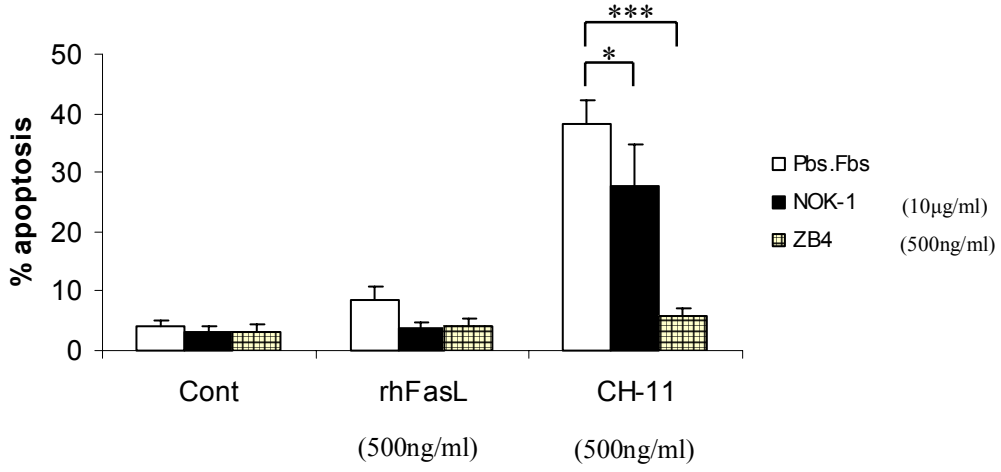
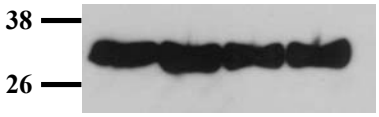


Figure 3.3. FasL and the neutrophil

(ai) Neutrophils (5 x 10^6/ml) in Pbs and 10% Fbs were incubated with varying concentrations of rhFasL for 3h and apoptosis assessed by Annexin V (n=1). (aii) Jurkat cells (5 x 10^6/ml) in RPMI and 10% Fbs were incubated with varying concentrations of rhFasL and apoptosis assessed at 3h by morphology (n=1). (b) Neutrophils (5 x 10^6/ml) in Pbs and 10% Fbs were pre-incubated for 1 hour in a shaking water bath with either Pbs and 10% Fbs, NOK-1(10µg/ml) or ZB4 (500ng/ml). They were then incubated for a further 3 hours in flexi-well plates with or without rhFasL (500ng/ml) or CH-11 (500ng/ml). Apoptosis was assessed by Annexin V (n=3). *$p<0.05$, ***$p<0.001$.

a)



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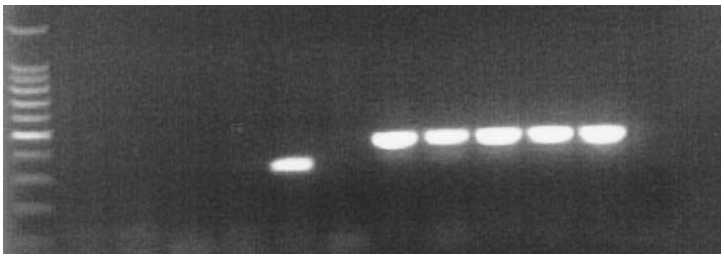
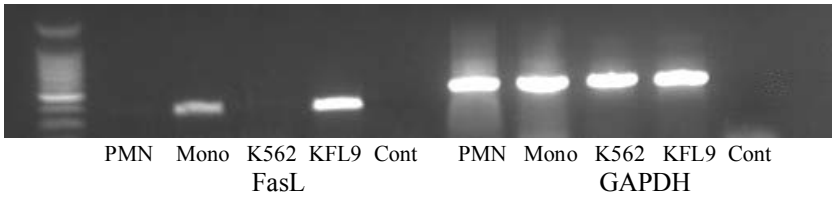


Figure 3.4 FasL protein and mRNA expression.

(a) The Jurkat cell line was grown to 1 x 10^6/ml and the cells harvested. The A549 cell line and FasL transfected A549 cell lines (PC3 and PC4) were grown to 90% confluency and harvested from plates using trypsin/EDTA. The Jurkat, A549, PC3 and PC4 cells were lysed, quantity of protein estimated and Western blotting carried out using 5µg/ml Clone 33 (Transduction) as described in the methods. (b) Total cellular RNA was extracted using the Trizol method from 5 x 10^6 cells: A549 cell lines stably transfected with FasL (PC1, PC3 and PC4), the parent cell line K562 and the K562 cell line stably transfected with FasL (KFL9). FasL and GAPDH mRNA was demonstrated by RT-PCR using specific primers.

a) mRNA levels by PCR



b) FasL surface expression

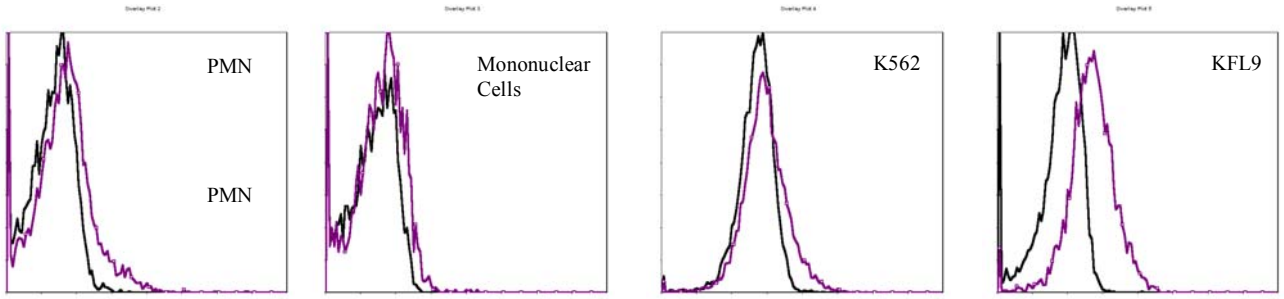


Figure 3.5. FasL expression by the neutrophil.

(a) Total cellular RNA was extracted, using the Trizol method, from 10 x 10^6 cells (fresh neutrophils (PMN), mononuclear cells (mono), the parent cell line K562 and the K562 cell line stably transfected with FasL, (KFL9). FasL and GAPDH mRNA was demonstrated by RT-PCR using specific primers. (b) Surface expression of FasL on neutrophils (PMN), mononuclear cells, K562 and KFL9s by flow cytometry. Isotype control antibody is shown as a blue curve. Specific binding of FasL (Alf-2.1a) is shown as a purple curve. Freshly isolated human cells were labelled with FITC-conjugated anti-human FasL antibody (Alf-2.1a) (1 in 50) or FITC-conjugated isotype control (1 in 50).

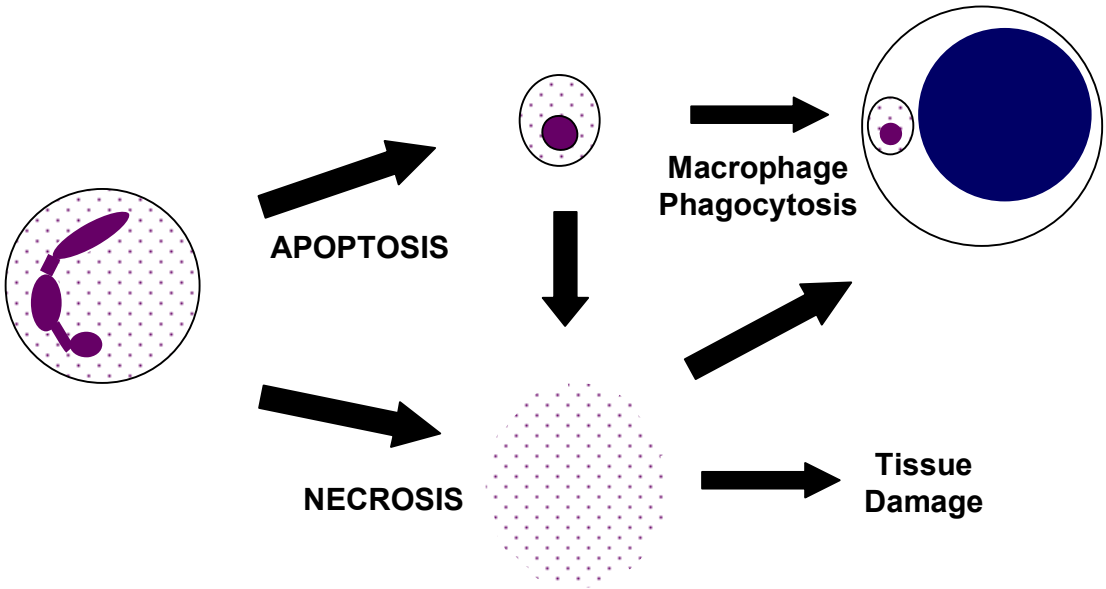


Diagram 3.1 The fate of an inflammatory neutrophi

The multilobed neutrophil accumulates at sites of inflammation. It can either "die" by apoptosis or necrosis. The apoptotic form of cell death results in cell shrinkage and DNA condensation but the plasma membrane remains intact. The apoptotic neutrophil is usually phagocytosed by a tissue macrophage but if this process is delayed or perverted it will undergo secondary necrosis. Necrosis is deleterious as it results in cell membrane damage and release of neutrophil granules which leads to tissue damage.

Chapter 4

Signalling in the neutrophil: to live or die

4.1 Introduction

In acute respiratory distress syndrome (ARDS), the balance of pro-inflammatory and anti-inflammatory mediators will help determine the fate of the neutrophil. In the inflammatory milieu, inflammatory mediators and cytokines are present which are produced locally by inflammatory cells, lung epithelial cells and fibroblasts (Ware and Matthay, 2000; Park *et al.*, 2001). In the bronchoalveolar lavage fluid (BALF) of patients with ARDS both pro- and anti-inflammatory mediators have been detected. Pro-inflammatory cytokines include tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 (Siler *et al.*, 1989; Suter *et al.*, 1992; Schutte *et al.*, 1996; Park *et al.*, 2001) while anti-inflammatory mediators include interleukin-10 (IL-10), soluble TNF receptor I (sTNF-RI), soluble TNF receptor II (sTNF-RII), interleukin-1 receptor antagonist (IL-1ra) and soluble interleukin-1 receptor II (sIL-1RII) (Suter *et al.*, 1992; Donnelly *et al.*, 1996; Park *et al.*, 2001).

The balance appears to favour anti-inflammatory cytokines during the early acute phase of ARDS which may be important in dampening the initial intense inflammation (Park *et al.*, 2001). During this early phase, BALF from ARDS patients has also been shown to delay neutrophil apoptosis *in vitro*; this inhibitory effect was largely attenuated by neutralizing granulocyte - colony stimulating factor (G-CSF) and granulocyte-macrophage - colony stimulating factor (GM-CSF) (Matute-Bello *et al.*, 1997). However our group found that BALF from patients with established ARDS was markedly pro-apoptotic for neutrophils (Donnelly & Haslett, unpublished observation).

Inflammatory agents, including the bacterial endotoxin, lipopolysaccharide (LPS) have also been found in BALF (Martin *et al.*, 1997). Endotoxin plays a major role in ARDS secondary to sepsis (Ware and Matthay, 2000). In experimental models,

intratracheal instillation of LPS produces a well-characterized response resembling acute lung injury in humans (Brigham and Meyrick, 1986; Xing *et al.*, 1994).

The fate of the neutrophil at an inflamed site will thus depend on the balance of pro- and anti-inflammatory mediators. Studies have investigated the effect of cytokines and bacterial products on neutrophil lifespan *in vitro*; most cytokines and bacterial products appear to prolong neutrophil survival but the studies sometimes differ in their results with respect to individual inflammatory mediators (Ward *et al.*, 1999).

In 1892, the bacterial product endotoxin was first discovered by Richard Pfeiffer, a co-worker of Robert Koch. Lipopolysaccharide (LPS) is released from Gram-negative bacteria and in large quantities, it can trigger a detrimental immune response leading to septic shock (Nogare and Yarbrough, 1990). However humans are continuously exposed to low levels of LPS which as an immunostimulatory molecule may be beneficial.

LPS binds to a TLR-4/MD-2 complex which transduces the signal intracellularly however this signal is amplified by the cellular receptor, CD14 (Miyake, 2004). Furthermore a serum component, LPS-binding protein, is an opsonin, enhancing the binding of LPS to CD14 (Wright *et al.*, 1990; Schumann, 1992). Although LPS has been shown to delay neutrophil apoptosis (Lee *et al.*, 1993), this effect has not always been seen (Dibbert *et al.*, 1999). It has been suggested that contaminating monocytes, due to differences in neutrophil preparation from human blood, may mediate the anti-apoptotic effect of LPS (Sabroe *et al.*, 2002).

Bacteria also produce proteins with an amino-terminal N-formylated methionine; the f-Met-Leu-Phe (fMLP) peptide is an important chemotactic factor for neutrophils and its receptor is a member of the serpentine receptor family. These are seven trans-membrane spanning, G-protein coupled receptors (Boulay *et al.*, 1990). *In vitro*, fMLP has variably been reported to have no effect or to prolong the neutrophil lifespan (Colotta *et al.*, 1992; Kettritz *et al.*, 1997; Ottonello *et al.*, 2002).

However, it is widely accepted that GM-CSF delays neutrophil apoptosis (Brach *et al.*, 1992; Colotta *et al.*, 1992; Lee *et al.*, 1993). At sites of inflammation, $CD4^+$

lymphocytes are usually the major source of GM-CSF which as well as activating macrophages locally, also has a distant action on bone marrow where it promotes the proliferation and differentiation of myeloid precursors (DeLamarter, 1988).

4.1.1 Signal Transduction Pathways

In the past 50 years, the field of signal transduction has been born and our knowledge has exploded. In the 1950s, Earl Sutherland and his colleagues discovered that the pathway downstream of adrenalin and glucagon receptors (both "serpentine" receptors) involved activation of the enzyme adenylate cyclase to generate adenosine cyclic 3´5´ monophosphate (cAMP), the "second messenger". This in turn activated protein kinase A. However, central to cell signalling was the discovery by Edwin Krebs and Ed Fischer, again in the 1950s, that the process of phosphorylation by kinases and dephosphorylation by phosphatases was pivotal to the control of cell behaviour (Hunter, 2000).

Another "second messenger" pathway downstream of serpentine receptors involves hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5 biphosphate by phospholipase C (until the 1970s, lipids were considered inert, structural components of cell membranes). This generates water soluble inositol 1,4,5-triphosphate which triggers a rise in intracellular Ca^{2+} which in turn activates many intracellular pathways. Phospholipase C also produces a second lipid soluble product, 1,2-diacylglycerol which activates one or more of the protein kinases C.

4.1.2 The protein kinase C pathway

The protein kinase C pathway appears to be involved in regulating spontaneous neutrophil apoptosis (Pongracz *et al.*, 1999). There are at least 11 isoenzymes which are subclassified into three groups, classical, novel and atypical depending on their mode of activation; the classical isoenzymes are -α, -β and -γ and their activation is dependent on diacylglycerol (DAG) and calcium, the novel isoenzymes are -δ, -ε, -η and -θ and they are activated by DAG alone while the activation of the atypical isoenzymes -ζ and -ι / λ are independent of DAG (Kent *et al.*, 1996).

4.1.3 The mitogen-activated kinase pathway

The mitogen-activated protein (MAP) kinase (MAPK) pathway is known to be an important mechanism by which cells control transcription (Seger and Krebs, 1995). It was originally discovered as an insulin-activated protein-serine kinase. Each pathway consists of a cascade of three protein kinases; a MAP kinase kinase kinase (MAP3K), which phosphorylates serine residues in the MAP kinase kinase (MAP2K) thereby activating it, to in turn phosphorylate both a serine and a tyrosine residue in the MAP kinase (Waskiewicz and Cooper, 1995). Once the terminal MAP kinase is activated, it can migrate into the nucleus where it phosphorylates and activates transcription factors. There are three known mammalian MAP kinase cascades: the stress activated protein kinase 1 (SAPK1)/C-Jun N-terminal kinase (JNK) cascade, the SAPK2/p38 cascade and the p42/p44 ERK cascade (Wallach *et al.*, 1999). The three MAP kinase cascades have been shown to be expressed in human neutrophils (Nick *et al.*, 1996). There are 4 isoforms of p38 MAPK (-α, -β, -δ and -γ) however human neutrophils, only express p38 MAPKα as the dominant isoform with lower levels of p38 MAPKδ (Nick *et al.*, 1999). LPS has been shown to stimulate the α isoform of p38 MAPK resulting in NF-κB activation which in turn stimulates adhesion and TNF-α synthesis (Nick *et al.*, 1999).

4.1.4 The phosphatidylinositol 3-kinase pathway

In neutrophils, the phosphatidylinositol 3 (PI 3)-kinase pathway is important in the GM-CSF-mediated prolongation of their lifespan (Vlahos *et al.*, 1995; Klein *et al.*, 2000; Cowburn *et al.*, 2002). PI-3 kinase activates protein kinase B/Akt which is an anti-apoptotic signalling pathway (Zundel and Giaccia, 1998). Phosphorylated Akt functions as a serine-threonine kinase and can phosphorylate Bad (Bcl-2/Bcl-X_L-antagonist causing cell death), a member of the Bcl-2 (B-cell leukaemia/lymphoma 2) family. Normally Bad promotes apoptosis by forming an inhibiting heterodimer with anti-apoptotic members of the Bcl-2 family. When Bad is phosphorylated, its inhibitory effect is lost as it dissociates from the heterodimer to leave the active anti-apoptotic Bcl-2 family proteins (Yang *et al.*, 1995). Prolongation of neutrophil apoptosis by GM-CSF was associated with increased Bad phosphorylation and decreased expression of Bad mRNA and this was inhibited by LY294002, a PI 3-

kinase inhibitor (Cowburn *et al.*, 2002). Furthermore, activation of the PI 3-kinase pathway, in a cell-dependent manner can also lead to p42/p44 ERK activation (Hawes *et al.*, 1996; Kilgour *et al.*, 1996).

4.1.5 The NF-κB pathway

The NF-κB pathway is evolutionarily conserved and is believed to be the central signalling pathway of activation in the innate immune system. The transcription factor, NF-κB, consists of homo- or heterodimers of the Rel family proteins (p50/NF-κB1, p52/ NF-κB2, p65/Rel A and c-Rel) which are sequestered in the cytoplasm due to their physical association with an inhibitory protein subunit termed (IκB) (Henkel *et al.*, 1993). Phosphorylation of IκB leads to its proteolytic breakdown in the proteasome and allows NF-κB to translocate to the nucleus (Finco *et al.*, 1994; Traenckner *et al.*, 1994) where it regulates transcription of many genes including those for pro-inflammatory cytokines. In the neutrophil, IκB-α has been found in the cytoplasm associated with p50/c-Rel, p50/Rel A and p65/c-Rel heterodimers however in the nucleus the complexes contained p65/Rel A and p50 (McDonald *et al.*, 1997).

The inflammatory mediators, LPS, fMLP and TNF-α have been shown to activate NF-κB (McDonald *et al.*, 1997). Furthermore we have previously shown that by blocking the translocation of an inducible form of NF-κB, neutrophil apoptosis induced by TNF-α, is accelerated (Ward *et al.*, 1999). However it is not known if Fas ligation results in NF-κB activation in human neutrophils.

Prostaglandins are traditionally thought to be pro-inflammatory mediators however cyclopentenone prostaglandins (Δ^{12} PGJ_2 and 15-deoxy-Δ^{12}, Δ^{14}- PGJ_2), which are natural metabolites of PGD_2 (diagram 4.1), have anti-inflammatory activity including inhibition of TNF-α induced IκBα degradation (Rossi *et al.*, 2000). Furthermore the resolution phase of pleurisy induced by carageenin in rats is associated with the production of prostaglandin D_2 (PGD_2) and $15dPGJ_2$ (Gilroy *et al.*, 2003).

4.1.6 The Fas/FasL Receptor pathway

The Fas receptor (CD95, APO-1) is a member of the TNF receptor family. These receptors are type I receptors and their cytoplasmic tails have a sequence motif termed the "death domain" (DD) (Feinstein *et al.*, 1995; Hofmann and Tschopp, 1995). This death domain interacts with adapter proteins which also bear a "death domain". The adapter protein "Fas-associated protein with death domain" (FADD) (Chinnaiyan *et al.*, 1995) has both a C-terminal DD and a N-terminal death-effector domain (DED) which allows it to interact with the DED of caspase 8 (MACH/FLICE/Mch5) (Boldin *et al.*, 1996; Fernandes-Alnemri *et al.*, 1996; Muzio *et al.*, 1996). Fas receptor activation thus induces the formation of a complex of proteins which was termed the death-inducing signalling complex (DISC) (Kischkel *et al.*, 1995). The formation of the DISC itself leads to activation of caspase-8 (Medema *et al.*, 1997; Ashkenazi and Dixit, 1999). Interestingly caspase-10 is also recruited to the DISC although its role in induction of apoptosis is less clear (Wang *et al.*, 1999; Kischkel *et al.*, 2001; Sprick *et al.*, 2002). Several other proteins have been shown to be recruited to the DISC including Daxx, FAP-1, FLASH, RIP, FAF1 and Dap3 but their role at present is unclear (Peter and Krammer, 2003).

Another protein with a DED, which has several names including FLIP, Casper and CASH, has been discovered (Bertin *et al.*, 1997; Goltsev *et al.*, 1997; Hu *et al.*, 1997; Inohara *et al.*, 1997; Irmler *et al.*, 1997; Shu *et al.*, 1997; Srinivasula *et al.*, 1997; Thome *et al.*, 1997; Rasper *et al.*, 1998). The γ-herpesviruses express a viral inhibitor, viral-FLICE - like inhibitory protein (v-FLIP) which was found to associate with Fas in the DISC but it inhibited apoptosis (Bertin *et al.*, 1997; Hu *et al.*, 1997; Thome *et al.*, 1997). Subsequently its cellular homologue termed c-FLIP was identified (Goltsev *et al.*, 1997; Inohara *et al.*, 1997; Irmler *et al.*, 1997; Shu *et al.*, 1997; Srinivasula *et al.*, 1997; Rasper *et al.*, 1998); it has both a short and a long form (c-$FLIP_S$ and c-$FLIP_L$). Both forms have tandem DED but c-$FLIP_L$ also has a domain which is homologous to the caspase-8 protease death domain however in c-$FLIP_L$ this domain has mutations which render it inactive. It would appear that c-$FLIP_L$ is pro-apoptotic at low concentrations but inhibitory at high levels of expression (Peter and Krammer, 2003). However c-$FLIP_S$ appeared to be only

inhibitory; its upregulation is associated with the development of resistance to Fas-mediated apoptosis in T-cells (Kirchhoff *et al.*, 2000). It has also been suggested that c-FLIP may activate the NF-κB and extracellular signal related kinase (ERK) pathway (Kataoka *et al.*, 2000).

We have used pharmacological inhibitors of these signal transduction pathways to examine the mechanisms by which the Fas/FasL pathway induces apoptosis in neutrophils and the mechanisms by which the inflammatory mediators, especially LPS, mediate their anti-apoptotic effects.

4.2 Results

4.2.1 Inflammatory mediators and signalling pathways

The inflammatory mediators, LPS, fMLP and GM-CSF, can prime or activate neutrophils (Gougerot-Podicalo *et al.*, 1996). LPS (0.1μg/ml), fMLP (10ng/ml) or GM-CSF (50U/ml) had no effect on spontaneous neutrophil apoptosis *in vitro* at 3 hours (figure 4.1a). However when neutrophils were cultured *in vitro* for 20 hours, LPS (0.1μg/ml) (36.9 ± 2.7%) and GM-CSF (50U/ml) (45.7 ± 3.6%) delayed neutrophil apoptosis (control, 71.6 ± 4.3%, $p<0.01$), while fMLP (10ng/ml) (79.1 ± 5.5%) had no significant effect on neutrophil apoptosis (control, 68.9 ± 5.3%). At 3 hours, all three inflammatory mediators, LPS (0.1μg/ml) (5.8 ± 1.4), fMLP (10ng/ml) (4.2 ± 0.9) and GM-CSF (50U/ml) (4.3 ± 0.8) attenuated CH-11- induced apoptosis (20.3 ± 3.2, n=3, $p<0.001$)(figure 4.1a).

4.2.2 The mitogen-activated protein kinase pathway

The p38 MAPK and p42/p44 ERK pathways are involved in intracellular signal transduction in neutrophils in response to LPS, fMLP and GM-CSF (Nick *et al.*, 1996; Nick *et al.*, 1997; Nolan *et al.*, 1999; Villunger *et al.*, 2000). PD98059 at a concentration of 50μM is a selective p42/p44 ERK inhibitor (Alessi *et al.*, 1995) while SB203580 at 10μM is a selective competitive inhibitor of the ATP-binding site on p38 MAPK (Cuenda *et al.*, 1995; Young *et al.*, 1997). PD98059 (50μM) had no effect on CH-11 induced apoptosis or on the LPS, fMLP or GM-CSF- mediated attenuation of CH-11- induced apoptosis (figure 4.1b). Initial results suggested that SB203580 (20μM) had no effect on the LPS, fMLP or GM-CSF- mediated attenuation of CH-11- induced apoptosis but SB203580 (11.2 ± 1.5%) attenuated CH-11- induced apoptosis (20.3 ± 3.2%, n=3, $p<0.001$) (fig 2a) suggesting that CH-11 may signal through the p38 MAPK pathway. However this was not reproducible; a fresh batch of SB203580 (54 ± 2.0%) augmented CH-11 – induced apoptosis (50.6 ± 1.6%, n=3, $p<0.05$) and SB203580 also partially reversed the LPS-mediated attenuation of CH-11 – induced apoptosis (control, 7.1 ± 0.7%; SB203580, 13.5 ± 0.7%, n=3, $p<0.001$) (figure 4.2b). The initial inhibition by SB203580 may have been due to contamination with endotoxin as it was an older batch. It has been

suggested that 20μM SB203580 also inhibits the JNK pathway whereas 5μM is specific for p38 MAPK (Cuenda *et al.*, 1995). We therefore repeated the experiment and found that 5μM SB203580 had no significant effect on CH-11 – induced apoptosis (control, 39.2 ± 7.7%; SB203580, 44.9 ± 5.5%) but partially inhibited the LPS-mediated attenuation of CH-11 – induced apoptosis (control 5.4 ± 1.1%, SB203580 9.1 ± 0.7%, n=3, $p<0.05$). In the neutrophil, the p38 MAPK may therefore be involved in LPS signalling while the JNK pathway may be an anti-apoptotic pathway; further investigation is required.

4.2.3 The phosphatidylinositol 3-kinase pathway

The role of PI 3-kinase in CH-11 – mediated neutrophil apoptosis is not clear. LY294002 is a reversible, ATP-competitive inhibitor of recombinant PI 3-kinase (Vlahos *et al.*, 1994). The IC_{50} for inhibition of PI 3-kinase by LY294002 is 10μM therefore this concentration was used (Davies *et al.*, 2000). We have shown that when neutrophils were cultured for 3 hours, LY294002 had no significant effect on spontaneous, CH-11-induced apoptosis or on the LPS-mediated attenuation of CH-11 – induced apoptosis (figure 4.3a). In neutrophils, the PI 3-kinase pathway is involved in GM-CSF signalling (Vlahos *et al.*, 1995; Klein *et al.*, 2000; Cowburn *et al.*, 2002) therefore we confirmed that LY294002 (10μM) did inhibit the GM-CSF mediated neutrophil survival at 20 hours (data not shown).

4.2.4 The protein kinase C pathway

Neutrophils have been shown to express the protein kinase C (PKC) isoenzymes –α, -β, -δ ((Majumdar *et al.*, 1991; Smallwood and Malawista, 1992) and –ζ (Dang *et al.*, 1995). However PKC-δ alone appears to be involved in the induction of spontaneous neutrophil apoptosis (Pongracz *et al.*, 1999). Ro318220 at 1μM is a broad-spectrum PKC inhibitor (Davis *et al.*, 1992; Davies *et al.*, 2000). Ro318220 (1μM) accelerated CH-11- induced neutrophil apoptosis (CH-11, 44.6 ± 8.9%; Ro318220 and CH-11, 64.3 ± 4.9%; n=3, $p<0.05$) and partially inhibited the LPS-mediated attenuation of CH-11- induced apoptosis (LPS.CH-11, 7.1 ± 1.1%; Ro318220 and LPS.CH-11, 44.2 ± 6.7%; n=3, $p<0.001$) (figure 4.3bi). This suggested that the PKC pathway was anti-apoptotic and that the inhibitory effect of LPS on neutrophil apoptosis was partially mediated by the PKC pathway. We therefore investigated the effects of the

specific isoenzyme inhibitors; Gö6976 (10nM) inhibits the classical PKC isoenzymes -α, -β and -γ (Thorp *et al.*, 1996) and Rottlerin (5μM) inhibits PKCδ (Gschwendt *et al.*, 1994). Rottlerin attenuated CH-11- induced apoptosis (control, 46 ± 9.8%; Rottlerin, 27.1 ± 11.7%; n=3, $p<0.05$) while Gö6976 had no effect (figure 4.2bii). However, neither Rottlerin nor Gö6976 had an effect on the LPS-mediated attenuation of CH-11- induced apoptosis (figure 4.2bii).

4.2.5 The NF-κB pathway

The inflammatory mediators, LPS, fMLP and TNF-α activate NF-κB in the neutrophil (McDonald *et al.*, 1997). Since IκB-α degradation is required for NF-κB translocation to the nucleus, we investigated the cytoplasmic protein levels of IκBα, in neutrophils, in response to inflammatory mediators and anti-Fas antibody, CH-11. At 30 minutes, LPS induced IκBα degradation whilst CH-11, fMLP and GM-CSF had no effect (figure 4.3a). It has been shown that GM-CSF stimulation of neutrophils does not induce IκBα degradation at any time point up to 2 hours and fMLP only induces IκBα degradation after 60 minutes (McDonald *et al.*, 1997). CH-11 had no effect on IκBα degradation when neutrophils were cultured for up to three hours (data not shown).

Our group has shown that the prostaglandin D_2 (PGD_2) metabolite 15-deoxy-Δ^{12}, Δ^{14}-PGJ_2 ($15dPGJ_2$) at a concentration of 10μM inhibits both LPS-mediated IκBα degradation in the neutrophil and the LPS-mediated survival effect on neutrophil apoptosis (Ward *et al.*, 2002). We thus proposed that $15dPGJ_2$ would also inhibit the LPS-mediated attenuation of CH-11 - induced apoptosis. However as $15dPGJ_2$ also activates perioxisome proliferating activating receptor-γ (PPAR-γ) (Jiang *et al.*, 1998; Ricote *et al.*, 1998), the specific PPAR-γ agonist ciglitazone (10μM) was used to determine if the LPS-mediated attenuation of CH-11- induced apoptosis involved the PPAR-γ pathway. Gliotoxin is also a potent and specific inhibitor of NF-κB (Pahl et al, 1996). We have shown that $15dPGJ_2$ (29.7 ± 8.8%) partially inhibited the LPS-mediated attenuation of CH-11- induced apoptosis (6.3 ± 2.5%, $p>0.05$) while ciglitazone had no effect (figure 4.4b). Furthermore, neither $15dPGJ_2$ nor ciglitazone affected CH-11- induced apoptosis (figure 4.4b). Due to the formation of the DISC

in the death receptor pathways, caspase-8 which is produced as a proenzyme (55/50 kDa doublet) is cleaved into smaller subunits of 40/36 kDa (doublet) and 23 kDa (Medema *et al.*, 1997). We showed that CH-11 and TNF-α/Gliotoxin induced caspase-8 degradation in neutrophils (figure 4.4c). The induction of caspase-8 degradation by CH-11 was inhibited by LPS and this LPS-mediated inhibition was reversed by $15dPGJ_2$ (figure 4.4c).

4.2.6 Expression of c-FLIP in the neutrophil

Our data suggest that the inhibitory effect of LPS on CH-11- induced apoptosis is associated with inhibition of caspase 8 degradation. Furthermore, pre-incubation with CH-11 reversed the anti-apoptotic effect of LPS (figure 4.5a). This raised the possibility that the inhibitory effect of LPS on CH-11- induced apoptosis may be mediated by the protein c-FLIP at the level of the DISC (diagram 4.2). Neutrophils constitutively expressed the 22kDa protein $cFLIP_s$ but did not express the 55kDa protein $cFLIP_L$ (figure 4.5b). Furthermore, the anti-FLIP antibody appeared to bind to a second protein which is approximately 24kDa and this protein may represent $cFLIP_s$ which has been post-translationally modified. After 15 minutes incubation, the 24kDa $cFLIP_s$ appeared to be degraded however autologous serum appeared to inhibit this degradation. LPS, at the concentration used in these experiments (0.1μg/ml), requires the lipopolysaccharide binding protein (LBP) found in serum, for its activity. Thus active LPS (LPS and Fbs) and fMLP appear to augment the levels of both the 22 and 24kda forms of $cFLIP_s$ at this time point (figure 4.5b). The quantity of protein in each sample is demonstrated by reprobing the blot for β-actin protein levels (figure 4.5c). The function of $cFLIP_s$ in the neutrophil is not known.

4.2.7 A strategy to immunoprecipitate the DISC and any other bound proteins

In order to investigate regulation of neutrophil apoptosis at the level of the DISC, we devised a strategy to use the cytoplasmic tail of the Fas receptor tagged to biotin to go "fishing" for proteins which bind to the Fas receptor in lysates of control neutrophils and LPS-stimulated neutrophils. The biotin tagged proteins could then be immunoprecipitated using streptavidin beads (diagram 4.3).

The first part of this approach involved making the "fishing rod". The cloned Fas receptor was obtained in the pCMV.Sport6 plasmid from LGC Promochem, UK (diagram 4.4a). Firstly we checked that the plasmid contained the Fas Receptor; using hind III restriction enzymes (diagram 4.4), we showed that the product obtained was the expected size 1342 base pairs (figure 4.6a). Using PCR (with the primers shown in diagram 4.5b), DNA encoding the cytoplasmic tail of the Fas receptor was produced. The DNA was then ligated into the Pinpoint Xa vector using the Pinpoint^{TM} Xa-1 T-Vector system (Promega, Madison, WI, USA). The Pinpoint Xa vector contains DNA encoding the Biotin purification tag (diagram 4.6). Competent *E. coli*, JM109, were then transformed as described previously. Initially 16 colonies were sampled and using *Hind III* restriction enzyme digest, "clone 4" was found to contain the FasR cDNA in the correct orientation (figure 4.5b). The cDNA was purified and 2µg sent to MWGBiotech for sequencing. The cloned sequence was verified as described in the methods. Protein production, by the transformed *E. coli* JM109 colonies grown up in Luria-Bertani broth, was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG). The *E.coli* containing no insert expressed a protein of 13kDa, those containing control cDNA expressed a protein of 27kDa and "clone 4" *E.coli* containing the FasR insert expressed a protein of 32kDa (figure 4.6a). The transformed *E.coli* were then cultured on a larger scale and induced to produce the biotinylated protein. The bacteria were then lysed using the lysozyme and detergent method although some biotinylated protein was lost and detected in the lysed bacterial cell pellet (figure 4.7bi). The bacterial cell lysate was applied to the prepared SoftLink™ monomeric avidin column and a sample of the flow-through showed that there was good binding of the biotinylated protein to the column (figure 4.7bii). Although the 1ml eluate contained 110µg protein there was significant degradation of the protein in the purification process and a large amount of the protein remained bound to the column (figure 4.7bii). Future work would involve transformation of a different *E.coli* strain (BL21) which is deficient in proteases. This may overcome the degradation problem and batch purification may overcome the problem with residual protein binding to the resin.

4.3 Discussion

The neutrophil is a professional phagocyte however in the absence of bacterial infection and at sites of inflammation, its lifespan is regulated by factors within the inflammatory milieu. If the neutrophils' lifespan is prolonged such that it undergoes secondary necrosis, this will exacerbate tissue injury. A strategy, used therapeutically in drug development, targets the signalling pathways used by these inflammatory mediators. We have explored these signalling pathways in human neutrophils particularly investigating those utilised by lipopolysaccharide (LPS) and the Fas/FasL pathway.

We, and others, have shown that inflammatory mediators including LPS and GM-CSF, not only prolong neutrophil survival *in vitro*, they also attenuate Fas-mediated neutrophil apoptosis (Colotta *et al.*, 1992; Lee *et al.*, 1993; Liles *et al.*, 1996). The LPS used in these studies was not purified therefore will contain a significant amount of bacterial lipopeptide (Hirschfeld *et al.*, 2000). The LPS will therefore signal through both TLR2 and TLR4 receptors on the neutrophil. Furthermore during the process of neutrophil extraction from whole blood, contaminating monocytes were not removed by negative selection. It has now been shown that purified LPS (a TLR4 agonist) also delays early constitutive apoptosis of purified neutrophils (Sabroe *et al.*, 2003). The mediator, fMLP had no effect on early (4h) or late (20h) spontaneous neutrophil apoptosis but attenuated Fas-induced neutrophil apoptosis. Protein kinases are differentially activated by inflammatory mediators. While fMLP has been shown to activate p42/44 ERK and p38 MAPK (Nick *et al.*, 1997), GM-CSF strongly activated the ERK cascade and PI 3-kinase pathway (Klein *et al.*, 2000; Cowburn *et al.*, 2002). However, we have shown that the p42/44 ERK pathway was not involved in the fMLP and GM-CSF-mediated attenuation of CH-11- induced apoptosis.

Early spontaneous and Fas-mediated neutrophil apoptosis appears to be independent of p38 MAPK. At a later time point (12 hours), other investigators have shown that the p38 MAPK inhibitor, SB203580, augments spontaneous and Fas-induced apoptosis; phosphorylated p38 MAPK was present constitutively in human

neutrophils and the activity of p38 MAPK was inhibited during spontaneous and CH-11- induced apoptosis (Alvarado-Kristensson *et al.*, 2002). Subsequently they have shown that Fas receptor stimulation results in protein phosphatase 2A (PP2A) activation which dephosphorylates p38MAPK and caspase 3 resulting in decreased p38 MAPK activity but increased caspase 3 activity and thus neutrophil apoptosis (Alvarado-Kristensson and Andersson, 2005). In murine neutrophils, SB203580 also accelerated spontaneous neutrophil apoptosis (Villunger *et al.*, 2000). Other groups have found differing results; Aoshiba *et al* (1999) found that SB203580 delayed spontaneous neutrophil apoptosis but had no effect on Fas-induced apoptosis. Furthermore, the p38 MAPK inhibitor SKF-86002, had no effect on spontaneous or Fas-induced apoptosis (Frasch *et al.*, 1998) however this inhibitor appears be less specific for p38 MAPK, also activating cyclooxygenase and 5-lipoxygenase (Griswold *et al.*, 1987).

LPS has been shown to prolong neutrophil survival however this effect was not apparent at early time points (4 hours). Our group and others have shown that this survival effect is partially mediated by p42/44 ERK (Nolan *et al.*, 1999; Klein *et al.*, 2001; Ward *et al.*, 2005). At early time points, the p42/44 ERK inhibitor, PD98059, had no effect on spontaneous or LPS-mediated neutrophil apoptosis.

The role of p38 MAPK in LPS-mediated neutrophil survival is less clear. LPS has been shown to activate the α-isoform of p38 MAPK resulting in neutrophil adhesion, NF-κB stimulation and TNF-α synthesis (Nick *et al.*, 1999). However two studies have shown LPS-mediated survival to be independent of p38 MAPK (Klein *et al.*, 2001; Ward *et al.*, 2005) and a third study confirmed that LPS activated p38 MAPK but showed that it was pro-apoptotic (Nolan *et al.*, 1999). Furthermore, we have shown, at early time points, that 5μM and 20μM SB203580 partially reversed the LPS mediated attenuation of CH-11 - induced apoptosis suggesting that p38 MAPK mediates an anti-apoptotic pathway. The inhibitor SB203580 at higher concentrations (20μM) also inhibits the JNK pathway (Cuenda *et al.*, 1995). It has been proposed, in human neutrophils, that JNK pathway activation, upon LPS stimulation, depends upon the physiological state of the neutrophil; in suspended

neutrophils LPS does not activate JNK (Nick *et al.*, 1999) however in adherent neutrophils JNK is activated (Arndt *et al.*, 2004).

The PI 3-kinase pathway is important in mediating the survival effect of GM-CSF in neutrophils. However at early time points it was not involved in LPS or Fas signalling (figure 4.4a). At later time points, LY294002 (10μM) reversed the survival effect of LPS (Ward *et al.*, 2005) and in murine PI 3 kinase$^{-/-}$ neutrophils both basal and LPS-stimulated cells showed increased rates of apoptosis compared to the wild type (Yang *et al.*, 2003). Although PI 3-kinase is usually considered an anti-apoptotic pathway in neutrophil biology, in one study LY294002 had no effect on LPS-mediated survival (Klein *et al.*, 2001) and another group have shown that LY294002 inhibited CH-11-induced apoptosis at later time points (Alvarado-Kristensson *et al.*, 2002).

The protein kinase C (PKC) pathway, in particular the PKCδ isoenzyme has been implicated in both spontaneous and Fas-induced neutrophil apoptosis (Khwaja and Tatton, 1999; Pongracz *et al.*, 1999). The PKC inhibitor, Ro318220(1μM), not only augmented spontaneous and CH-11- induced apoptosis, it also partially reversed the LPS-mediated attenuation of CH-11- induced apoptosis. However, the specific PKCδ inhibitor, Rottlerin significantly attenuated CH-11 induced apoptosis but had no effect on the LPS-mediated attenuation. These inhibitors have subsequently been shown to inhibit many protein kinases and therefore valid conclusions cannot be drawn from these data alone (Davies *et al.*, 2000). Other groups have shown that the protein kinase C pathway is important in spontaneous and Fas-induced neutrophil apoptosis by demonstrating that in the former, caspase 3 and in the latter, caspase 8 mediate PKCδ cleavage and activation (Khwaja and Tatton, 1999; Pongracz *et al.*, 1999).

The NF-κB family of transcription factors mediate many of the cellular responses to inflammatory mediators. In the neutrophil, the prototypic TNF-α death receptor pathway induces both death in the neutrophil via the caspases and life via NF-κB activation (Ward *et al.*, 1999). In the neutrophil, LPS also activate NF-κB through p38 MAPKα (Nick *et al.*, 1999) (diagram 4.2). Furthermore in cell lines, both

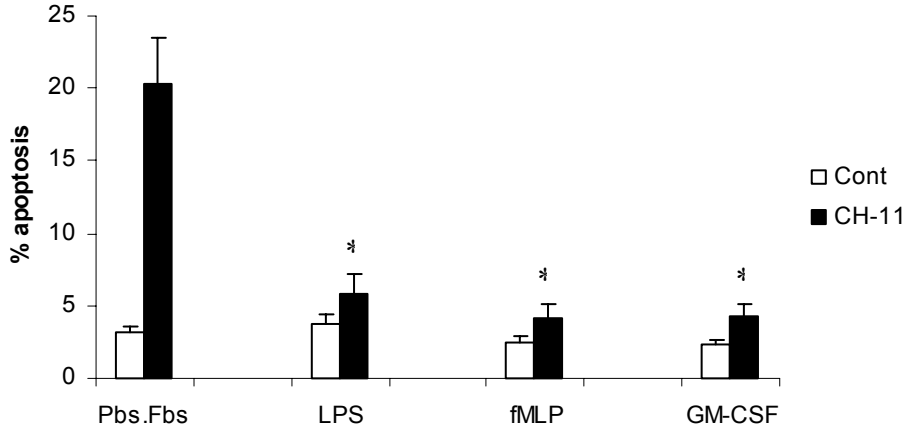
sensitive and resistant to apoptosis induced by Fas ligation, anti-CD95 antibody activated NF-κB (Ponton *et al.*, 1996). In human bronchiolar epithelial cells and monocytes, Fas ligation has also been associated with NF-κB activation (Hagimoto *et al.*, 1999; Park *et al.*, 2003) However in human neutrophils we have shown that while LPS induced IκBα degradation, CH-11 had no effect. Thus Fas ligation in the neutrophil does not activate NF-κB. The cytoplasmic adapter protein FLIP is thought to be important in regulating the apoptotic/non-apoptotic switch at the level of the DISC (Wajant *et al.*, 2003). In endothelial cells, cellular FLIP appears to protect against LPS-induced apoptosis and suppresses NF-κB activation (Bannerman D *et al*, 2004). We have shown that neutrophils only express the short form of cellular FLIP ($cFLIP_S$) and its role at present is not clear.

An alternative strategy looked at inhibiting the NF-κB pathway. There are several natural and synthetic NF-κB inhibitors. The PGD_2 metabolite $15dPGJ_2$ is known to inhibit LPS-mediated IκBα degradation (Ward *et al.*, 2002). We found that $15dPGJ_2$ partially inhibited the LPS-mediated attenuation of CH-11 – induced apoptosis and this was associated with $15dPGJ_2$ partially inhibiting the LPS-mediated attenuation of caspase-8 degradation. Furthermore at early time points, the cellular concentration of $cFLIP_S$ was differentially regulated by LPS and CH11. Our data suggested that LPS inhibited Fas signalling in the human neutrophil at the level of the DISC. We therefore devised a strategy to determine the proteins which bind to the cytoplasmic tail of the Fas receptor upon Fas ligation and to determine if this protein binding is altered by LPS. Initially DNA encoding the cytoplasmic tail of the Fas receptor was cloned into the Pinpoint Xa vector in order to produce a biotin-tagged protein. The 32kDa biotin-tagged Fas receptor cytoplasmic tail was successfully produced however we were subsequently unable to batch purify the protein without significant proteolytic breakdown. Other investigators have subsequently managed to immunoprecipitate the neutrophil DISC using anti-Fas receptor monoclonal antibodies (Apo-1 and Fas B-10) and protein A/G (Daigle *et al.*, 2002; Scheel-Toellner *et al.*, 2004). This alternative strategy could therefore be utilised to examine the effects of LPS on the Fas signalling pathway.

We have shown that pre-incubation with GM-CSF inhibits Fas-mediated apoptosis: conversely GM-CSF-mediated survival is inhibited by simultaneous activation of the Fas receptor (Daigle *et al.*, 2002). Elegant studies have shown that the Fas receptor death domain contains a tyrosine residue which upon phosphorylation binds the inhibitory phosphatase Src homology domain 2 (SH-2)-containing tyrosine phosphatase-1 (SHP-1). As previously described GM-CSF stimulates anti-apoptotic pathways which include tyrosine phosphorylation of Lyn; this is prevented by concurrent Fas receptor ligation and it is proposed that SHP-1 mediates this inactivation (Daigle *et al.*, 2002). On the other hand, pre-incubation with GM-CSF has been shown to interfere with the recruitment of FADD to the DISC upon Fas receptor ligation (Kotone-Miyahara *et al.*, 2004).

Thus the regulation of neutrophil lifespan is complex depending not only on factors within the inflammatory milieu but within the cell itself, there is cross-talk between survival and death pathways.

a)



b)

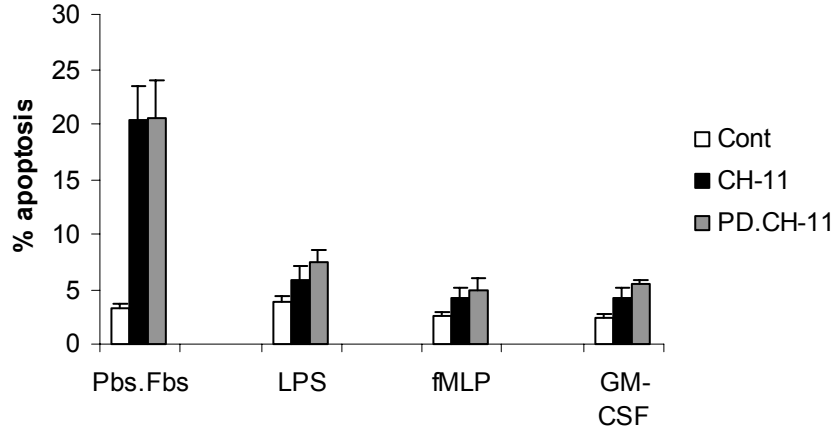
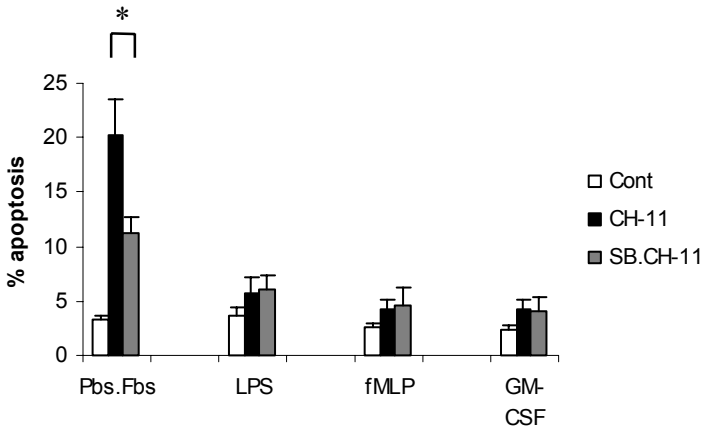


Figure 4.1. The p42/44 ERK pathway, CH-11, inflammatory mediators and neutrophil apoptosis

(a) Effect of LPS, fMLP and GM-CSF on spontaneous and CH-11 induced neutrophil apoptosis. Neutrophils (5 x 10^6/ml) in Pbs and 10% Fbs were pre-incubated in a shaking water bath for 1 hour with LPS (0.1μg/ml), fMLP (10ng/ml) or GM-CSF (50U/ml). They were then incubated for a further 3 hours in flexible well plates with CH-11 (500ng/ml) and apoptosis was assessed by Annexin V binding. * $p<0.01$ compared with CH-11 control values. (b) The effect of pre-incubation with PD98059 (50μM) on the LPS, fMLP and GM-CSF attenuation of CH-11 induced apoptosis. Neutrophils (5 x 10^6/ml) in Pbs were pre-incubated for 1 hour in a shaking water bath with Pbs or PD98059 (50μM). They were then incubated for a further 3 hours in flexible well plates with 10% Fbs and either Pbs $\pm$ CH-11 (500ng/ml), LPS (0.1μg/ml) $\pm$ CH-11 (500ng/ml), fMLP (10ng/ml) $\pm$ CH-11 (500ng/ml) or GM-CSF (50U/ml) $\pm$ CH-11 (500ng/ml). Apoptosis was assessed by Annexin V binding. All values represent mean $\pm$ SEM of n = 3 experiments; each performed in duplicate.

a)



b)

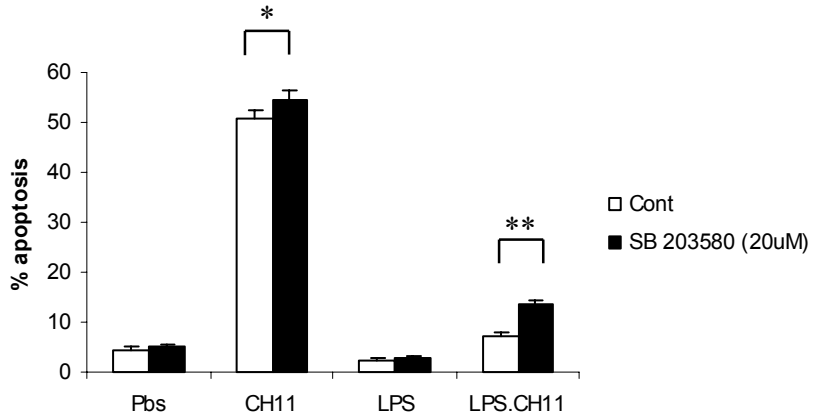


Figure 4.2. The p38 MAPK pathway, CH-11, inflammatory mediators and neutrophil apoptosis

(a) The effect of pre-incubation with SB203580 (20μM) on the LPS, fMLP and GM-CSF-mediated attenuation of CH-11 induced apoptosis. Neutrophils (5 x 10^6/ml) in Pbs were pre-incubated for 1 hour in a shaking water bath with Pbs or SB203580 (20μM). They were then incubated for a further 3 hours in flexible well plates with 10% Fbs and either Pbs ± CH-11 (500ng/ml), LPS (0.1μg/ml) ± CH-11 (500ng/ml), fMLP (10ng/ml) ± CH-11 (500ng/ml) or GM-CSF (50U/ml) ± CH-11 (500ng/ml). Apoptosis was assessed by Annexin V binding. (b) The effect of pre-incubation with a new batch of SB203580 (20μM) on the LPS – mediated attenuation of CH-11 induced apoptosis. Neutrophils (5 x 10^6/ml) in Pbs were pre-incubated for 1 hour in a shaking water bath with Pbs or SB203580 (20μM). They were then incubated for a further 3 hours in flexible well plates with 10% Fbs and either Pbs ± CH-11 (500ng/ml) or LPS (0.1μg/ml) ± CH-11 (500ng/ml). Apoptosis was assessed by Annexin V binding. All values represent mean ± SEM of n = 3 experiments; each performed in duplicate. *$p<0.05$, **$p<0.001$.

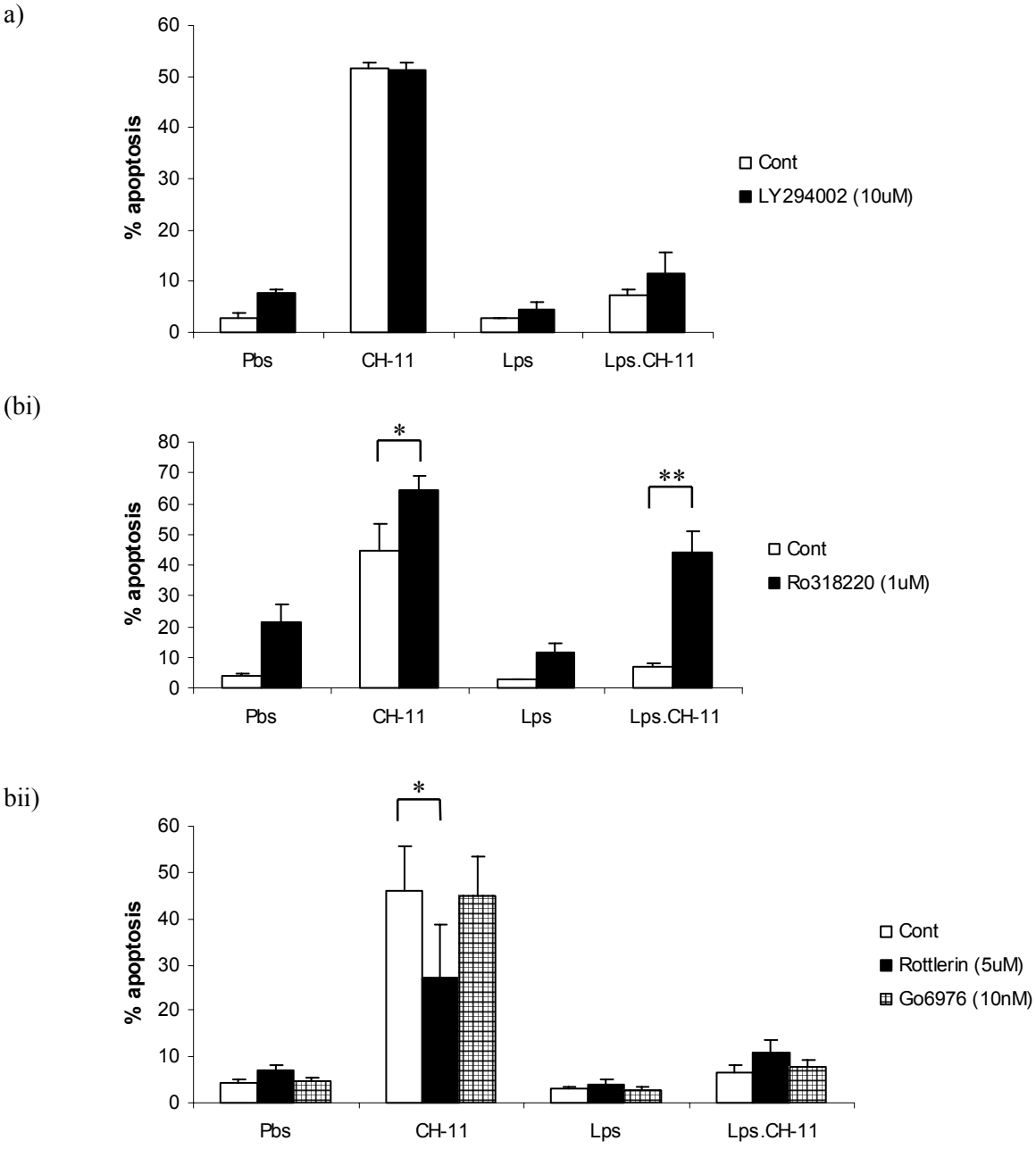


Figure 4.3. The PI3-K/PKC pathway, CH-11, LPS and neutrophil apoptosis

The effect of pre-incubation with (a) the PI3-kinase inhibitor, LY294002, (bi) the PKC inhibitor, Ro318220, (bii) the PKCδ inhibitor, Rottlerin and PKC (classical isoenzyme) inhibitor, Go6976 on the LPS-mediated attenuation of CH-11 - induced apoptosis. Neutrophils (5 x 10^6/ml) in PBS were pre-incubated for 1 hour in a shaking water bath with PBS or (a) LY294002 (10μM) or (bi) Ro318220 (1μM) or (bii) Rottlerin (5μM), Go6976 (10nM). They were then incubated for a further 3 hours in flexible well plates with 10% FBS and either PBS, CH-11 (500ng/ml), LPS (0.1μg/ml) or LPS (0.1μg/ml) and CH-11 (500ng/ml). Apoptosis was assessed by Annexin V binding. All values represent mean $\pm$ SEM of n = 3 experiments; each performed in duplicate. *$p<0.05$, **$p<0.001$.

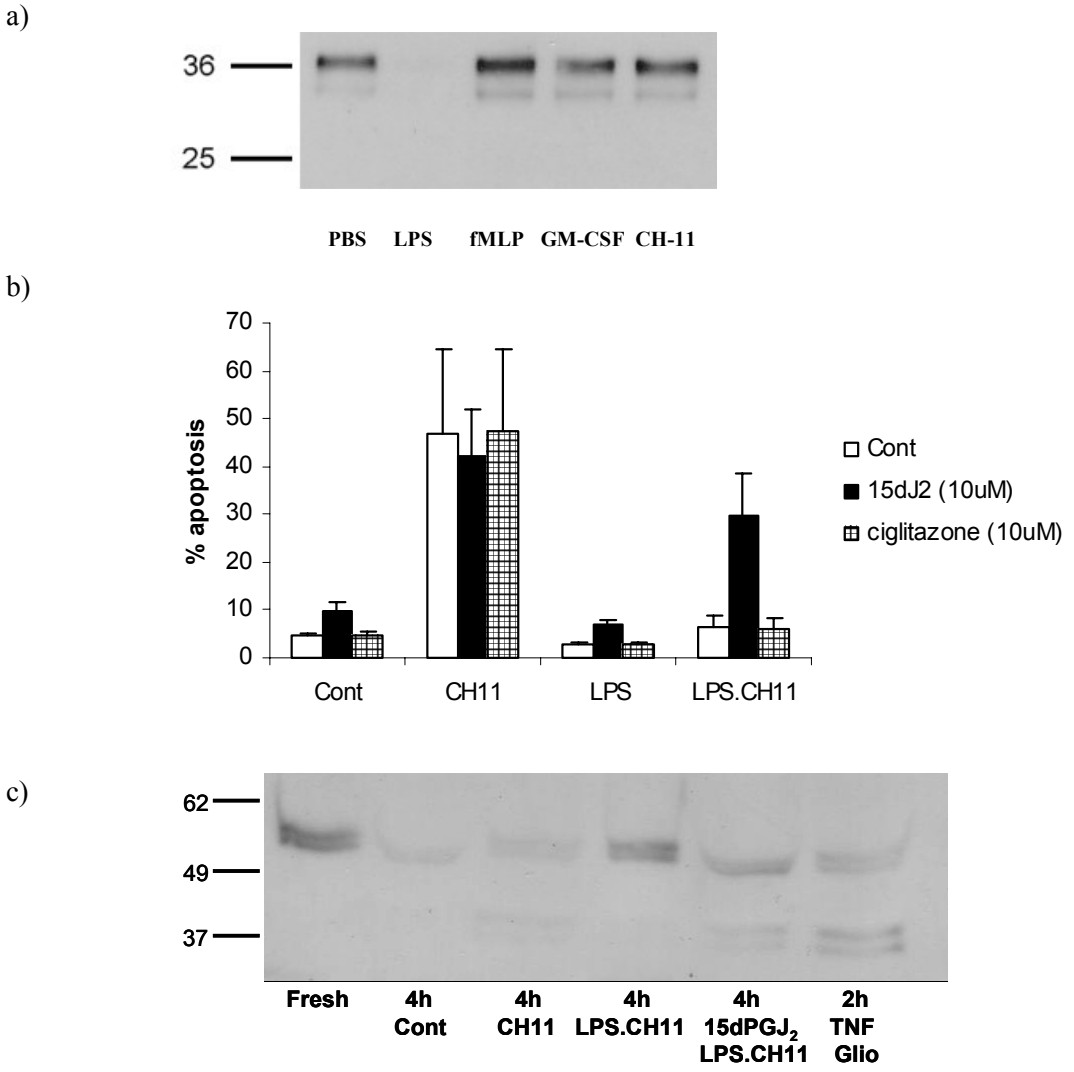
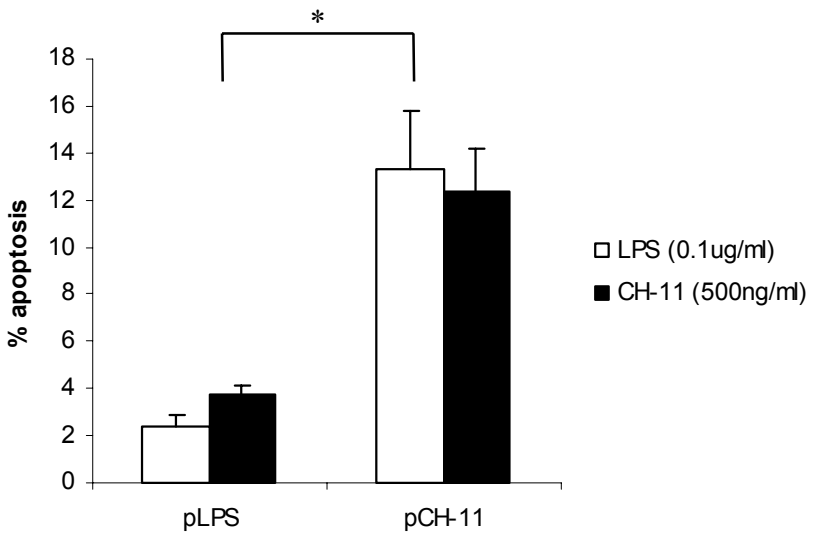


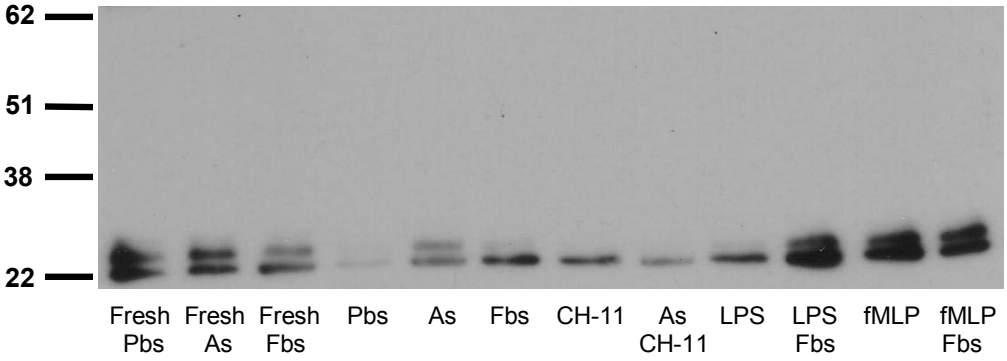
Figure 4.4. **The NF-κB pathway and neutrophil apoptosis**

(a) IκBα protein in neutrophils. Neutrophils (5 x 10^6/ml) in Pbs and 10% Fbs were incubated in a shaking water bath with PBS, LPS (0.1μg/ml), fMLP (10ng/ml), GM-CSF (50U/ml) or CH-11 (500ng/ml) for 30 minutes. Cell lysates were prepared and Western blotting carried out using IκBα antibody (1:500). (b) Effect of the prostaglandin D_2 metabolite, 15-deoxy-Δ^{12}, Δ^{14}- PGJ_2 ($15dPGJ_2$) on LPS-mediated attenuation of CH-11- induced neutrophil apoptosis. Neutrophils (5 x 10^6/ml) in Pbs were pre-incubated in a shaking water bath with $15dPGJ_2$ (10μM) and ciglitazone (10μM) for 1 hour. They were then incubated for a further 3 hours in flexible well plates with 10% FBS and either PBS, CH-11 (500ng/ml), LPS (0.1μg/ml) or LPS (0.1μg/ml) and CH-11 (500ng/ml). Apoptosis was assessed by Annexin V binding. (c) Caspase 8 protein in neutrophils. Neutrophils (5 x 10^6/ml) in Pbs were pre-incubated in a shaking water bath for 30 min with Pbs or $15dPGJ_2$ (10μM). They were then incubated for a further 4 hours with 10% Fbs and either Pbs, CH-11 (500ng/ml) or LPS (0.1μg/ml) and CH-11 (500ng/ml). Neutrophils (5 x 10^6/ml) in Pbs and 10% Fbs were incubated in a shaking water bath for 2 hours with TNF-α (10ng/ml) and gliotoxin (Glio) (2μg/ml). Cell lysates were prepared and Western blotting carried out using caspase-8 antibody (4μg/ml).

a)



bi)



bii)

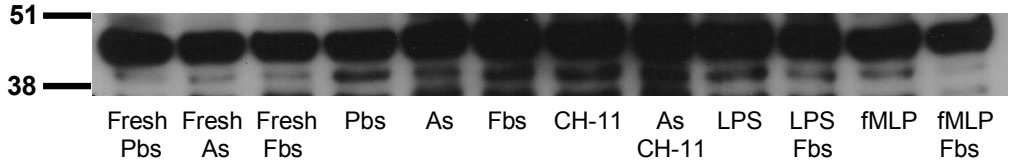
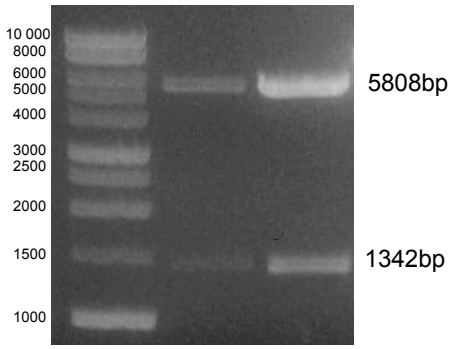


Figure 4.5. **cFLIP, CH-11, inflammatory mediators and neutrophil apoptosis**

(a) The effect of pre-incubation with CH-11. Neutrophils (5 x 10^6/ml) in Pbs and 10% Fbs were incubated in a shaking water bath with LPS (0.1μg/ml) or CH-11 (500ng/ml) for 60 minutes. They were then incubated for a further 3 hours in flexible well plates with either CH-11 (500ng/ml) or LPS (0.1μg/ml). Apoptosis was assessed by Annexin V binding. All values represent mean ± SEM of n = 3 experiments; each performed in duplicate. *$p<0.01$. (b) The effect of serum, anti-Fas antibody (CH-11) and inflammatory mediators on cFLIP protein levels in human neutrophils. Neutrophils (5 x 10^6/ml) in Pbs ± 10% Fbs or 10% autologous serum (As) were incubated in a shaking water bath for 15 min with CH-11 (500ng/ml) or LPS (0.1μg/ml) or fMLP (10ng/ml). Cell lysates were prepared and Western blotting carried out using (bi) anti-human FLIP (1:1000). (bii) The blot was stripped and reprobed with anti-human β-actin (1:100).

a)



b)

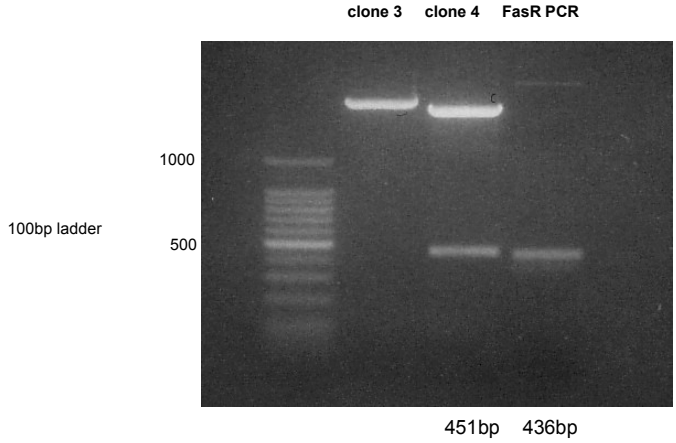
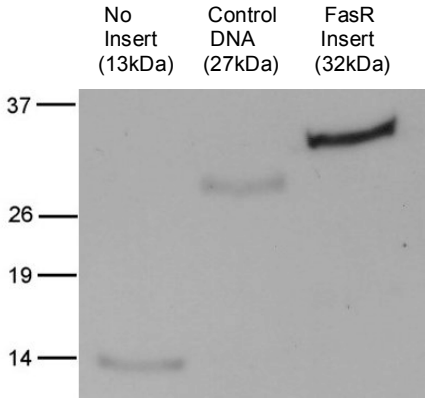


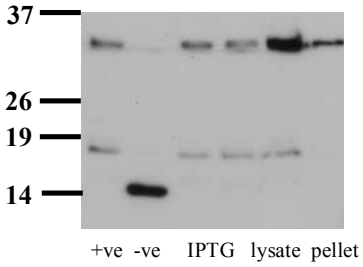
Figure 4.6. Hind III restriction digest of cDNA

(a) Hind III restriction digest of purified pCMV.Sport6 vector containing Fas receptor cDNA. The predicted size of the linearised plasmid is 5808bp and the Fas receptor insert is 1342bp (diagram 4.4). (b) Hind III restriction digest of cDNA purified from transformed JM109 cells. The predicted size of the product is 451bp (diagram 4.6). In lane 3, using PCR and the primer set shown in diagram 4.5, is the Fas receptor cytoplasmic tail DNA (436bp).

a)



bi)



bii)

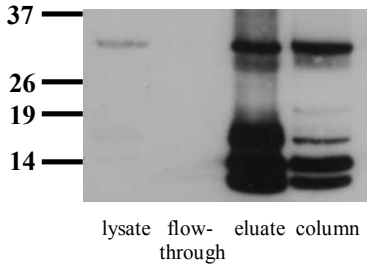


Figure 4.7. Biotinylated Fas protein expression

(a) Expression of biotinylated proteins by transformed *E.Coli* JM109. *E.Coli* JM109 were transformed with Pinpoint^{TM} Xa plasmid containing no insert, control DNA supplied by Promega and the FasR cytoplasmic tail DNA (clone 4, figure 4.6) and grown up in Luria-Bertani broth. Cell lysates were prepared and Western blotting carried out using HRP-Streptavidin (1:2500). (bi) *E.Coli* JM109 transformed with Pinpoint^{TM} Xa plasmid containing FasR cytoplasmic tail DNA (+ve, lane 1) and no insert (-ve, lane 2) were cultured on a large scale having been induced with IPTG to produce protein (lanes 3 and 4). The bacteria were lysed using lysozyme and detergent (lane 5) and cell debris pelleted (lane 6). Western blotting of the samples was carried out using HRP-Streptavidin (1:2500). (bii) Protein purification using the SoftLink™ monomeric avidin resin column. The lysate (lane 1) was applied to the column and a sample of the flow-through collected (lane 2). The protein was then eluted (lane 3) from the column (lane 4). Western blotting of the samples was carried out using HRP-Streptavidin (1:2500).

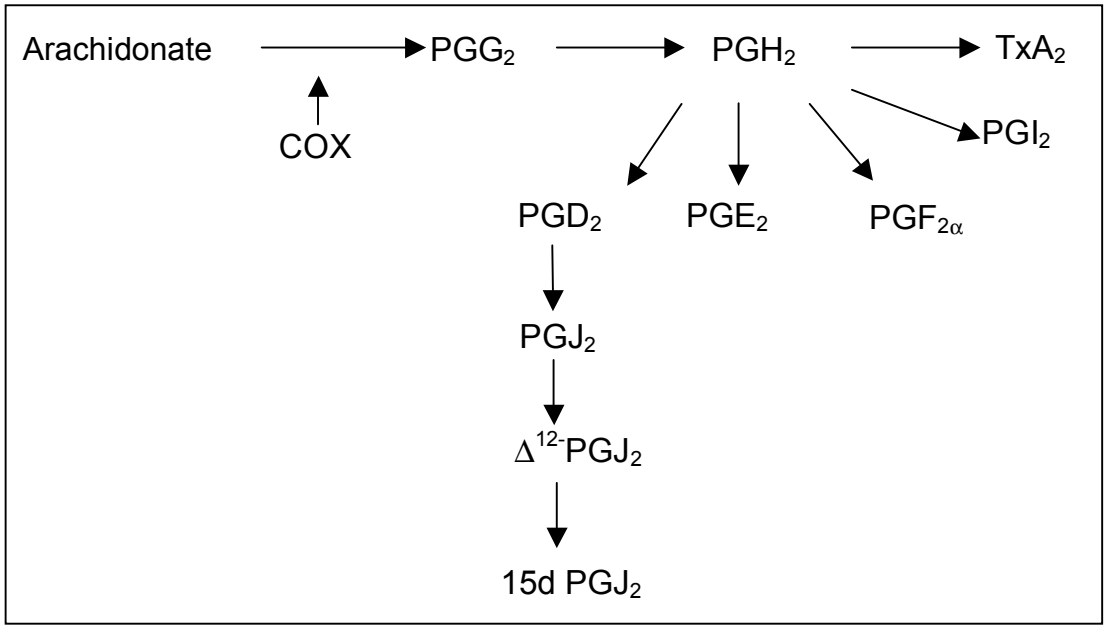


Diagram 4.1. The prostaglandin (PG) pathway

Prostaglandins are primarily derived from arachidonate which phospholipases release from membrane phospholipids. Arachidonate is converted to an unstable intermediate PGG_2 by cyclooxygenase (COX). This is subsequently converted by specific prostaglandin synthetases to PGD_2, PGE_2, $PGF_{2\alpha}$, prostacylin (PGI_2) and thromboxane A_2 (TxA_2). *In vitro*, PGD_2 spontaneously converts into the cyclopentone prostaglandins of the J series, PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-Δ^{12}, Δ^{14}- PGJ_2 (15d PGJ_2). (Schibata et al, 2002).

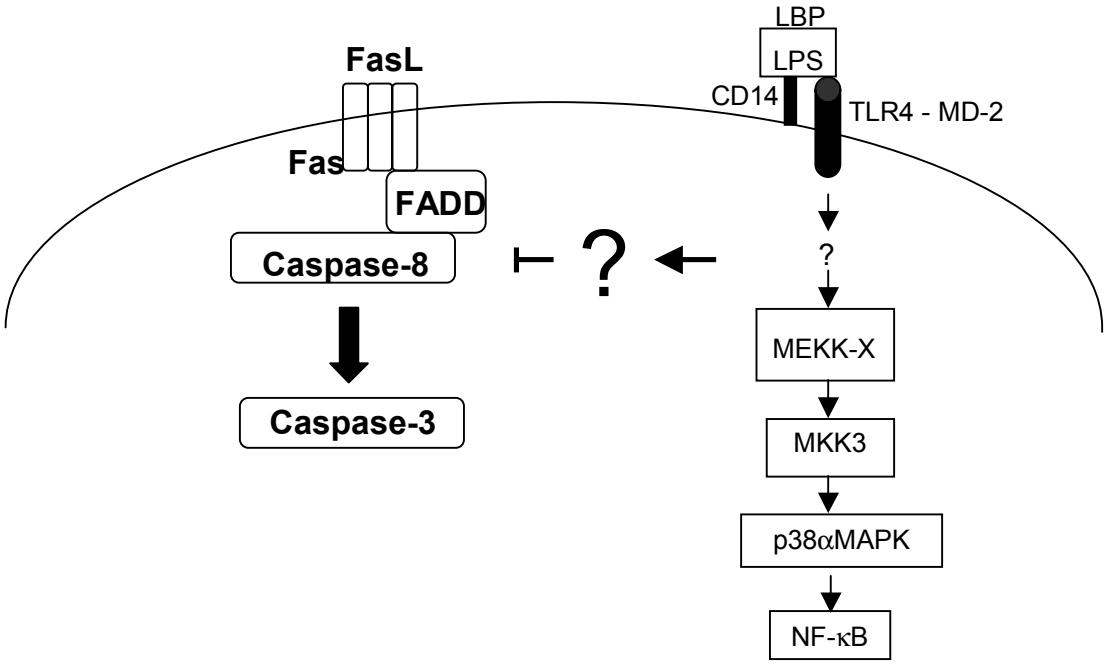


Diagram 4.2. Schematic diagram depicting proposed signalling pathway by which LPS inhibits the Fas receptor pathway in human neutrophils.

LPS binds to a TLR4-MD-2 complex which transduces the signal intracellularly. The cellular receptor, CD14 amplifies this signal (Miyake, 2004). Furthermore a serum component, LPS-binding protein, enhances the binding of LPS to CD14 (Wright *et al.*, 1990). This leads to the activation of the MKK3/p38αMAPK cascade which results in NF-κB activation. The pathway upstream of MKK3 (MEKK-X) is not yet known (Nick *et al.*, 1999). The binding of FasL to Fas results in trimerization/oligomerization of the receptor and recruitment of the adaptor protein Fas-associated death domain containing protein (FADD) and caspase-8 to form the death-inducing signalling complex (DISC) (Kischkel *et al.*, 1995) which in turn initiates the caspase cascade resulting eventually in activation of the effector caspases including caspase-3 (Earnshaw *et al.*, 1999).

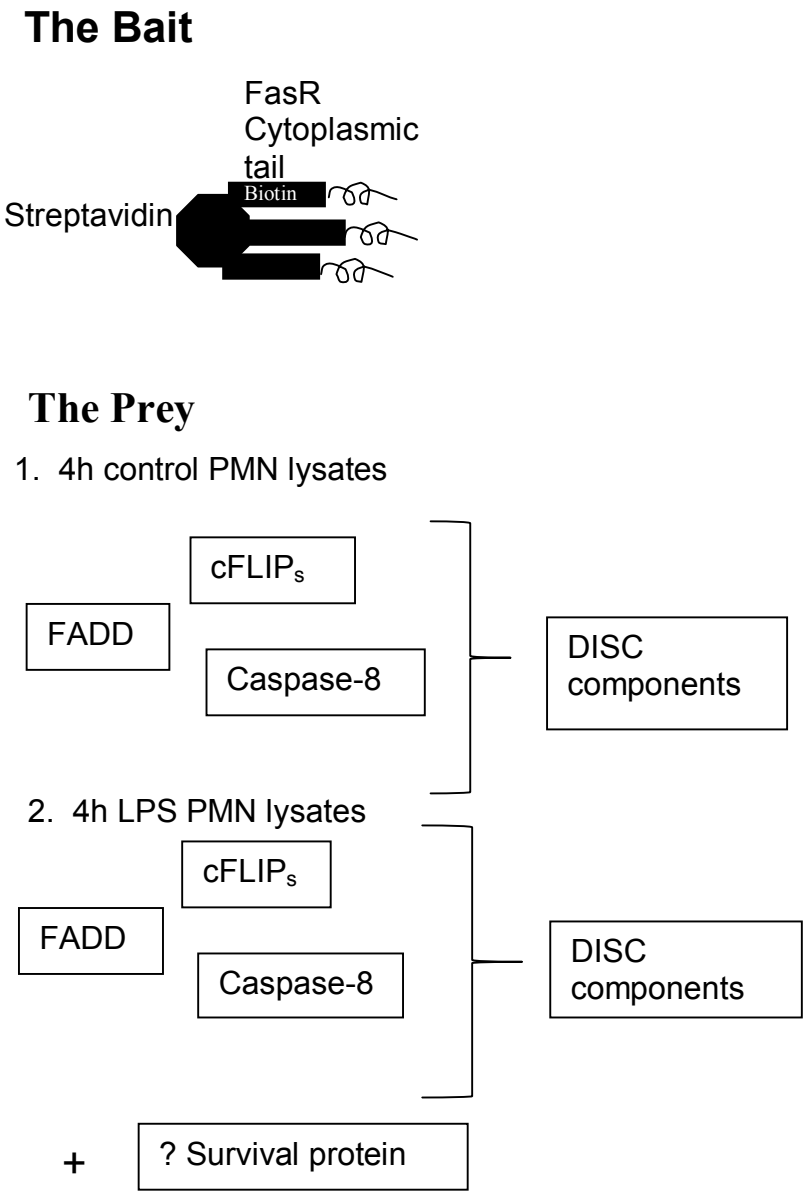
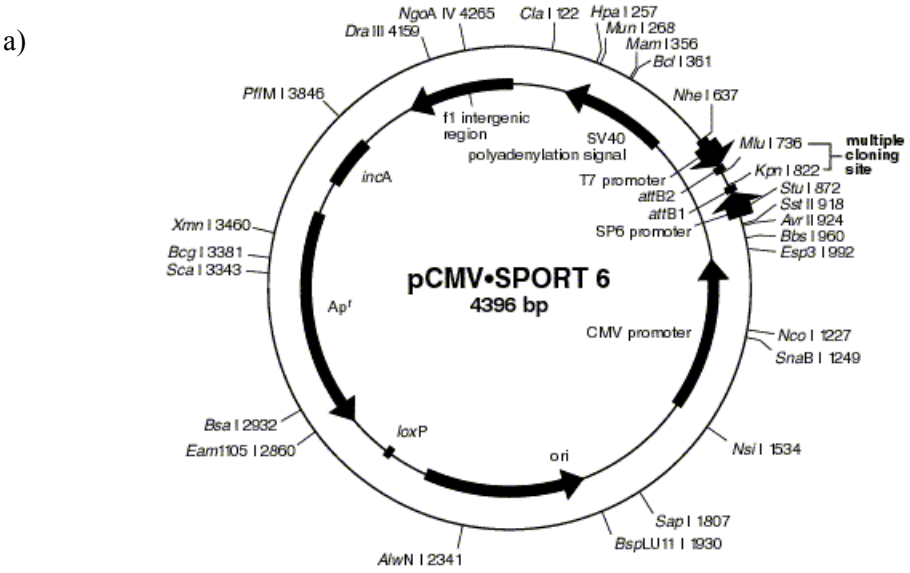


Diagram 4.3. Schematic diagram of the proposed experiment using a Fas receptor cytoplasmic tail tagged with biotin to discover what human neutrophil proteins bind to the cytoplasmic tail.

In this proposed experiment, neutrophils would be incubated for 4h under control conditions and stimulated with LPS. Cell lysates would then be prepared as described in the methods. Fas receptor stimulation results in the cytoplasmic tail forming a death-inducing signalling complex (DISC). It is proposed that when the cells are stimulated with LPS the binding of $cFLIP_s$ or other "survival protein(s)" to the DISC is differentially regulated. The biotin-tagged proteins would then be immunoprecipitated using streptavidin-coated magnetic beads.

a)



pCMV•SPORT 6 multiple cloning site and primer binding regions: 641-917 (The sequence listed here is the (-) strand.)

700

M13/pUC Forward 23-Base Sequencing Primer
5′-CC CAGTCACGAC GTTGTAAAAC G-3′→

T7 Promoter Primer
5′-TAATACGACT CACTATAGGG-3′→

attB2

5′-GCAGTTTTCC CAGTCACGAC GTTGTAAAAC GACGGCCAGT GCCTAGCTTA TAATACGACT CACTATAGGG ACCACTTTGT ACAAGAAAGC TGGGTACGCG TAAGCTTGGG CCCCTCGAGG GATACTCTAG AGCGGCCGCCC

|---------- T7 promoter ---------|

Mlu I | Hind III | Apa I | Xho I | Xba I | Not I

attB1

Mlu I* 800 PinA I

cDNA Insert CGGACGCG TGGGTCGACG ATATCCCGGG AATTCCGGAC CGGTACCAGC CTGCTTTTTT GTACAAACTT GTTCTATAGT GTCACCTAAA TAGGCCTAAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCT-3′

|-------- SP6 promoter --------|

Sal I | EcoR V | Sma I | EcoR I | Rsr II | Kpn I

←3′-GATATCA CAGTGGATTT A-5′
SP6 Promoter Primer

←3′-GGACAC ACTTTAACAA TAGGCGA-5′
M13/pUC Reverse 23-Base Sequencing Primer

* This *Mlu* I restriction site is contained within the *Sal* I adapter introduced into the vector upon ligation of the cDNA insert.

b)

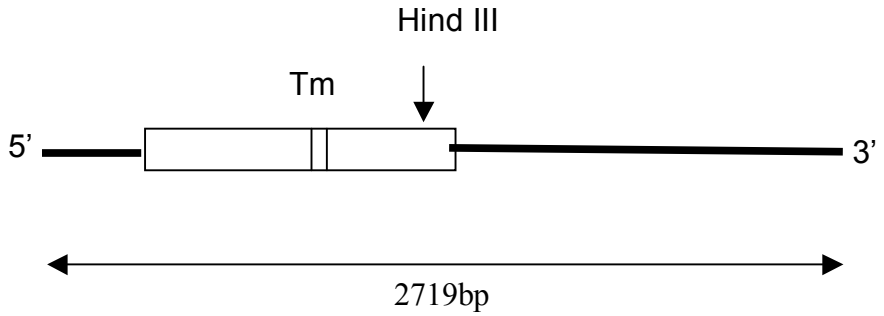
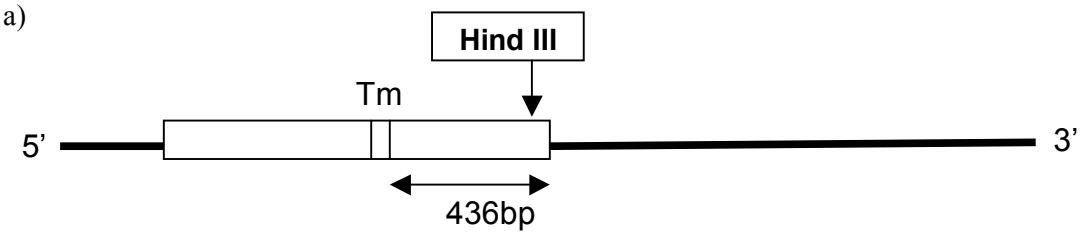


Diagram 4.4. The pCMV.Sport6 plasmid and cloned Fas receptor

(a) pCMV-Sport 6 vector map. (b) Schematic representation of human Fas receptor DNA. The open box represents the open reading frame within which the small box indicates the transmembrane region (Tm). The Hind III restriction enzyme site is shown at 1305bp (GenBank: NM_000043).

a)

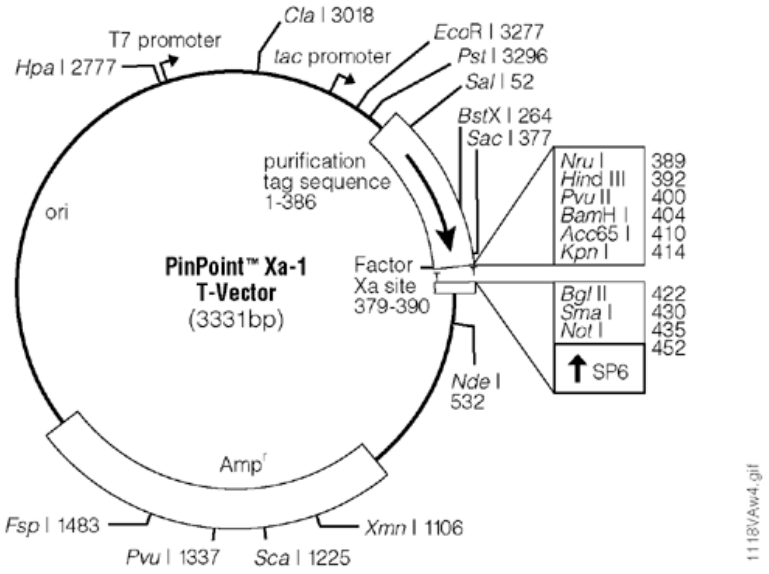


b)

AAGAGAAAGGAAGTACAGAAAACATGCAGAAAGCACAGAAAGGA
AAACCAAGGTTCTCATGAATCTCCAACCTTAAATCCTGAAACAG
TGGCAATAAATTTATCTGATGTTGACTTGAGTAAATATATCACC
ACTATTGCTGGAGTCATGACACTAAGTCAAGTTAAAGGCTTTGT
TCGAAAGAATGGTGTCAATGAAGCCAAAATAGATGAGATCAAGA
ATGACAATGTCCAAGACACAGCAGACAGAAAGTTCAACTGCTTC
GTAATTGGCATCAACTTCATGGAAAGAAAGAAGCGTATGACACA
TTGATTAAAGATCTCAAAAAAGCCAATCTTGTACTCTTGCAGAG
AAAATTCAGACTATCATCCTCAAGGACATTACTAGTGACTCAGA
AAATTCAAACTTCAGAAATGAAATCCAAAGCTTGGTCTAG

Diagram 4.5. The cytoplasmic tail of the human Fas receptor

(a) Schematic representation of the 436bp cytoplasmic tail of the human Fas receptor (GenBank: NM_000043) with a Hind III restriction enzyme site at 423bp. (b) The sequence of the cytoplasmic tail of the human Fas receptor (Itoh et al, 1991). The primer sequences are shown in red.



+

a) FasR cytoplasmic tail (436bp)

b) Control DNA

c) No insert

Diagram 4.6. Schematic representation of DNA ligation into the Pinpoint™ Xa vector

(a) FasR cytoplasmic tail DNA (436bp), (b) control DNA and (c) no DNA.

Chapter 5

The Macrophage and Neutrophil Apoptosis

5.1 Introduction

Lipopolysaccharide (LPS) is a common infectious trigger in the human lung and its action is utilized in animal models of acute lung injury. LPS activates macrophages to produce cytokines however the cytokine milieu differs depending on the inflammatory trigger (Lucas *et al.*, 2003).

In addition to cytokines and other inflammatory mediators, the Fas-Fas ligand pathway may also be important at sites of inflammation. Monocytes and macrophages constitutively express the Fas receptor (Liles *et al.*, 1996; Kiener *et al.*, 1997; Kiener *et al.*, 1997). Mononuclear cells have mRNA for FasL (figure 3.5) and upon activation T-lymphocytes and monocytes express FasL (Kiener *et al.*, 1997; Bossi and Griffiths, 1999). Macrophages do not constitutively express FasL however when they phagocytose opsonized zymosan or apoptotic neutrophils, its surface expression is induced (Brown and Savill, 1999). Furthermore sFasL was found in the supernatants from these stimulated macrophages and these conditioned supernatants also induced apoptosis in neutrophils (Brown and Savill, 1999).

However the cytokine milieu produced by macrophages differs depending whether particulates or effete cells have been phagocytosed. When macrophages phagocytosed latex beads although the conditioned supernatant contained sFasL, it was unable to induce apoptosis in neutrophils. Furthermore the pro-apoptotic activity of the conditioned supernatant from macrophages which have phagocytosed apoptotic neutrophils was only partially due to sFasL (Brown and Savill, 1999).

We have further explored the soluble factors produced by LPS-activated macrophages and studied their effect on neutrophil apoptosis.

5.2 Results

Initially the experimental conditions were optimized using the monocytic cell line THP-1.

5.2.1 THP-1 and neutrophil co-culture

The THP-1 cells are derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia (European Collection of Cell Cultures). They are grown in suspension but can differentiate into a macrophage phenotype using fibronectin and phorbol 12-myristate 13-acetate (PMA) (Auwerx, 1991). As PMA can trigger degranulation of neutrophils (Dewald *et al.*, 1983), after 24 hours, the differentiated THP-1 cells were washed and grown for a further 24 hours in fresh medium (RPMI and 10% FBS). The cells were activated with differing concentrations of LPS (0.01, 0.1 and 1μg/ml) however no difference in activity of the conditioned media was seen. The cells were therefore stimulated with LPS (1μg/ml).

Initially the cells were stimulated with LPS for 4 hours. When neutrophils (PMN) were co-cultured with LPS-activated THP-1 cells, there was a non-significant trend suggesting that activated THP-1 cells induced neutrophil apoptosis maximally (10.9 $\pm$ 2.8%; control, 3.0 $\pm$ 1.3%) in the presence of their conditioned media (figure 5.1a). The conditioned media alone had no effect on neutrophil apoptosis (figure 5.1a). When the cells were stimulated with LPS for 20 hours and apoptosis assessed after 4 hours incubation, activated THP-1 cells, significantly and maximally, induced neutrophil apoptosis (7.7 $\pm$ 1.2 %; control, 3.2 $\pm$ 0.8%, $p<0.05$) in the presence of their conditioned media (figure 5.1b). In the presence of conditioned media alone, there was a non-significant trend suggesting that it increased neutrophil apoptosis (5.8 $\pm$ 1.1%; control, 2.8 $\pm$ 0.3%). After 8 hours incubation, THP-1 cells prolonged neutrophil survival (THP, 1.2 $\pm$ 0.7%; PMN, 6.4 $\pm$ 0.6%, $p<0.01$) and both LPS-activated THP-1 cells and conditioned media alone non-significantly attenuated neutrophil apoptosis (figure 5.1b). After removing the cell supernatants, the plates were fixed with glutaraldehyde (2.5%) and the myeloperoxidase of neutrophils stained with 0.1mg/ml dimethoxybenzidine (DMB) and 0.03% (v/v) hydrogen

peroxide. A limitation of these experiments is that viable neutrophils did adhere to THP-1 cells so rates of apoptosis in co-culture experiments are probably over-estimated however there was no evidence of phagocytosis of apoptotic neutrophils.

5.2.2. The effect of conditioned supernatants from LPS-stimulated, human peripheral blood-derived macrophages (HDM)

Human peripheral blood-derived mononuclear cells (HDMC) differentiate *in vitro* into macrophages under certain experimental conditions. After the HDMCs ($4x10^6$/ml in IMDM) have been plated onto plastic tissue culture ware for 1 hour, the monocytes adhere such the lymphocytes can be washed off. In the presence of autologous serum, over 5 days, the monocytes differentiate into macrophages (figure 5.2b). When the cells are stimulated with LPS (1μg/ml) for 20 hours, transfer of the conditioned supernatants (CM) induces neutrophil apoptosis (45 $\pm$ 3.6%; control, 3.3 $\pm$ 0.4%, $p<0.001$). If the CM is fractionated through 50kD filters, neutrophil apoptosis is induced entirely by the fraction containing proteins with a mass greater than 50kD ($50kD^+$) (46.0 $\pm$ 0.7%; $50kD^-$, 6.1 $\pm$ 1.3%, $p<0.001$). CM transferred from unstimulated HDM did not induce neutrophil apoptosis (1.76 $\pm$ 0.65%, n=3, data not shown)

In order to examine the proteins which are present in the conditioned media we entered the field of proteomics. We planned to utilize two techniques to maximize resolution of the different proteins. The first was 2D gel electrophoresis coupled with mass spectrometry to determine the protein identity. At the same time, we had the opportunity to utilise the newer surface-enhanced lased desorption and ionization – time of flight (SELDI-TOF) technique which provides rapid protein profiling analysis (Ciphergan). Serum contains many soluble proteins therefore to maximize resolution of the proteins produced by the LPS-stimulated macrophages, we optimized a serum-free experiment. Macrophage-SFM supplemented with GM-CSF (500U/ml) has been shown to support human peripheral blood macrophage function comparable to conventional serum-based media (Geissler *et al.*, 1989; Vincent *et al.*, 1992). We have shown that human peripheral blood monocytes maintained in the presence of serum form more giant cells however the absence of serum does not alter their phenotype; both serum and serum-free macrophages expressed CD64, HLA-

DR, FcγRI and CD14 receptors on their surface (figure 5.2b). Lipopolysaccharide-binding protein (LBP) was required in the absence of serum to enable LPS (1μg/ml) to activate the HDMs such that their conditioned supernatants induced neutrophil apoptosis (control mean, 7.7%; CM mean 23.9%, n=2) (figure 5.3a). The optimal concentration of LBP was found to be 15.9μg/ml (data not shown). The $50kD^{+}$ CM fraction from serum-free HDMs was again pro-apoptotic (mean 15.5%, control mean, 7.7%, n=2) (figure 5.3a). These $50kD^{+}$ and $50kD^{-}$ conditioned supernatants were stored at -70°C. Subsequently 2D electrophoresis of the supernatants was carried out as described in the methods (figure 5.3b). Unfortunately we do not currently have access to automated software to comprehensively analyze the 2D gels but our subsequent work suggested TNF-α was important. The supernatants were also sent for SELDI-TOF mass spectrometry analysis but there was a problem with their software and they were unable to analyse the specimens.

Our group has previously shown that the conditioned supernatant from macrophages which have phagocytosed apoptotic neutrophils is able to induce apoptosis in fresh neutrophils (Brown and Savill, 1999). We therefore investigated the conditioned media of LPS-stimulated monocyte-derived macrophages which have phagocytosed apoptotic neutrophils. The cytokine content of the conditioned supernatants was determined using flow cytometry and a cytokine bead assay, as described in the methods. LPS-stimulated macrophages produced large quantities of TNF-α (3627 $\pm$ 717 pg/ml), IL-6 (2836 $\pm$ 1093 pg/ml) and IL-8 (>5000 pg/ml) (n=3, figure 5.4a). At this time point (20h), macrophages which have phagocytosed apoptotic neutrophils produced minimal amounts of these cytokines and phagocytoses of apoptotic neutrophils also appears to downregulate the LPS-induced secretion of TNF-α (range, 6.7-18.1 pg/ml), IL-6 (range, 14.9-169.5 pg/ml) and IL-8 (range, 789.5-5000 pg/ml) (n=2, figure 5.4a). Furthermore, at this time point the conditioned supernatants from neither control monocyte-derived macrophages, which had phagocytosed apoptotic neutrophils nor those which had been concurrently stimulated with LPS and phagocytosed apoptotic neutrophils, induced apoptosis in freshly isolated neutrophils (figure 5.4b).

As there was significant production of TNF-α by the LPS-activated macrophages and TNF-α (EC_{50} 2.8ng/ml) can induce apoptosis in neutrophils at early time points (Murray *et al.*, 1997), we used a neutralizing anti-human TNF-α antibody to try to block the activity of TNF-α in the conditioned supernatants. The concentration of neutralizing antibody required to block neutrophil apoptosis induced by recombinant human TNF-α (50ng/ml) was determined to be 3 to 30μg/ml. Anti-TNF-α antibody was therefore used at a concentration of 3μg/ml in subsequent experiments (figure 5.5a). Neutrophil apoptosis induced by the conditioned supernatants from LPS-stimulated monocyte-derived macrophages was completely inhibited by neutralizing anti-TNF-α antibody (3μg/ml) (CM, 19.3 $\pm$ 3.8%; anti-TNF.CM, 1.0 $\pm$ 0.5%, $p<0.001$) (figure 5.5b). The apoptotic effect of the conditioned media was also lost with heating (HI) at 95°C for 5 minutes (HI.CM, 0.4 $\pm$ 0.2%, $p<0.001$) (figure 5.5b). However the neutralizing anti-Fas antibody, ZB4, had no effect (figure 5.5b). At early time points (4h), the conditioned supernatant induced apoptosis however at later time points (8h), it induced survival (figure 5.5c). Our group has previously shown this temporal effect on neutrophil apoptosis with recombinant TNF-α (Murray *et al.*, 1997) therefore this is consistent with our finding that neutralizing anti-TNF-α antibody inhibits the effect of the conditioned supernatant.

This suggests that TNF-α is important in the induction of neutrophil apoptosis by LPS-stimulated monocyte-derived macrophages. However phagocytosis of apoptotic neutrophils downregulates TNF-α production and in association with adoption of this anti-inflammatory phenotype, its conditioned supernatant was no longer able to induce apoptosis in bystander neutrophils. This is likely to be important as the neutrophil will have to function as the main defence against infection whilst the macrophage is “hypoimmune”.

5.3 Discussion

It has been known for many years that macrophages are important in innate immunity however their role in inflammatory diseases has only recently become more apparent. They appear to be central to the switch from inflammation to resolution and as we have shown, the inflammatory mediators which they produce and their effect on bystander neutrophils will vary depending on the macrophage stimulant.

Lipopolysaccharide-stimulated monocyte-derived macrophages produced a "cytokine soup" which induced neutrophil apoptosis at early time points. In this "soup", soluble TNF-α appeared to be important in mediating this apoptotic effect which could be abolished by neutralizing anti-TNF-α antibodies. To confirm the role of TNF-α, future work would assess the effect of blocking antibodies to the TNF receptors (TNFR1 and TNFRII) as these are also known to block TNF-α –stimulated neutrophil apoptosis (Murray *et al.*, 1997).

As TNF-α appears to be the soluble pro-apoptotic factor, it must be in its trimerized form in the "cytokine soup" as only the fraction containing proteins greater than 50kD was able to induce apoptosis. Wound-derived macrophages from rats have also been shown to induce neutrophil apoptosis mediated by TNF-α but cell to cell contact was required (Meszaros *et al.*, 2000). Furthermore in a murine model, intraperitoneal injection of *Leishmania major* induced neutrophil recruitment. Subsequent neutrophil apoptosis was shown to be induced by membrane-bound TNF-α present on macrophages (Allenbach *et al.*, 2006).

At later time points we showed that the "cytokine soup" from LPS-stimulated macrophages delayed neutrophil apoptosis which may be mediated by TNF-α since recombinant TNF-α also delays neutrophil apoptosis at later time points (Murray *et al.*, 1997). However in the lung, other cytokines including GM-CSF and macrophage migration inhibitory factor (MIF) may play a role. MIF has recently been shown to delay neutrophil apoptosis (Baumann *et al.*, 2003).

In the lung, GM-CSF appears to play a critical role in the normal functioning of alveolar macrophages. It is important in the terminal differentiation of macrophages through induction of the transcription factor PU.1 (Shibata *et al.*, 2001; Berclaz *et al.*, 2002). Furthermore, the alveolar macrophages of GM-CSF knockout mice are unable to produce TNF-α in response to LPS (LeVine *et al.*, 1999) and have defects in cellular adhesion and phagocytosis (Paine *et al.*, 2001). Consequently the knockout mice have severely impaired clearance of pulmonary bacterial and fungal pathogens (LeVine *et al.*, 1999; Paine *et al.*, 2000). The knockout mice also developed a condition that resembled human pulmonary alveolar proteinosis where eosinophilic lipoproteinaceous material and large foamy macrophages accumulate in the alveoli. It is now known that this is due to defective catabolism of the surfactant proteins by alveolar macrophages and that the acquired form of pulmonary alveolar proteinosis in humans is due to GM-CSF neutralizing autoantibodies (Kitamura *et al.*, 1999; Yoshida *et al.*, 2001).

If the LPS-stimulated monocyte-derived macrophages phagocytosed apoptotic neutrophils, the macrophages appeared to switch to a more quiescent phenotype and their "cytokine soup" was unable to induce neutrophil apoptosis. The interaction of LPS, apoptotic cells and murine macrophages has been extensively studied. LPS alone triggers TNF-α release over 24 hours. While phagocytosis of apoptotic cells augments the TNF-α at early time points (4-6 hours), at later time points TNF-α production is inhibited (Lucas *et al.*, 2003).

Monocyte-derived macrophages which have phagocytosed apoptotic neutrophils express FasL (Brown and Savill, 1999) however the Fas/FasL pathway does not appear to be important in inducing neutrophil apoptosis when the macrophages are stimulated with LPS. In neutrophils, Fas ligation does not lead to NF-κB activation (figure 4.4a) however in monocytes NF-κB is activated upon Fas ligation and Fas ligation in human macrophages stimulates TNF-α production (Park *et al.*, 2003).

It has been shown in primary human macrophages that interruption of Fas signalling, either at the Fas receptor or by expression of dominant-negative FADD, suppresses LPS-induced TNF-α secretion. In the macrophage, LPS signals through the same

pathway as in the neutrophil (diagram 4.2). FADD and MyD88 have death domains which promote protein-protein interaction and it was shown in primary macrophages that Fas ligation suppressed the interaction of FADD with MyD88 by sequestering FADD in the DISC (Ma *et al.*, 2004). The reverse may be true in neutrophils; perhaps MyD88 interacts with FADD preventing it forming the DISC and thus LPS inhibits Fas-induced neutrophil apoptosis.

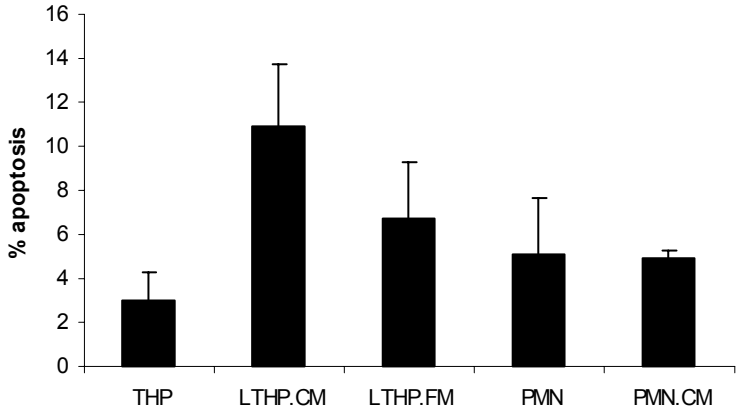
In rheumatoid arthritis (RA), macrophage number and cytokine expression, including TNF-α, strongly correlates with disease symptoms and joint destruction (Feldmann *et al.*, 1996; Mulherin *et al.*, 1996; Tak *et al.*, 1997). In keeping with this, inhibition of TNF-α by humanized monoclonal anti-TNF-α antibodies (infliximab) or neutralizing anti-TNF-α proteins (etanercept), ameliorate the symptoms and reduce joint destruction in RA (Bathon *et al.*, 2000; Lipsky *et al.*, 2000). This has led to widespread use of these agents however the incidence of tuberculosis in treated patients has increased four-fold (Wolfe *et al.*, 2004). *Mycobacterium tuberculosis* finds a haven in macrophages and it is thought, by many in the field, that macrophage apoptosis is therefore required to initiate killing of the organism. As this is a TNF-α -dependent process (Rojas *et al.*, 1999), latent TB may become reactivated during anti-TNF-α treatment. However in an animal model of immunosuppression-related gram-negative bacteraemia, anti-TNF-α treatment reduced circulating bioactive TNF-α but failed to reduce organ damage and mortality (Lechner *et al.*, 1997). It would be interesting to know why anti-TNF-α therapies have proved successful in rheumatoid arthritis, Crohn's disease and inflammatory ocular diseases but so far have not shown benefit in pulmonary disorders including ARDS and sarcoidosis. Is this a function of the phenotype of the alveolar macrophage?

Proteomics in comparison to the study of the genome is in its infancy. At a cellular level 2D gel databases for alveolar macrophages and human monocytes have been constructed. This has allowed analysis of protein changes during differentiation and also the effect of smoking, on protein levels in alveolar macrophages, has been determined (Wu *et al.*, 2005). There is also increasing interest in the use of proteomics to find markers of disease. In the field of respiratory medicine, 2D gel

electrophoresis has been used to examine differential protein expression in lung biopsies and samples from patients with cystic fibrosis, idiopathic pulmonary fibrosis, mesothelioma, asbestosis, hypersensitivity pneumonitis and acute lung injury (Lenz *et al.*, 1993; Lindahl *et al.*, 1996; Wattiez *et al.*, 2000; Griese *et al.*, 2001; Bowler *et al.*, 2003). In idiopathic pulmonary fibrosis and hypersensitivity pneumonitis while there was a relative decrease in the quantity of surfactant-A, there was a relative increase in transferrin, transthyretin, α_1-antitrypsin and immunoglobulin (Wattiez *et al.*, 2000). In cystic fibrosis patients there was a relative increase in α_1-antitrypsin and lower molecular weight isoforms of surfactant-A (Griese *et al.*, 2001). In acute lung injury, as expected, due to loss of the alveolar-capillary barrier, there was leakage of high molecular weight proteins such as albumin, transferrin and immunoglobulin into the BALF. As there is loss of type II pneumocyte function in ALI, there was also a relative decrease in surfactant-A. Acute phase proteins such as serum amyloid A were increased. Interestingly these investigators found that another acute phase protein orosomucoid was post-translationally modified in ALI and putatively it may reduce neutrophil inflammation by binding E-selectin and P-selectin (Bowler *et al.*, 2003).

In summary, it would appear that TNF-α is important in the interaction between inflammatory neutrophils and macrophages. In rodents, membrane-bound TNF-α is required for macrophages to induce neutrophil apoptosis however in a human *in vitro* system, we have shown that soluble TNF-α is important. However alveolar macrophages have specialized to optimize their function for operating in a unique microenvironment. It would therefore be interesting to study, *in vitro* and *in vivo*, the interaction between neutrophils and alveolar macrophages.

a)



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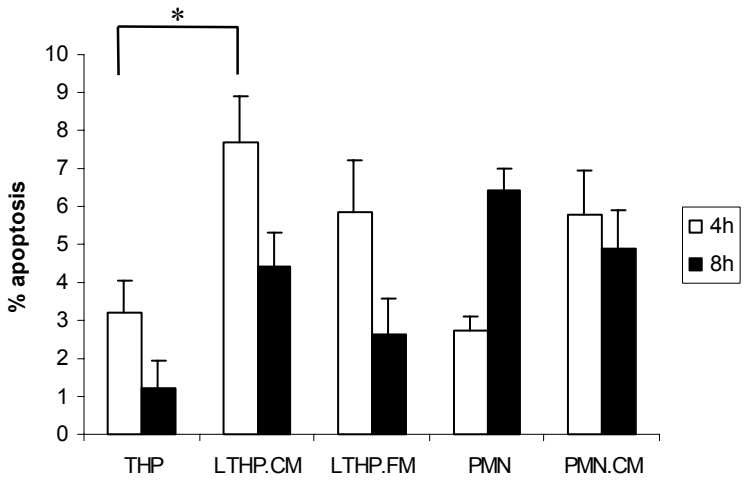


Figure 5.1. THP-1 and Neutrophil (PMN) Co-culture.

THP-1 ($1x10^6$/ml) in RPMI and 10% Fbs adhered to fibronectin in 12-well plates and were stimulated with PMA (1ng/ml) for 24h. Cells were washed and cultured for 24h in RPMI and 10% Fbs before stimulation with LPS (1µg/ml). PMN ($2x10^6$/ml) were cultured with THP-1 (THP), LPS-stimulated THP-1 (L.THP.CM), LPS-stimulated THP-1 washed, with fresh media (LTHP.FM), control PMN (PMN) and PMN with conditioned media transferred from LPS-stimulated THP-1 (PMN.CM). PMN apoptosis was assessed by Annexin V. All values represent mean $\pm$ SEM of n = 3 experiments; each performed in duplicate. *$p<0.05$. (a) THP-1 cells were stimulated with LPS for 4 hours. (b) THP-1 cells were stimulated with LPS for 20 hours and PMN apoptosis assessed after 4 and 8 hours incubation.

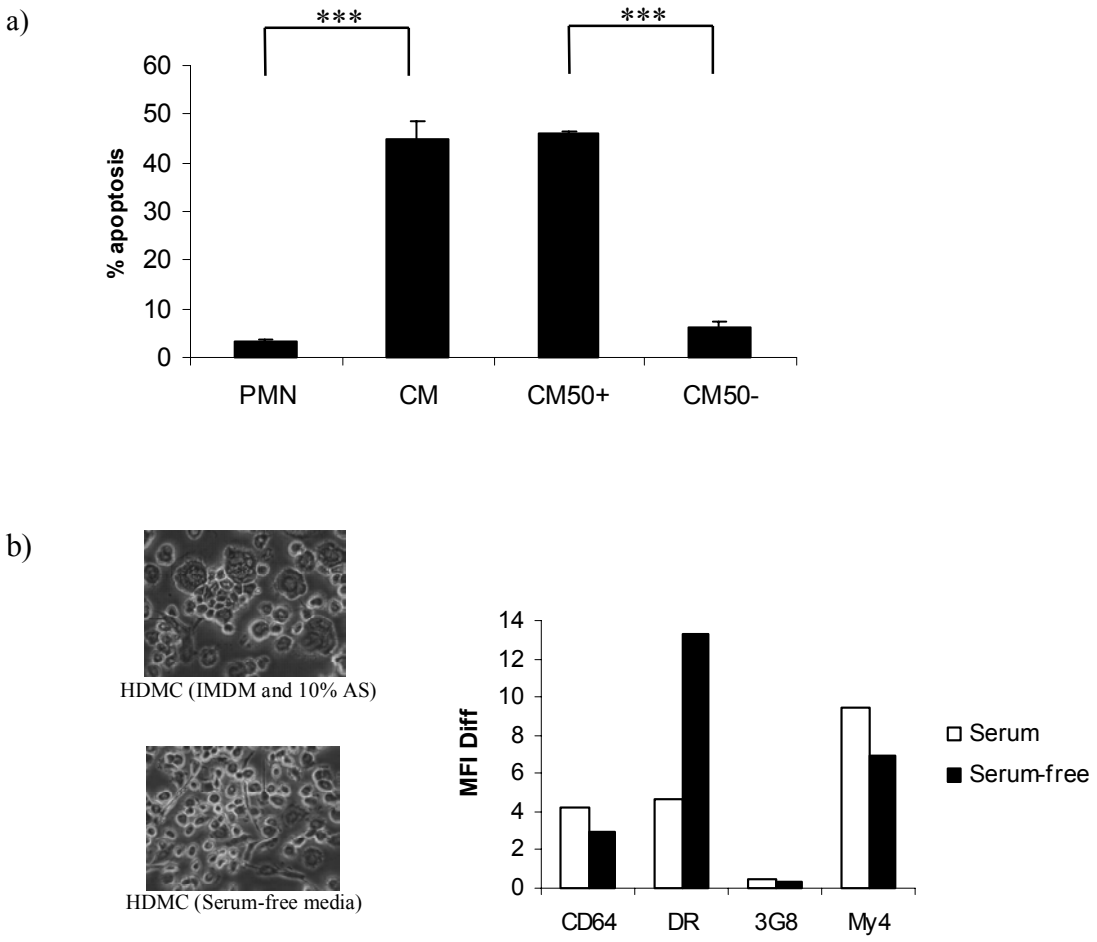
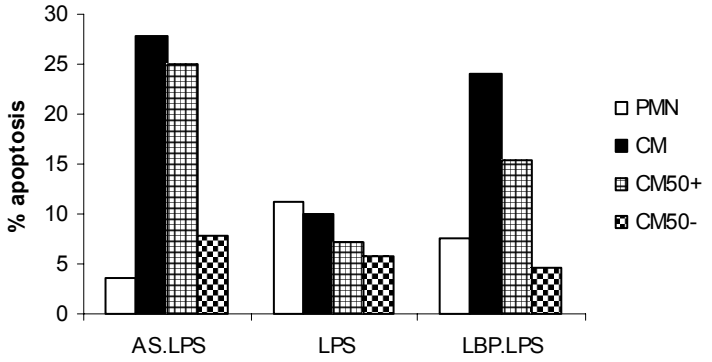


Figure 5.2. The effect on neutrophil (PMN) apoptosis of conditioned supernatants from LPS-stimulated human peripheral blood-derived macrophages (HDM)

(a) Human peripheral blood-derived mononuclear cells ($4x10^6$/ml) differentiated in 48 well-plates (IMDM with 10% AS) for 5 days, were stimulated with LPS (1μg/ml) for 20h. The conditioned supernatants (CM) were fractionated through 50kD Centricon filters. PMN ($2x10^6$/ml) were cultured in the 48-well plates with IMDM + conditioned supernatants for 4 hours. Apoptosis assessed by morphology (n=3). ***$p<0.001$. (b) HDMCs ($4x10^6$/ml) were differentiated in 48-well plates with IMDM and 10% autologous serum (serum) or macrophage serum-free medium supplemented with GM-CSF (500U/ml) for 5 days. They were trypsinized and the cells were labelled with anti-CD64 (1:50), anti-DR (1:50), 3G8 (1:50), My4 (1:50) and isotype control antibodies. Binding was assessed by flow cytometry as described in the methods.

a)



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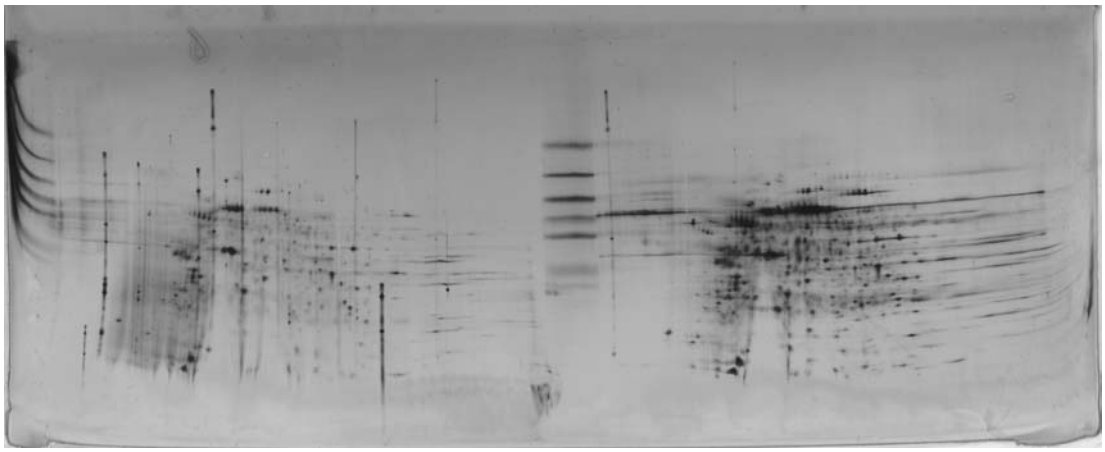
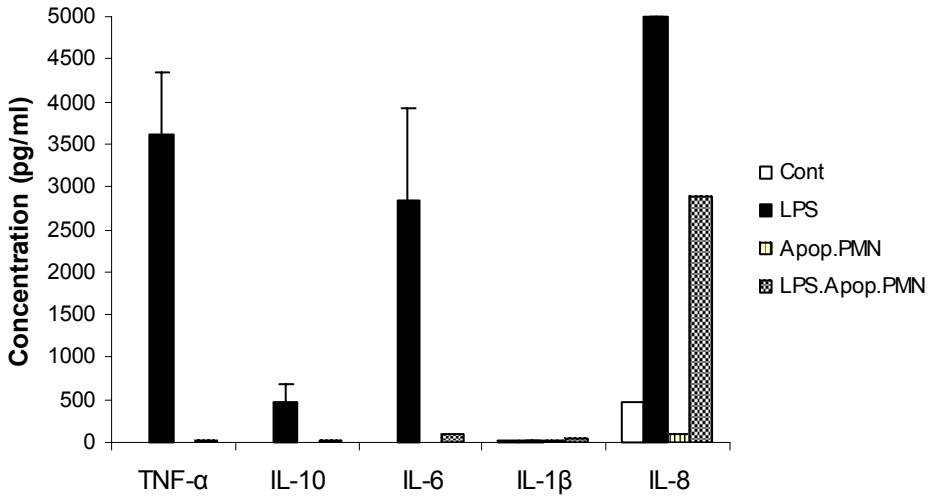


Figure 5.3. The effects of fractionation of the conditioned supernatants from LPS-stimulated HDM

(a) Human peripheral blood-derived mononuclear cells ($4x10^6$/ml) were differentiated in 48 well-plates in serum (IMDM with 10% AS) or serum-free conditions (Macrophage-SFM with GM-CSF (500U/ml) for 5 days and stimulated for 20 hours with LPS (1µg/ml) $\pm$ 10% autologous serum (AS) or lipopolysaccharide-binding protein (LBP) (15.9µg/ml). The conditioned supernatants (CM) were fractionated through 50kD Centricon filters. PMN ($2x10^6$/ml) were incubated in 48-well plates with IMDM $\pm$ conditioned supernatants for 4 hours. Apoptosis was assessed by morphology (n=2). (b) 2D electrophoresis of the conditioned supernatants was carried out as described in the methods. This is a representative blot (n=2).

a)



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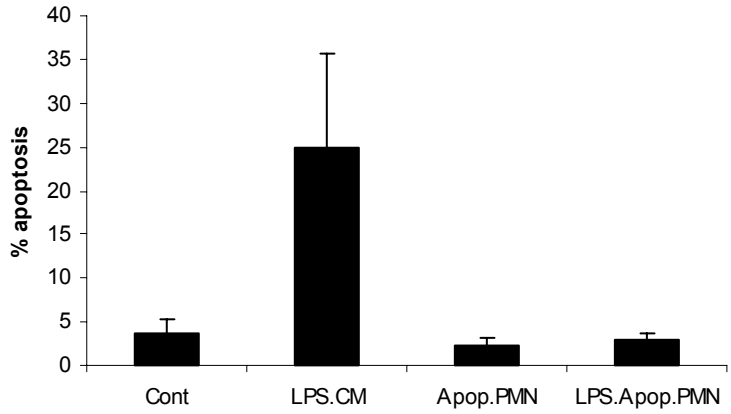
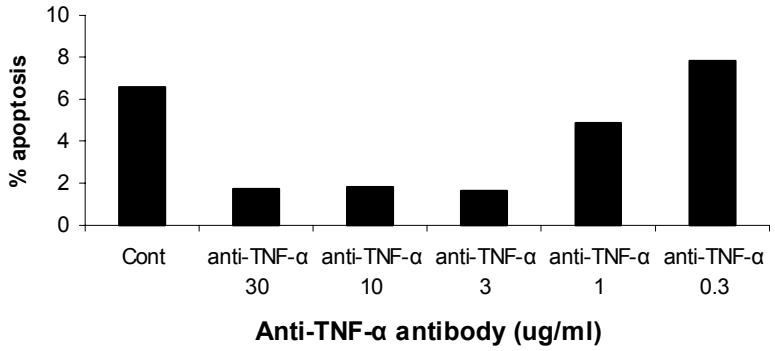


Figure 5.4. The effect of phagocytosis of aged human neutrophils by HDM.

Human peripheral blood-derived mononuclear cells ($4x10^6$/ml) differentiated in 48 well-plates (IMDM with 10% AS) for 5 days, were cultured for 20h ± LPS (1μg/ml) ± aged neutrophils (PMN ($4x10^6$/ml) in IMDM and 10% AS which had been incubated overnight) (Apop.PMN). The supernatants were removed and centrifuged to remove cellular debris. (a) A cytokine bead assay was used to determine concentrations of various inflammatory cytokines in these supernatants as described in the methods. (b) PMN ($2x10^6$/ml) in IMDM and 10% AS were incubated with the supernatants for 4 hours and apoptosis assessed by morphology (n=3).

a)



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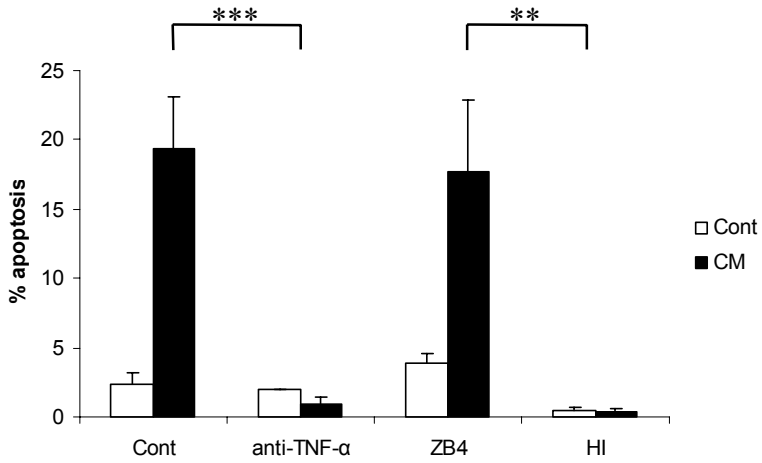

c)

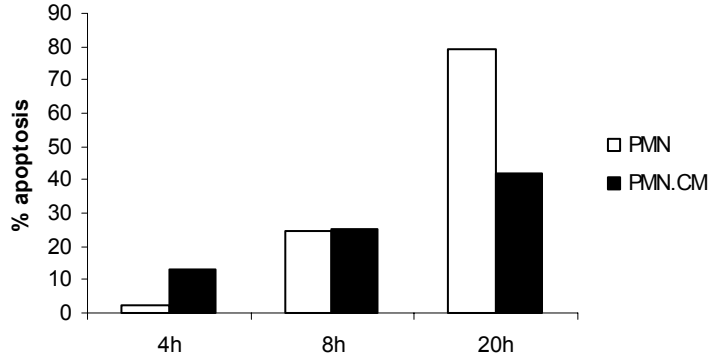

Figure 5.5. TNF-α, CM from LPS-stimulated HDM and neutrophil apoptosis

(a) PMN ($2x10^6$/ml) in IMDM and 10% AS were pre-incubated with varying concentrations of anti-human TNF-α antibody in a shaking water bath for 1 hour. They were then incubated, in 96 well-plates, with recombinant human TNF-α (50ng/ml) for 4 hours and apoptosis assessed by Annexin V (n=2). (b) HDMCs ($4x10^6$/ml) differentiated in 48 well-plates (IMDM with 10% AS) for 5 days, were stimulated for 20h with LPS (1μg/ml). The conditioned supernatants (CM) were harvested. PMN ($2x10^6$/ml) in IMDM and 10% AS were pre-incubated with IMDM $\pm$ anti-human TNF-α antibody (3μg/ml) or ZB4 (500ng/ml) in a shaking water bath for 1 hour. They were then incubated, in 96 well-plates, $\pm$ IMDM or conditioned supernatant (CM) or heat-inactivated CM for 4 hours and apoptosis assessed by morphology (n=3, **$p<0.01$, *** $p<0.001$). (c) PMN ($2x10^6$/ml) in IMDM and 10% AS were incubated with CM for varying time points and apoptosis assessed by morphology (n=1).

Chapter 6

General Discussion

The alveolar milieu and the activation state of the neutrophil are important determinants of neutrophil apoptosis. Inflammatory neutrophils from LPS treated rats were found to be resistant to the induction of apoptosis by anti-Fas antibody (Watson *et al.*, 1997). These studies have also shown *in vitro* that the inflammatory mediators fMLP, lipopolysaccharide (LPS) and GM-CSF inhibited Fas-induced neutrophil apoptosis by a mechanism independent of the ERK pathway. At early time points, the LPS-inhibition of Fas-induced neutrophil apoptosis did not appear to involve the, PI3-kinase or PKC pathways but this inhibition may be partially mediated by p38 MAPK and NF-κB. The natural prostaglandin metabolite, $15dPGJ_2$, is an NF-κB inhibitor (Ward *et al.*, 2002) and *in vitro*, these studies confirmed that $15dPGJ_2$ attenuated the LPS-mediated inhibition of Fas-induced neutrophil apoptosis and associated caspase-8 degradation. Our studies of signalling pathways were limited by only using kinase inhibitors. Although these inhibitors are widely used in scientific studies, their specificity for a particular enzyme is never 100% (Davies *et al.*, 2000). Therefore these inhibitors should only be used as fishing tools and further work, using immunoblot analysis of phosphorylation of individual kinases and/or direct kinase assays, is required to elucidate whether individual signalling pathways are indeed involved. In studying these pathways, it is also important to be aware of each inhibitors' mechanism of action. As SB203580 is a competitive inhibitor of the ATP-binding site on p38 MAPK, it does not affect the phosphorylation of p38 MAPK but affects phosphorylation of its downstream target heat shock protein 27 (HSP27) (Cuenda *et al.*, 1995).

Consistent with this observation, $15dPGJ_2$ was shown to have a protective role in a carageenin model of ALI, (Mochizuki *et al.*, 2005). However, in a LPS model of ALI, although $15dPGJ_2$ reduced NF-κB activation, $15dPGJ_2$ enhanced lung injury (Inoue *et al.*, 2003). In endoxaemia-induced ALI, inflammatory neutrophils show

increased NF-κB activation compared to peripheral blood neutrophils which leads to their expression of pro-inflammatory mediators including IL-1β, IL-8 and TNF-α (Shenkar and Abraham, 1999). In contrast to $15dPGJ_2$, in a LPS model of ALI, anti-oxidants have been shown to suppress NF-κB activation and subsequent neutrophilic inflammation (Blackwell *et al.*, 1996).

In neutrophils, p38 MAPK partially mediated the anti-apoptotic effect of LPS on Fas-induced neutrophil apoptosis. In murine models, inhibition of p38 MAPK prior to haemorrhagic shock or endotoxaemia, did not decrease lung injury (Arcaroli *et al.*, 2001) whereas systemic administration of a p38 MAPK inhibitor after intra-tracheal LPS reduced neutrophilic inflammation in the lung (Nick *et al.*, 2000). In humans, a p38 MAPK inhibitor has also prevented the deleterious effects of endotoxaemia (Branger *et al.*, 2002).

In studies of the Fas/FasL pathway, the most commonly used activating anti-Fas antibody is CH-11. We found that the potency of individual batches of CH-11 decreased over time even despite aliquoting the antibody to reduce freeze/thaw cycles. Therefore a single experiment was repeated over a short period to minimize this effect. This observation explains the wide variation in the apoptosis-inducing effect of CH-11 seen in our studies. However there was less inter-subject variation in the neutrophil response to CH-11 unlike the large inter-subject variation in neutrophil response to TNF-α which other groups have found (Walmsley *et al.*, 2004).

The inhibitory effect of LPS was prevented if the Fas-signalling pathway was first activated. We have been unable to dissect the signalling pathways involved using a modified immunoprecipitation technique however the adapter molecule cFLIP is a potential candidate. In acute lung injury, contrary to our hypothesis, other groups have now shown that the Fas/FasL pathway actually has a pro- rather than anti-inflammatory role. In a murine model, intratracheal instillation of a Fas-activating antibody induced neutrophilic lung inflammation. There are several reasons why activation of the Fas/FasL pathway may be pro-inflammatory *in vivo*. As well as inducing apoptosis, in human airway epithelial cells and human monocyte-derived

macrophages, Fas can activate NF-κB leading to secretion of pro-inflammatory cytokines including IL-8 (Hagimoto *et al.*, 1999; Park *et al.*, 2003). The soluble form of FasL (sFasL) is also chemotactic for neutrophils and induces apoptosis in distal lung epithelial cells (Matute-Bello *et al.*, 1999). However a recent study suggests that the Fas-expressing alveolar epithelium appears to be the primary target in this model (Matute-Bello *et al.*, 2005). In patients with ALI/ARDS the expression of Fas/FasL is also upregulated in lung tissue (Albertine *et al.*, 2002). The lung injury induced by Fas/FasL can be ameliorated by the analogue of the natural FasL antagonist, decoy receptor 3 (Wortinger *et al.*, 2003). After haemorrhagic shock and sepsis, Fas-small interfering RNA (siRNA) but not caspase-8 siRNA, reduced the extent of lung injury including pulmonary epithelial cell apoptosis and neutrophilic inflammation (Perl *et al.*, 2005). Thus Fas-small interfering RNA (siRNA) might be a novel treatment for sepsis-induced ALI (Wesche *et al.*, 2005).

In the lung, the alveolar macrophage is not only important in bacterial phagocytosis, it also phagocytoses apoptotic neutrophils and is important in regulating the cytokine profile of the alveolar milieu. We have shown that LPS-activated monocyte-derived macrophages induce neutrophil apoptosis at early time points due to their production of soluble TNF-α. We have also confirmed that these LPS-activated macrophages which have phagocytosed apoptotic neutrophils downregulate their production of pro-inflammatory cytokines such that their conditioned supernatants are no longer able to induce neutrophil apoptosis. In quiescent macrophages, phagocytoses of apoptotic neutrophils appears to stimulate FasL expression and their conditioned supernatants containing sFasL induce neutrophil apoptosis at later time points than studied here (Brown and Savill, 1999). We found that Fas/FasL was not involved in the neutrophil apoptosis induced by LPS-activated monocyte-derived macrophages at early time points. The cellular microenvironment may also be important in determining the pattern of cytokines released by LPS-activated macrophages and also their effect on neutrophil apoptosis. In rat alveolar macrophages cultured *in vitro*, increasing the CO_2 concentration decreased early TNF-α release (Lang *et al.*, 2005). It would be interesting to investigate the response of human alveolar macrophages under different culture conditions.

Elegant proteomic studies have not only shown how alveolar macrophages differ from their precursor blood monocytes but also how they differ in various lung diseases (Wu *et al.*, 2005). In ARDS, alveolar macrophages have been phenotyped and whilst there was no evidence of resident alveolar macrophage proliferation, early BALF samples showed abundant monocyte-like alveolar macrophages associated with elevated MCP-1 levels. In patients who demonstrated less lung injury manifest by better gas exchange, MCP-1 levels declined earlier associated with a switch in the BALF from the predominance of monocyte-like alveolar macrophages to the mature alveolar macrophage phenotype (Rosseau *et al.*, 2000). In studying alveolar macrophages *in vitro*, mimicking their lung microenvironment is important. For example, human alveolar macrophages have only been shown to express nitric oxide synthase type-2 (NOS2) when co-cultured with human alveolar type-II epithelial cells in the presence of IFN-γ. Surfactant protein A (SP-A) is also important for alveolar macrophage function however its production of nitric oxide appeared to be independent of SP-A (Pechkovsky *et al.*, 2002). Future work would therefore involve the study of human alveolar macrophages from different stages of ARDS, in the presence of different cytokines and to examine the effect of co-culture with human alveolar type-II epithelial cells.

There are many large randomised clinical control trials investigating management options in the treatment of sepsis, ALI and ARDS. In severe sepsis, recombinant activated protein C (rhAPC) has been shown to improve mortality (Ely *et al.*, 2003). As well as being a natural anticoagulant, inhibiting activated factor V and VIII of the clotting cascade, activated protein C also has anti-inflammatory properties including inhibition of neutrophil adhesion to vascular endothelium (Uchiba *et al.*, 1996) and reduction in LPS-stimulated production of TNF-α by monocytes (Grey *et al.*, 1994). Recently the effect of rhAPC on neutrophil function after LPS has been instilled in a human lung segment was determined. Neutrophil accumumation in response to endobronchial LPS was reduced and isolated blood and BALF neutrophils showed reduced chemotaxis in response to IL-8 *in vitro*. Neutrophil function was otherwise normal in these healthy volunteers treated with rhAPC (Nick *et al.*, 2004). However

the main anti-inflammatory effect of rhAPC in sepsis is probably due to decreased endothelial cell stimulation.

Mortality in ARDS has improved. Lung protective ventilation strategies are now universally applied in the management of patients with ALI (ARDS, 2000). The aetiology of ventilator-induced lung injury has largely been determined in the laboratory. Application of physical forces to cultured alveolar epithelial cells stimulates production of cytokines, chemokines and other inflammatory mediators (Dreyfuss and Saumon, 1998). A conservative strategy to fluid management has also proved beneficial in improving lung function and central nervous system function but there was no benefit in mortality (Wiedemann *et al.*, 2006). No benefit from prone ventilation or treatment with recombinant surfactant has been shown (Spragg *et al.*, 2004; Fan *et al.*, 2005).

In summary, we, like others, have refuted the earlier findings of FasL expression on neutrophils and the Fas/FasL pathway does not appear to be important in spontaneous neutrophil apoptosis. At inflammatory sites, neutrophils are unlikely to be susceptible to Fas-induced apoptosis as inflammatory mediators inhibit Fas-induced neutrophil apoptosis *in vitro*. Furthermore there are many studies suggesting that activation of the Fas/FasL pathway on non-myeloid cells in the lung appears to be pro-inflammatory. Cross-talk between intracellular survival and death pathways in the neutrophil and the role of cFLIPs is worthy of further study. The use of small interfering RNA in the field of medical therapeutics is rapidly developing and these pathways are attractive targets to enhance neutrophil apoptosis in the inflamed lung. Further elucidation of the interaction between the alveolar macrophage and neutrophil at different time points in the course of acute lung injury may also lead to development of different therapeutic strategies.

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