# Studies relating to molecular mechanism of CD44 augmented macrophage phagocytosis of apoptotic neutrophils.

**Sharon Vivers**

## DECLARATION

This thesis and the research described herein is solely my own work. Any collaborative work or assistance from others is explicitly acknowledged at the relevant point within the text. No part of this work has been, or is being submitted for any other degree or qualification.

Sharon Vivers, 2005

# ABSTRACT

Apoptosis of neutrophil granulocytes is an essential process in the resolution of the inflammatory response, providing a safe mechanism for down-regulation of neutrophil function and clearance of potentially harmful inflammatory cells by macrophages. However, in chronic inflammatory conditions, the rate of cell death by apoptosis can exceed the macrophage clearance capacity leading to secondary necrosis, resulting in release of harmful cellular contents and damage to the surrounding tissues. There are many possible ways in which the rate and capacity of macrophage clearance of apoptotic cells may be influenced, including soluble mediators such as cytokines and glucocorticoid hormones or interactions with extracellular matrix components. CD44 is a surface receptor, which has been implicated in cellular adhesion to extracellular matrix proteins including hyaluronan. CD44 has also been shown to augment macrophage phagocytosis of apoptotic neutrophils after cross-linking surface receptors by a bivalent antibody. The aim of this thesis was to characterise the mechanism underlying this augmentation further and to investigate potential mechanisms responsible for the observed changes in macrophage phagocytic capacity. Ligation of CD44 by bivalent antibody was shown to exert a prolonged effect upon augmentation of macrophage phagocytosis of apoptotic neutrophils, suggesting that augmentation of macrophage phagocytosis by cross-linking of CD44 was unlikely to be mediated by intra-cellular signalling and might involve physical alterations to the CD44 receptor. However, analysis of the surface expression and distribution of CD44 by immunofluorescence microscopy did not support this hypothesis. To further characterise the mechanism of cross-linking of CD44 on macrophage phagocytosis, cation depletion studies were carried out. Data presented in this thesis demonstrates that augmentation of phagocytosis following CD44 cross-linking involved two components. My data indicate that CD44 cross-linking results in augmentation of macrophage phagocytosis via both a cation dependent and cation independent component. Depletion of divalent cations reduces the level of CD44 augmented phagocytosis but does not entirely block it. Treatment of macrophages with a variety of inhibitors and antibodies allowed

identification of the molecule responsible for the cation dependent component – CD32 (FcγRII). CD44 antibodies were shown to be acting as a bridge between CD32 on the apoptotic neutrophil and CD44 on the macrophage. This highlighted the importance of using $F(ab')_2$ fragments instead of whole antibodies for functional studies. To investigate potential signalling mechanisms involved in the divalent cation-dependent effect resulting in CD44 augmented phagocytosis anti-CD44 $F(ab')_2$ fragments were generated and conjugated to 6μm microspheres, which were used to cross-link CD44 on the macrophage surface. Immunofluorescent microscopy was then used to image changes in cellular distribution of signalling molecules in response to CD44 cross-linking. The data presented in this thesis implicates redistribution of ezrin, actin, PKC and Rac2 in the augmentation of macrophage phagocytosis of apoptotic neutrophils following CD44 cross-linking. Western blot and inhibition studies indicated that ERK did not play a role in CD44 augmented phagocytosis. In summary, the studies presented in this thesis represent an analysis of the cellular and molecular events associated with augmentation of phagocytosis of apoptotic neutrophils. Whilst no gross alterations in CD44 distribution were seen, these studies suggest that the irreversible nature of the augmentation reflects redistribution of key cytoskeletal and signalling elements within the macrophage. Together, these studies provide a firm foundation for future studies and highlight the potential for modulation of phagocyte capacity for clearance of apoptotic cells in treatment of inflammatory disease.

## DEDICATION

This thesis is dedicated to my parents, David and Janet Vivers.

## ACKNOWLEDGEMENTS

My biggest thank you must go to both Ian Dransfield and Simon Hart, who guided and supported me during my PhD Studentship.

A huge thanks to Trevor Walker and Carol Ward for their help with the antibody microsphere system and the ERK assays, respectively. Both were a constant source of helpful advice and encouragement.

I am incredibly grateful to Linda Wilson for sharing her expertise in confocal microscopy and to Shonna Johnson, for her patience and support during my many days in the flow cytometry lab.

Thank you to all inhabitants of Room A7 (past and present), who tolerated me for the last three years. A special thanks to Lorna Murray, Emma Taylor and Karen Alexander – my agony aunts and great friends!

Thank you to the many lab outsiders who helped me through the PhD experience, especially Lindsay Learmonth, David Barnes and Steven Dewar. The fact that you are still talking to me after all my moaning is a testament to our friendship.

Lastly, but by no means least, I cannot express how grateful I am for having the wonderful companionship of Graeme Martinow. His support and love over the last three years, especially when things were difficult, has been one of my greatest assets.

## FUNDING

This work was supported by a Medical Research Council PhD Studentship.

Laboratory facilities were also provided by the MRC.

## Table of Contents

### CHAPTER 1: INTRODUCTION

1. The inflammatory response....12
1.1 Initiation of inflammation....12
1.2 Progression of the inflammatory response....15
1.3 Resolution of inflammation....16
2. Origins and development of the neutrophil....16
2.1 Neutrophil function....17
2.2 Neutrophil apoptosis (programmed cell death)....18
3. The mononuclear phagocyte system....19
3.1 Macrophage origin....21
3.2 Macrophage morphology and physiology....22
3.3 Macrophage function and phagocytosis....23
3.4 Clearance of apoptotic cells by macrophages....26
3.5 Regulation of phagocytosis of apoptotic cells....31
4. Structure and function of CD44....34
4.1 CD44 and hyaluronan binding....38
4.2 CD44 in inflammation and disease....41
4.3 CD44 and clearance of apoptotic neutrophils....42
AIMS....43

### CHAPTER 2: MATERIALS & METHODS

Reagents and antibodies....44
Preparation of CD44 F(ab')2 antibody fragments....44
Cell isolation....45
Preparation of human autologous serum....46
Assessment of macrophage phagocytosis using flow cytometry....46
Assessment of macrophage phagocytosis using plate assay....47
The effects of antibody/ligand inhibitors upon phagocytosis....48
Preparation of macrophage lysates....48
Western blotting for p44/p42 MAPK (Erk1/2) activity....49

Inhibition of p44/42 MAPK (Erk1/2)..........................................49
Time-lapse microscopy migration assay....................................49
Immunofluorescence microscopy of CD44 surface expression..........50
Antibody biotinylation.......................................................50
Dot blotting.................................................................51
Preparation of antibody conjugated microspheres.........................51
Analysis of microsphere binding by flow cytometry........................51
Analysis of microsphere binding by phase-contrast microscopy.........52
Preparation of Fc specific goat anti-mouse secondary antibody.........52
Imaging cytoskeletal changes and
signalling events after CD44 stimulation .....................................52

**CHAPTER 3: CHARACTERISATION OF CD44-AUGMENTED PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS**

INTRODUCTION...............................................................54
RESULTS & DISCUSSION...................................................56
Time-course of CD44 augmented phagocytosis....................56
Time-course of CD44 cross-linking effect...........................58
CD44-augmented phagocytosis in cation deplete conditions......61
Antibody/Ligand panel...................................................63
Inhibition of CD32 on macrophages and neutrophils................65
Generation of anti-CD44 $F(ab')_2$ fragments............................67
Effect of anti-CD44 $F(ab')_2$ fragments on
macrophage phagocytosis...............................................68
CD43 and macrophage phagocytosis...................................71
CD44-augmented phagocytosis in serum-free conditions...........74
CD44 stimulation and macrophage migration..........................75
SUMMARY......................................................................77

**CHAPTER 4: SIGNALLING IN RESPONSE TO CD44 CROSS-LINKING ON THE MACROPHAGE SURFACE**

INTRODUCTION...............................................................79

RESULTS & DISCUSSION........................................................85

CD44 surface expression..................................................85

CD44 surface expression timecourse...................................87

Migration of macrophages in response to treatment with $F(ab')_2$ fragments.........................90

Microsphere assay to investigate macrophage signalling...........91

Microsphere internalisation..................................................94

Macrophage signalling in response to $F(ab')_2$ coated microspheres..............................................96

Actin.......................................................................96

Talin & Vinculin...........................................................96

Rac2.......................................................................99

Protein Kinase C.........................................................102

ERK.......................................................................102

Ezrin......................................................................108

SUMMARY..................................................................108

**CHAPTER 5: FUTURE DIRECTIONS..........................................110**

**REFERENCES...............................................................116**

## ABBREVIATIONS

| | |
|---|---|
| cAMP | cyclic adenosine monophosphate |
| CARD | Caspase activation and recruitment domain |
| CRP | C-reactive protein |
| DD | Death domain |
| DED | Death-effector domain |
| DISC | Death induced signalling complex |
| ECM | extracellular matrix |
| ERM | ezrin/radixin/moesin |
| ERK | extracellular signal-related protein kinases |
| FcR | immunoglobulin Fc receptor |
| FGF | fibroblast growth factor |
| FITC | fluorescein isothiocyanate |
| GAP | GTPase activating protein |
| GEF | guanine nucleotide exchange factors |
| GM-CSF | granulocyte/macrophage colony stimulating factor |
| HBS | Hepes buffered saline |
| HBSS | Hank's buffered salt solution |
| HRP | horseradish peroxidase |
| ICAM | intercellular adhesion molecule |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| IMDM | Iscove's modified Dulbecco's medium |
| LPS | lipopolysaccharide |
| MAP Kinase | mitogen-activated protein kinase |
| MBP | mannan binding protein |
| MCP-1 | macrophage chemotactic protein -1 |
| M-CSF | macrophage colony stimulating factor |
| MHC | major histocompatibility complex |

| | |
|---|---|
| MMP | matrix metalloproteinase |
| MW | molecular weight |
| PAGE | polyacrylamide gel electrophoresis |
| PAMP | pathogen-associated molecular patterns |
| PBS | phosphate buffered saline |
| PDGF | platelet derived growth factor |
| PGE | prostaglandin E |
| PS | phosphatidylserine |
| PSR | phosphatidylserine receptor |
| ROS | reactive oxygen species |
| SDS | sodium dodecyl sulphate |
| TLR | Toll like receptor |
| TNF | tumour necrosis factor |
| VCAM | vascular cell adhesion molecule |

# Chapter 1
# INTRODUCTION

## 1. The Inflammatory Response

Inflammation is a physiological response to infection or cellular injury. The primary aim of an inflammatory response is to remove or isolate the infectious organism and limit tissue damage. Once the infectious organism is removed, resolution of the inflammatory response involves repairing any tissue damage and clearance of unwanted leukocytes. Initiation of an acute inflammatory response is rapid and may last for days or a few weeks. Cornelius Celsus in the first century AD described the "cardinal signs" of inflammation as rubor (redness), calor (heat), dolor (pain) and tumor (swelling), emphasising the importance of changes in the microcirculation during inflammation. These complex changes involve a series of events such as increased vascular permeability and blood flow, exudation of plasma proteins and migration of leukocytes into the inflammatory site. Infiltrating leukocytes can clear invading organisms while also contributing to tissue repair. They can also co-ordinate the inflammatory response by producing cytokines to promote or dampen the response and influence progression to an adaptive immune response.

### *1.1 Initiation of inflammation*

The initial stages of inflammation involve the acute-phase response and activation of the complement, coagulation and kinin pathways.

The acute-phase response is initiated by inflammatory stimuli to alter the concentration of specific plasma proteins (Gabay & Kushner, 1999). Release of pro-inflammatory cytokines such as tumour necrosis factor α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6) by tissue macrophages and other cells stimulates hepatocytes to synthesise and secrete acute-phase proteins. Important acute-phase

proteins include C-reactive protein (CRP) and Mannan-binding protein (MBP). C-reactive protein binds to membrane phospholipids present in microbial membranes and acts as an opsonin. It can also activate the classical complement pathway (Mortensen, Osmand et al., 1976). Mannan-binding protein binds to mannose sugars found on bacterial and fungal surfaces but not on mammalian cells (Ezekowitz & Stahl, 1988). It activates the lectin pathway, which is similar to the classical complement pathway (Ideda, Sannoh et al., 1987). MBP also acts as an opsonin, flagging the opsonised particle for phagocytic uptake (Kuhlman, Joiner et al., 1989)

The complement pathway consists of a number of plasma proteins organised into two distinct activation pathways; the classical and the alternative pathways. Antibody-antigen complexes initiate the classical pathway, whereas bacteria, viruses, fungi and some aggregates of immunoglobulin activate the alternative pathway (Lachmann & Hughes-Jones, 1984). Both these pathways ultimately result in the formation of the Membrane Attack Complex (MAC), which lyses infectious organisms by creating pores in the organism's membrane (Muller-Eberhard, 1986). In addition to producing the MAC, proteins of the complement cascade can also act as chemoattractants (e.g. C3a and C5a) and opsonins (e.g. C3b). (Goldstein, Kaplan et al., 1976; Orr, Varani et al., 1978)

Kinins such as bradykinin are important mediators in the inflammatory response. They are liberated from precursor molecule (kininogens) by the action of several proteases known as kininogenases. Kininogens are synthesised by the liver and are released into the plasma. The kinins are potent vasoactive basic peptides and their properties are wide ranging, including the ability to increase vascular permeability, cause vasodilation, and the contraction of smooth muscle, and to act as co-factors in the coagulation pathway (Regoli & Barabe, 1980). Initiation of the kinin pathway occurs in response to the exposure of Hageman factor to negatively charged surfaces during injury (Griffin, 1978). Hageman factor becomes activated and in turn activates the kininogenase prekallikrein by converting it to kallikrein (Kaplan & Austen, 1971). Kallikrein then breaks down kininogens to release bradykinin (Scott, Silver et al., 1985).

The coagulation pathway produces thrombin, which in addition to producing blood clots, can also alter vascular permeability and generate chemoattractants. Activation of the coagulation cascade occurs in response to tissue injury. Kallikrein, activated by the kinin pathway cleaves Factor XII, which then activates Factor XI, a process that is calcium dependent (Davie, Fujikawa et al., 1979). Activation of Factor X occurs and leads to the formation of thrombin from prothrombin. Thrombin can then cleave fibrinogen to generate fibrin and a blood clot.

Mediators from all three of these pathways can activate local macrophage populations, leading to release of proinflammatory mediators such as TNF$\alpha$ and chemoattractant molecules that promote leukocyte recruitment into a site of tissue injury.

The first cell type recruited to a site of inflammation is the neutrophil granulocyte. Neutrophils can be rapidly recruited from the blood in response to chemoattractant molecules released from stromal cells, microorganisms or dying cells, such as MIP-2 and CXCR2 which have been shown to be released from apoptotic cells in peritoneal inflammation (Iyoda & Kobayashi, 2004). Neutrophils initially adhere to the endothelial wall through interactions between selectins and their ligands (e.g. Sialyl le$^x$, P-selectin, glycoprotein ligand-1) which results in "rolling" adhesion. If the neutrophil becomes activated by chemoattractants (e.g. platelet activating factor, IL-8, f-met-leu-phe, C5a) or bacterial products (e.g. lipopolysaccharide), then tighter binding occurs and the cell begins to migrate in an integrin-dependent manner between endothelial cells through modified tight junctions (diapedesis) (Parkos, 1997;Wagner & Roth, 2000). Once in the site of inflammation, the neutrophil exerts its antimicrobial and cytotoxic effects by various mechanisms. Primarily, the neutrophil phagocytoses invading micro-organisms, damaged cells and tissue debris. However, the neutrophil also has the capacity to release granule contents and generate reactive-oxygen metabolites that can result in tissue damage. As inflammation progresses, circulating monocytes are actively recruited to continue the inflammatory response and assist in tissue repair.

### *1.2 Progression of the Inflammatory Response*

Circulating monocytes and T lymphocytes are recruited to the site of injury in cases of prolonged inflammation. They rapidly cross the endothelial barrier in a similar manner to neutrophils using selectins, integrins and immunoglobulin superfamily members to become tethered and diapedese (Albelda, Smith et al., 1994). Once the monocyte leaves the circulation, it differentiates in a tissue specific manner to form a tissue macrophage that has both phenotypic and functional properties. The macrophage becomes activated in response to pro-inflammatory cytokines, bacterial components and binding of immune complexes or opsonised particles resulting in an increased ability to phagocytose and promoting the secretion of soluble mediators of inflammation such as IFN-γ, TNF-α, IL-1 and MCP-1 (Mosser, 2003). Macrophages have also been shown to induce apoptosis in neutrophils and lymphocytes indicating their importance in mediating the progression and ultimate resolution of inflammation (Meszaros, Reichner et al., 2000;Brown & Savill, 1999).

During inflammation, extensive tissue remodelling is mediated by the macrophage through the production of matrix metalloproteinases (MMPs) both directly and indirectly. Macrophages have been shown to up-regulate the activity of MMPs with gelatinase, caseinase and elastinase activity; in particular MMP-2, MMP-7, MMP-9 and MMP-12 (Song, Ouyang et al., 2000). Macrophages can also secrete collagenases (MMP1 and MMP-13) in combination with a number of other enzymes such as elastase and hyaluronidase (Nathan, 1987;Mariani, Sandefur et al., 1998), which break down host tissues. Tissue remodelling is a dynamic process, which removes damaged tissue and allows the immune system to remove any remaining infectious particles. However, this process has to be tightly regulated to prevent excessive tissue damage and lead to resolution of inflammation.

### *1.3 Resolution of Inflammation*

In most cases, acute inflammatory responses will resolve leaving little or no permanent damage. During resolution of the inflammatory response, macrophages play a vital role in clearance of apoptotic cells and tissue regeneration. Clearance of apoptotic cells is essential to limit inflammation as many inflammatory cells, such as neutrophils, are unable to exit the tissues and may then undergo secondary necrosis leading to release of cellular contents and further host tissue damage (Savill, 1997). Upon ingestion of apoptotic cells, macrophages become less responsive to pro-inflammatory stimuli e.g. LPS and begin to produce anti-inflammatory mediators such as transforming growth factor-β (TGF-β), IL-10 and prostaglandin $E_2$ (Fadok, McDonald et al., 1998;Voll, Herrmann et al., 1997).

As acute inflammation begins to resolve, the process of wound repair begins. The fibrin clot formed during the initiation of the inflammatory response becomes replaced by granulation tissue. Macrophages at the site of inflammation produce fibronectin, which is a chemoattractant for fibroblasts (Tsukamoto, Helsel et al., 1981). Fibroblasts infiltrate the granulation tissue and act to reconstruct the extracellular matrix through production of a wide range of extracellular matrix proteins including collagen and fibronectin (Gabbiani, 2003). Macrophages also play a role through secretion of growth factors and cytokines such as TGF-β, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) that assist with angiogenesis and tissue rebuilding (Wahl, Hunt et al., 1987;Wahl, Wong et al., 1989).

## 2. Origins and development of the Neutrophil

Neutrophils are produced in the bone marrow and are the most predominant leukocyte found in blood (Bainton, 1980). In the bone marrow, the neutrophil undergoes several stages of development before becoming terminally differentiated and released into the blood. Neutrophils originate from a stem cell pool developing into a granulocyte precursor called a myeloblast, which is a relatively

undifferentiated cell with a round nucleus and no granules. This stage is followed by the promyelocyte and myelocyte stages, during which two types of granule develop (Bainton, Ullyot et al., 1971). The first granule, the azurophil or primary granule, forms during the promyelocyte stage and contains peroxidase. The second, the specific or secondary granule, forms later during the myelocyte stage and is peroxidase negative. The two final neutrophils precursors, the metamyelocyte and band form are non-proliferating cells that eventually become mature neutrophils with the characteristic multi-lobed nuclear morphology.

### *2.1 Neutrophil Function*

The main function of neutrophils is phagocytosis - ingesting and destroying target micro-organisms and dead cells through Fcγ receptors and complement receptors (Witko-Sarsat, Rieu et al., 2000). This process is thought to induce the neutrophil to undergo programmed cell death or apoptosis, as described below, during which it becomes unable to respond to external stimuli, causing downregulation of chemotaxis, phagocytosis and degranulation (Hart, Ross et al., 2000). The neutrophil contains cytoplasmic granules, which contain microbicidal proteins, proteases including collagenase, gelatinase, proteases and plasma proteins that can inhibit the growth of the invading micro-organism. Furthermore, the neutrophil can generate reactive oxygen species (ROS) through NADPH oxidase action in the cell membrane, which can also effectively kill micro-organisms (DeLeo, Allen et al., 1999). The cell also generates and secretes pro-inflammatory cytokines, which act to recruit further inflammatory cells (lymphocytes and monocytes) to the site (Lapinet, Scapini et al., 2000).

*2.2 Neutrophil Apoptosis (programmed cell death)*

Once neutrophils have been recruited to a site of inflammation, they do not migrate to the lymph nodes like monocytes and lymphocytes, but die locally (Savill, 1994). It was originally thought that neutrophils died by undergoing necrosis - cell death in response to injury, which results in disruption of the plasma membrane and release of intracellular contents (Hurley, 1983). However, it is now apparent that neutrophils undergo a controlled form of cell death called apoptosis, which was first described by Kerr and colleagues in 1972 (Kerr, Wyllie et al., 1972). In contrast to necrosis, apoptosis maintains cellular membrane integrity preventing release of intracellular contents and limiting the potential for causing tissue damage. Phagocytes can then recognise these apoptotic cells and clear them from the inflammatory site.

During apoptosis, cells undergo a series of characteristic morphological and biochemical changes. These include cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and formation of apoptotic bodies (Kerr, Wyllie, & Currie, 1972;Wyllie, Kerr et al., 1980). The main effector proteins in apoptosis are a family of proteolytic enzymes called cysteinyl aspartate-specific proteases (caspases). Upon activation, the combined actions of caspases result in cellular changes, which produce the apoptotic phenotype (Earnshaw, Martins et al., 1999).

Triggering of the apoptosis pathway occurs by a number of distinct pathways, including interaction of a death receptor with its respective extracellular ligand. Death receptors are cell-surface receptors belonging to the tumour necrosis factor (TNF) receptor superfamily, which trigger apoptosis in response to ligand binding (Nagata, 1997). Currently, six members of the death receptor family are known including Fas (Apo-1 or CD95) and TNF-R1 (p55 and CD120a). Ligation of the death receptor by their ligand i.e. Fas-L (CD95L) or TNF$\alpha$ promotes receptor oligomerisation leading to recruitment of a number of adaptor proteins (Wallach, Varfolomeev et al., 1999). Adaptor proteins contain domains such as the death domain (DD), death-effector domain (DED) and the caspase activation and recruitment domain (CARD), which allow them to link the receptor with initiator caspases (caspase-8 and caspase-10) through the formation of the death induced

signalling complex (DISC). The downstream signal of the DISC complex has been classified into two types (Scaffidi, Fulda et al., 1998). In type I cells, large amounts of caspase-8 are activated at the DISC as a result of their close proximity and intrinsic proteolytic activity. This is followed by rapid activation of effector caspases (e.g. caspase-3, caspase-6 and caspase-7) resulting in apoptosis. In type II cells, DISC formation is greatly reduced and caspase activation occurs mainly downstream of the mitochondria. This pathway involves the Bcl-2 family of proteins, which can either be pro-apoptotic (e.g. Bax) or anti-apoptotic (e.g.Bcl-2). Caspase-8 cleaves Bid, a pro-apoptotic member of the Bcl-2 family, to give truncated-Bid (t-Bid). tBid translocates to the mitochondria and promotes release of cytochrome c, apoptosis inducing factor (AIF) (Susin, Zamzami et al., 1996) and second mitochondria-derived activator of caspases/direct IAP binding protein with low PI (Smac/DIABLO)(Du, Fang et al., 2000;Verhagen, Ekert et al., 2000). Once in the cytosol, cytochrome c binds to apoptosis-activating factor 1 (Apaf-1) and procaspase-9 to form a complex called the apoptosome. Procaspase-9 becomes active and triggers activation of caspase-3.

Engagement of these intracellular pathways effectively uncouples the apoptotic cell from environmental stimuli and limits effector functions. In addition, there are surface membrane alterations that may determine recognition and internalisation by phagocytes. These changes will be considered in more detail in section 3.4.

## 3. The Mononuclear Phagocyte System

Macrophages represent only one part of a host-wide collection of cells, which are termed the mononuclear phagocyte system. The mononuclear phagocyte system can be defined as a population of cells derived from progenitor cells in the bone marrow, which differentiate to form monocytes that circulate in the blood and then enter into tissues where they become resident macrophages (van Furth & van Zwet, 1992). Information on the bone marrow origin of macrophages was gained from studies with chimeras (van Furth, Goud et al., 1982). Studies with labelled cells revealed

that peritoneal macrophages, Kupffer cells, pulmonary macrophages and spleen macrophages derive from circulating blood monocytes, indicating their common lineage (Spector, Walters et al., 1965;Crofton, Diesselhoff-den Dulk et al., 1978;van Furth & Diesselhoff-den Dulk, 1984). However, many of these studies based their observations upon morphological similarities and expression of specific enzymes and receptors, the validity of which is controversial (Hume, Ross et al., 2002;McKnight & Gordon, 1998). For example, the F4/80 antigen has been shown to be difficult to detect upon certain populations of macrophage-like cell populations, such as alveolar macrophages and macrophages of the lymphoid organs (Gordon, Crocker et al., 1986). Moreover, Langerhans cells in the skin were originally thought to be tissue macrophages as they express macrophage markers (Hoefsmit, Duijvestijn & Kamperdijk, 1982.). However, they do not phagocytose and it is now thought that they are a specialised type of dendritic cell, indicating an overlap between the properties of different cell types.

| Tissue | Cell Name |
|---|---|
| Connective tissue | histiocytes |
| Skin | Langerhans cells |
| Liver | Kupffer cells |
| Spleen | red pulp macrophages |
| Bone | osteoclasts |
| Lung | alveolar macrophages |
| Central nervous system | Microglia |

**TABLE 1**
***Cells belonging to the mononuclear phagocyte system.***

The phenotypic plasticity of macrophages can also make identifying members of the mononuclear phagocyte system difficult. Resident macrophages adapt to their local environment in order to perform specific functions (Gordon, 1995) and are phenotypically different from recruited macrophages. However, some resident macrophages, for example microglial cells, have been shown to undergo activation in certain disease states, indicating the flexible nature of macrophage-like cell populations (Kreutzberg, 1996).

### *3.1 Macrophage origin*

Macrophages are tissue resident phagocytic cells that form part of the mononuclear phagocyte system (van Furth, Langevoort et al., 1975). Macrophages are derived from circulating monocytes and their characterisation is essential for investigation of their development and function. There are numerous methods that allow this to be carried out. One of the redundant methods for identification of monocytes was the use of positive staining for non-specific esterase with acetate as substrate. The presence of peroxidase positive granules can also be used to discriminate between resident macrophages and exudate macrophages, which have migrated from the circulation to a site of inflammation and have not lost their peroxidase activity by degranulation (van Furth, Hirsch et al., 1970). With the development of monoclonal antibodies against surface receptors and intracellular antigens the complexities of monocyte-macrophage differentiation have been revealed. A number of broadly expressed "markers" have been defined including such as F4/80 antigen on murine cells, CD14 on human monocytes and CD68 on human tissue macrophages (Austyn & Gordon, 1981; Pulford, Sipos et al., 1990; Ugolini, Nunez et al., 1980). In addition, differences in levels of expression of specific markers have been proposed for monocytes differing in location and activation state. For example, in comparison to nonactivated cells, activated peritoneal macrophages express more Mac-2 antigen and activated alveolar macrophages express more CD11b/CD18 (CR3) (Nibbering, Leijh et al., 1987).

Macrophages represent the terminally differentiated end stage of the mononuclear phagocyte system. The development of mononuclear phagocytes begins in the bone marrow with the maturation of granulocyte/monocyte-colony-forming units into monoblasts, promonocytes and finally, into monocytes (Gordon, Crocker, Morris, Lee, Perry, & Hume, 1986). The production of monocytes *in vivo* is controlled by a number of growth factors. IL- 3, granulocyte/macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) promote cell division

by the monocyte precursors, whereas prostaglandin E (PGE) and interferon α and β (IFNα/β) inhibit cell division (Sachs, 2005).

In the absence of tissue injury or infection, monocytes emigrate at a steady rate from the bone marrow into the blood and then migrate into the tissues where they become local tissue macrophages (Meuret & Hoffmann, 1973). However, production of monocytes in the bone marrow increases during inflammation leading to an increased number of monocytes in the blood. This has been shown to be a result of the action of the monokine, factor increasing monocytopoiesis (FIM), which is produced by macrophages at a site of inflammation. It then acts as a long range regulator to promote production of monocytes by the bone marrow (Annema, Sluiter et al., 1992). As circulating monocytes enter sites of inflammation they come into contact with inflammatory mediators, which promote their maturation into macrophages with potent secretory and endocytic capacity.

### *3.2 Macrophage morphology and physiology*

The morphology of macrophages can be easily observed by allowing them to adhere and spread during *in vitro* culture. The single kidney-shaped nucleus is surrounded by abundant cytoplasm containing a complex cytoskeleton of actin filaments and microtubules, which is responsible for the formation of ruffles and pseudopods. Macrophages are extremely active metabolically and constantly pinocytose. It is estimated that macrophages turn-over the equivalent of their entire plasma membranes every 30 min (Steinman, Mellman et al., 1983). In most cases these pinosomes become primary lysosomes. The surface of the macrophage consists of a wide variety of proteins including class I and class II molecules of the major histocompatibility complex (MHC) and receptors for molecules such as immunoglobulin, complement and specific bacterial components. These receptors play an essential role in recognition and internalisation of infectious organisms.

Macrophages are also powerful secretory cells, which have been shown to produce over 100 different molecules (Nathan, 1987). These include components of the

complement cascade, coagulation factors, proteases, acid hydrolases, cytokines and growth factors. Some of these, such as lysozyme are secreted constitutively; however, most others are produced in response to occupancy of macrophage surface receptors. Certain ligand-receptor interactions can result in macrophage activation and triggering of the respiratory burst. The respiratory burst is generated by the formation of an NADPH oxidase on phagosomal membranes and results in the production of reactive oxygen compounds such as superoxide anions and through the action of catalase and peroxidases, hydrogen peroxide and hydroxyl radicals (Forman & Torres, 2002)

### *3.3 Macrophage function and phagocytosis*

Macrophages are capable of a number of diverse functions including phagocytosis, antigen presentation, cytokine and chemokine release and tissue repair and remodelling. Elie Metchnikov first observed the process of phagocytosis in the late nineteenth century (Cotran R.S., Kumar V. et al., 1989). Since then, there has been much progress in the understanding of this process. Macrophages recognise micro-organisms and apoptotic cells through a variety of different cell surface receptors. Engagement of specific receptors induces specific signalling cascades within the cell, which controls subsequent responses. These receptors include toll-like receptors (TLRs), complement receptors and immunoglobulin receptors (FcRs).

Toll-like receptors have evolved to recognise conserved products present on the surface of bacteria, viruses and fungae (Janeway, Jr., 1992). These products are termed pathogen-associated molecular patterns (PAMPs) and are unique to microorganisms. PAMPs include molecules such as lipopolysaccharide (LPS), peptidoglycan and lipoteichoic acids. In response to binding PAMPs, TLRs activate a signalling pathway leading to activation of NF-κB and MAP kinases (Li, Carpio et al., 2001;Alexopoulou, Holt et al., 2001;Chu, Ostertag et al., 1999) and induction of various genes that function in host defense (Thoma-Uszynski, Stenger et al., 2001).

Complement receptors recognise opsonins produced by the complement pathway such as C3b, iC3b and C4b. Opsonins bind to the surface of microorganisms and act as a bridge between the microorganim and the phagocyte. Macrophages express three receptors for opsonic fragments of complement – CR1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (Law, 1988). CR1 is a single chain transmembrane molecule with an extracellular lectin-like ligand binding domain (Ghiran, Barbashov et al., 2000). It is unable to mediate internalisation of an opsonised particle without additional signals; although ligation of CR1 has been shown to enhance Fc-receptor mediated phagocytosis (Ghiran, Barbashov, Klickstein, Tas, Jensenius, & Nicholson-Weller, 2000). CR3 and CR4 are integrins, which consist of a common beta chain (CD18) paired with a specific alpha chain (CD11b or Cd11c) (Ehlers, 2000). Internalisation of an opsonised microorganism by CR3 requires additional signalling to increase the number of receptors at the cells surface (Berger, O'Shea et al., 1984;Sengelov, Kjeldsen et al., 1993) and the affinity of the receptors (Jones, Schofield et al., 1998); which is then sufficient to trigger phagocytosis. In addition to complement components, TNFα, LPS and fibronectin have all been shown to stimulate phagocytosis through CR3 (Wright & Griffin, Jr., 1985; Pommier, Inada et al., 1983).

Microorganisms opsonised by immunoglobulin can be recognised by Fc receptors present on the surface of macrophages (Ravetch & Bolland, 2001). The most widely studied of these are the FcγRs which recognise the Fc portion of IgG antibodies. FcγRs can be divided into two categories; receptors that contain immunoreceptor tyrosine-based activating motif (ITAM) motifs in their intracellular domains that activate phosphorylation cascades, and receptors that contain immunoreceptor tyrosine-based inhibitory motif (ITIM) motifs, which recruit phosphatases to inhibit signalling (Daeron, 1997). Activating receptors with high affinity (FcγR1/CD64) and low affinity (FcγRIIA/CD32 and FcγRIIIA/CD16) for IgG undergo phosphorylation in response to receptor clustering as a result of ligand binding (Fitzer-Attas, Lowry et al., 2000). Once activated the receptors recruit Syk, an SH2 domain containing kinase (Crowley, Costello et al., 1997; Kiefer, Brumell et al., 1998), which subsequently can recruit a number of adaptor molecules such as SLP-

76 and BLNK (Bonilla, Fujita et al., 2000) that initiate pathways to promote cytoskeletal alterations, pseudopod extension and engulfment of the opsonised microorganism (May, Caron et al., 2000;Lennartz, Yuen et al., 1997;Kusner, Hall et al., 1999). In contrast, FcγRIIB, the inhibitory receptor, regulates engulfment of opsonised microorganisms via recruitment of the phosphatase SHIP that blocks signalling through PI-3 kinase (Marshall, Booth et al., 2001). Therefore, the relative expression of activating and inhibiting receptors determines the threshold for phagocytosis and inflammatory responses to IgG opsonised particle.

In studies of the mechanisms of phagocytosis via different receptors, it has been observed that uptake by complement receptors is very different to uptake by Fc receptors. For CR-mediated phagocytosis, bound particles appear to sink into the phagocytes surface, suggesting that formation of a phagocytic pit occurs (Kaplan, 1977). However, phagocytosis by Fc-receptors involves membrane "zippering" as lamellipodia extend around the particle (Griffin, Jr., Bianco et al., 1975). These differences can be accounted for by differential recruitment of cytoskeletal components to phagosomes. During Fc-receptor mediated phagocytosis, recruitment of Syk and enrichment of cytoskeletal proteins including actin, talin and paxillin occurs near the phagosome membrane and phagocytosis can be entirely inhibited by tyrosine kinase inhibitors (Allen & Aderem, 1996). In contrast, during CR-mediated phagocytosis, cytoskeletal proteins formed discrete foci over the phagosome surface and tyrosine kinase inhibition fails to prevent phagocytosis (Allen & Aderem, 1996).

Internalisation of micro-organisms by macrophages generally results in release of pro-inflammatory cytokines and activation of antimicrobial mechanisms. Most phagocytes including macrophages kill internalised micro-organisms through the production of reactive superoxide ions produced by the formation of NADPH oxidase on phagosomal membranes (DeLeo, Allen, Apicella, & Nauseef, 1999). There are also a number of oxygen-independent proteins present in macrophage granules to assist in microbial killing including lactoferrin and lysozyme. Lactoferrin is an 80kDa iron binding protein which can inhibit growth of microorganisms by sequestering iron required for microbial respiration (Arnold, Russell et al., 1982). Lysozyme is a 14kDa cationic enzyme that hydrolyses

glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid residues in peptidoglycan, a component of bacterial cell walls (Laible & Germaine, 1985). Lysozyme is highly effective against many Gram-positive species but appears to be ineffective against Gram-negative bacteria unless potentiated by co-factors such as hydrogen peroxide and ascorbic acid (Ellison, III & Giehl, 1991).

### *3.4 Clearance of apoptotic cells by macrophages*

There are a number of molecular receptors that have been suggested to contribute to recognition of apoptotic cells by macrophages. Many of these receptors play a critical role in innate immune responses including lectins (McGreal, Martinez-Pomares et al., 2004), integrins (Savill, Hogg et al., 1992; Dransfield, Buckle et al., 1990), scavenger receptors (Peiser, Mukhopadhyay et al., 2002), oxidised LDL receptors (Miller, Viriyakosol et al., 2003), complement receptors (Carroll, 1998) and the LPS receptor, CD14 (Fenton & Golenbock, 1998). Interestingly, uptake of bacterial pathogens via these receptors results in an inflammatory response and stimulation of an adaptive immune response (Zhang, Broser et al., 1995; Aderem, 2003). However, uptake of apoptotic cells is generally considered to be non-inflammatory leading to dampening down of the immune response (Kurosaka, Takahashi et al., 2003; Haslett, 1992).

One of the best characterised mechanisms for apoptotic cell recognition by phagocytes is through the exposure of phosphatidylserine on the apoptotic cell. As cells undergo apoptosis, they lose phospholipid asymmetry leading to exposure of phosphatidylserine on the cell surface (Fadok, de Cathelineau et al., 2001). Indeed, use of annexin V binding to exposed phosphatidylserine is one of the most commonly used methods to detect the level of apoptosis within a cell population (Zhang, Gurtu et al., 1997). This change has been shown to be required for recognition and engulfment to occur. Plasma membrane asymmetry is maintained in viable cells by an aminophospholipid translocase, which is thought to be a $Mg^{2+}$-dependent ATPase (Bevers, Comfurius et al., 1999). This ATPase transports any

phospatidylserine, which may have reached the outer side back to the inner side of the plasma membrane. However, during apoptosis there is activation of "scramblase" proteins, which can move phospholipids across the membrane and increase surface exposure of phosphatidylserine (Sims & Wiedmer, 2001). Moreover, when apoptosis commences, the aminophospholipid translocase activity becomes downregulated and oxidation of phosphatidylserine alters its ability to be transported back to the inner membrane (Kagan, Fabisiak et al., 2000; Tyurina, Shvedova et al., 2000). These changes result in decreased transport of phosphatidylserine from the outer to the inner plasma membrane.

The method of recognition of phosphatidylserine by macrophages is not yet completely understood but a potential receptor for phosphatidylserine expressed on the macrophage surface has been identified and cloned (Fadok, Bratton et al., 2000). However, there is some controversy regarding the nature of the proposed phosphatidylserine receptor. Recent research investigating developmental defects in phosphatidylserine receptor (PSR) knockout mice has yielded interesting, yet conflicting results. Li and colleagues reported that PSR-deficient mice showed abnormal development in the brain, lungs and eyes leading to death within hours of birth (Li, Sarkisian et al., 2003). They also indicated that PSR-deficient macrophages did not clear apoptotic cells indicating the role of PSR in apoptotic cell clearance. However, it is interesting to note that macrophage clearance was not completely inhibited suggesting that the PSR is not essential for apoptotic cell clearance. A further study investigating haematopoiesis in PSR-deficient animals highlighted developmental defects in erythrocytes and T-lymphocytes (Kunisaki, Masuko et al., 2004). Finally, Bose and colleagues supported the observations that ablation of PSR function resulted in impaired development of organs, however, clearance of apoptotic cells was shown to be normal (Bose, Gruber et al., 2004). They also compared reactivity of cells from wild-type and knockout animals with mAb 217, the antibody originally used to isolate the phosphatidylserine receptor. They observed no difference in reactivity and any staining was restricted to the membrane of fetal-liver macrophages. Therefore, it seems likely that the proposed phosphatidylserine receptor encodes a protein that is essential during development

but is not the primary phosphatidylserine receptor involved in apoptotic cell clearance. This is supported by recent research suggesting that the *psr* gene encodes a nuclear protein (Cikala, Alexandrova et al., 2004; Cui, Qin et al., 2004). Alternatively, it is possible that there is no specific receptor for phosphatidylserine and that other bridging proteins interact with phosphatidylserine on the apoptotic cell.

Other receptors have been suggested to recognise phosphatidylserine including CD14, CD36 and the LDL-receptor related protein (CD91) (Henson, Bratton et al., 2001; Savill, Dransfield et al., 2002). Moreover, soluble substances can also interact with PS such as thrombospondin (Savill, Hogg, Ren, & Haslett, 1992; Stern, Savill et al., 1996), Growth Arrest Specific Gene-6 (Gas-6) (Nakano, Ishimoto et al., 1997), Milk Fat Globule Factor-E8 (MFG-E8) (Couto, Taylor et al., 1996) and Protein S (Anderson, Maylock et al., 2003). Both thrombospondin and MFG-E8 contain aminophospholipid binding sites that bind PS and an RGD motif that binds $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins. Gas-6 and Protein-S interact directly with PS on the apoptotic cell and Mer and Rse receptor tyrosine kinases on the phagocyte (Anderson, Maylock, Williams, Paweletz, Shu, & Shacter, 2003; Scott, McMahon et al., 2001).

A number of other surface changes on apoptotic cells, which promote recognition and uptake by phagocytes, have been investigated. In humans, deficiency of C1q is the foremost known genetic risk factor for systemic lupus erythematosis (SLE), a human inflammatory condition characterised by circulating immune complexes and autoantibodies in the blood (Navratil, Korb et al., 1999). C1q-deficient mice develop an SLE-like condition with immune complex deposition in the tissues and resulting glomerulonephritis. Other complement components have also been implicated in the process of apoptotic cell clearance. Complement binding to the apoptotic cell surface would allow apoptotic cell recognition by macrophage complement receptors CR1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18). Antibody blockade of complement receptors CR3 and CR4 has been shown to inhibit phagocytosis of serum-exposed apoptotic cells (Mevorach, Zhou et al., 1998; Mevorach, 2000).

Antibodies and immune complexes are also potential candidates for opsonisation of apoptotic cells. Patients with antiphospholipid syndrome (APLS) produce antibodies against phospholipid-associated proteins, which can bind to PS. These autoantibodies have been shown to bind apoptotic cells *in vitro* resulting in increase in uptake by phagocytosis (Pittoni, Ravirajan et al., 2000; Balasubramanian, Chandra et al., 1997). Recently, IgG containing immune complexes have been shown to opsonise apoptotic neutrophils through FcγR interactions (Hart, Jackson et al., 2003). Fc receptor-mediated phagocytosis is classically pro-inflammatory; raising the possibility that opsonisation of apoptotic cells with IgG complexes may influence the resolution of an inflammatory response by altering the balance of macrophage cytokine production. However, it is possible that Fc receptor ligation in combination apoptotic cell recognition molecules will engage specific signal transduction pathways that act to down-regulate the pro-inflammatory responses normally associated with FcγR ligation.

Other potential opsonins present within serum for apoptotic cells include pentraxins and collectins. Pentraxins such as C-reactive protein (CRP) and serum amyloid protein (SAP) are acute-phase proteins, which can bind chromatin, small nuclear ribonucleoproteins and C1q (Volanakis & Wirtz, 1979). CRP can bind to late apoptotic human lymphocytes, increasing the level of binding of C1q, factor B and iC3b (Gershov, Kim et al., 2000). SAP has been reported to bind phosphatidylethanolamine on apoptotic lymphoma cells (Familian, Zwart et al., 2001). Collectins, mannan binding lectin (MBL) and surfactant protein A (SP-A) are lectins which form structures similar to C1q and recognise high mannose carbohydrates and other pathogen-associated molecular patterns (PAMPs).

In addition to the phosphatidylserine receptor, a number of receptors have been reported to mediate their binding and uptake by macrophages and other phagocytic cell types. The first to be investigated was the recognition of N-acetylglucosamine, N-acetylgalactosamine, and galactose groups on apoptotic lymphocytes by an uncharacterised macrophage lectin (Duvall, Wyllie et al., 1985). Subsequently, an asialoglycoprotein receptor expressed on hepatocytes was shown to contribute to recognition of apoptotic hepatocytes by their neighbours (Dini, Autuori et al., 1992).

Moreover, an asialoglycoprotein receptor has been identified on macrophages, which can possibly assist in uptake of apoptotic cells (Ii, Kurata et al., 1990).

Scavenger receptors and integrins have also been identified as contributing to the recognition of apoptotic cells. In Drosophila, a member of the SR-B family, Croquemort, has been shown to participate in removal of apoptotic cells (Franc, Heitzler et al., 1999). In human monocyte-derived macrophages, CD36 was found to associate with the $\alpha v\beta 3$ vitronectin receptor and mediate binding of apoptotic cells via thrombospondin, a soluble molecule that acts as a bridge between the phagocyte and the apoptotic cell. This interaction has also been suggested to be essential for PS mediated uptake by human macrophages (Fadok, Warner et al., 1998). Blockade of CD36 resulted in eliminating uptake of apoptotic neutrophils by both PS stimulated and unstimulated macrophages, indicating that CD36 plays an important role in uptake of apoptotic neutrophils by human macrophages.

Recently, work by Hoebe and colleagues has shown that CD36 can act in combination with Toll-like receptors 2 and 6 (TLR2/6) as a sensor of microbial diacylglycerides (Hoebe, Georgel et al., 2005). Since CD14 and TLR4 act together to mediate recognition of LPS (Poltorak, Ricciardi-Castagnoli et al., 2000), this presents an interesting possibility that there is a signalling "bridge" between the response to apoptotic cells and the innate immune response to microorganisms.

The LPS receptor, CD14 has also been suggested to be involved in uptake of apoptotic cells (Devitt, Moffatt et al., 1998; Devitt, Parker et al., 2004). The monoclonal antibody 61D3 was found to bind to the surface of human monocyte derived macrophages and inhibited their ability to interact with apoptotic cells. Identification of the epitope recognised by 61D3 implicated a region of the GPI-linked plasma membrane receptor CD14 that is closely associated with the LPS binding region. However, unlike binding of LPS, CD14-mediated binding of apoptotic cells did not result in release of inflammatory cytokines, suggesting that CD14 has the potential to generate both pro- and anti- inflammatory signals. The ligand of CD14 has been suggested to be an altered form of ICAM-3 (Moffatt, Devitt

et al., 1999b). ICAM-3 has been suggested to undergo a conformational change associated with apoptosis that allows recognition by macrophage CD14.

### *3.5 Regulation of phagocytosis of apoptotic cells*

If the rate of cell death by apoptosis is such that macrophage clearance capacity is exceeded, apoptotic cells may become necrotic, resulting in release of harmful cellular contents and damage to the surrounding tissue (figure 1.1). In support of this suggestion, treatment of mice with anti-Fas antibody triggered a massive wave of apoptosis in the liver, and the animals developed extensive hepatic necrosis and died (Ogasawara, Watanabe-Fukunaga et al., 1993). Similarly, induction of apoptosis in the rat lung led to pulmonary fibrosis (Hagimoto, Kuwano et al., 1997). It is likely that in these situations that the hepatic and pulmonary macrophages respectively were unable to clear the load of apoptotic cells with which they were faced. Similar situations could arise if neutrophil apoptosis was to be deliberately induced as part of a therapeutic strategy for reducing neutrophil numbers in inflammatory diseases.

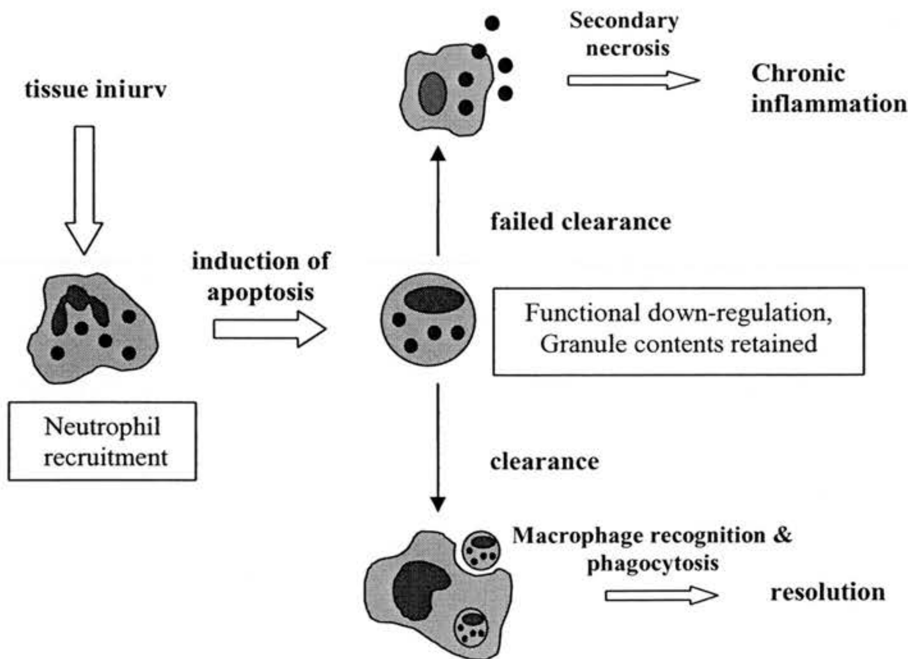



**FIGURE 1.1**
***Neutrophil apoptosis and phagocytic clearance by macrophages in the resolution of inflammation.***

Future strategies for manipulation of inflammatory cell apoptosis in treatment of disease may therefore have to include a way of concurrently augmenting the clearance efficiency of local macrophages.

Macrophage phagocytic capacity in vitro may be influenced by soluble mediators such as cytokines (Ren & Savill, 1995) and prostaglandins (Rossi, McCutcheon et al., 1998), or by glucocorticoid hormones (Giles et al., 2000; Hart et al., 2000; Liu, Cousin et al., 1999). In addition, interaction of surface adhesion molecules with neighbouring cells and extracellular matrix components may profoundly influence many aspects of cellular behaviour, including phagocytosis (Brown, 1986).

Some inflammatory mediators, including prostaglandins, signal through activation of adenylate cyclase and elevation of intracellular cyclic AMP levels. Prostaglandins can influence the immune response in a number of different ways, but prostaglandins $PGE_2$ and $PGD_2$ are capable of inhibiting macrophage phagocytosis of apoptotic neutrophils *in vitro* (Rossi, McCutcheon, Roy, Chilvers, Haslett, & Dransfield, 1998). Moreover, treatment of macrophages with a membrane permeable analogue of cAMP also inhibited the uptake of apoptotic neutrophil (Rossi, McCutcheon, Roy, Chilvers, Haslett, & Dransfield, 1998). Elevated levels of cAMP had no effect on FcγR-mediated phagocytosis, indicating specificity for inhibiting apoptotic cell uptake. Elevation of cAMP concomitantly induced phenotypic alterations in the macrophage, including changes in the localisation of actin and talin into discrete structures. Similar results have been observed when staining for vinculin, paxillin and tyrosine phosphorylated proteins, suggesting that cAMP may uncouple adhesion receptors from cytoplasmic cytoskeletal elements leading to observed morphological alterations.

Corticosteroids are used widely in the treatment of inflammatory diseases and are known to inhibit inflammatory cell recruitment and dampen cellular responsiveness. They also have diverse effects on inflammatory cell apoptosis, promoting apoptosis of thymocytes and eosinophils while inhibiting neutrophil apoptosis (Kato, Takeda et al., 1995). Treatment of human macrophages with glucocorticoids markedly increases their capacity to phagocytose apoptotic neutrophils *in vitro* (Liu et al.,

2000). The effect of glucocorticoids requires several hours of treatment and is inhibited by the protein synthesis blocker cycloheximide (Giles, Ross et al., 2001). Also, phagocytic effects are not restricted to apoptotic target cells of the neutrophil lineage. Human monocytes treated with the glucocorticoid dexamethasone *in vitro* mature into a homogeneous population of macrophages with a typically smaller, more rounded appearance than untreated cells. This is associated with reduced expression of p130Cas (Giles, Ross, Rossi, Hotchin, Haslett, & Dransfield, 2001), an adaptor molecule, which is predicted to bind to Crk/DOCK180 (ced-2/ced-5) complexes that are important for cell adhesion. Disruption of these complexes in combination with reduced phosphorylation and recruitment of paxillin and Pyk2 to sites of adhesion may account for the changes in the cell morphology and phagocytic capacity that accompany steroid hormone treatment (Giles, Ross, Rossi, Hotchin, Haslett, & Dransfield, 2001). Despite the rounded appearance of dexamethasone-treated cells they remain highly membrane active and can be observed rapidly contracting and expanding cellular processes. Increased membrane protrusion may be attributable to increased levels of active GTP-bound Rac in the dexamethasone treated macrophages (Giles, Ross, Rossi, Hotchin, Haslett, & Dransfield, 2001).

Cytokines present at sites of inflammation have the potential to modulate cell behaviour, including phagocytosis of a wide range of targets. A whole host of cytokines including GM-CSF, TGF-β1, IFNγ, IL-1β and TNFα have been shown to increase macrophage uptake of apoptotic neutrophils after four hours' incubation (Ren & Savill, 1995). GM-CSF, IFNγ, IL-1β and TNFα all play important roles in the initiation and amplification of an immune response. Moreover, they are present at sites of inflammation where monocytes mature into macrophages. Therefore, these cytokines may assist in preparing macrophages to clear senescent neutrophils once they undergo apoptosis.

The adhesive state of a macrophage can modulate phagocytic potential. Adhesion of macrophages to fibronectin, vitronectin or collagen VI increased the proportion of macrophages that ingested apoptotic neutrophils (McCutcheon, Hart et al., 1998). It is possible that the binding of a specific repertoire of macrophage adhesion receptors can influence the potential for phagocytosis of apoptotic neutrophils. Thus,

macrophage production and deposition of matrix molecules *in vitro* may play a role in regulating observed levels of phagocytosis of apoptotic cells in experimental systems. Macrophage adhesion to fibronectin may involve CD44, which can be ligated via heparin binding sites. Heparin completely inhibited fibronectin augmentation of macrophage phagocytosis (McCutcheon, Hart, Canning, Ross, Humphries, & Dransfield, 1998). Despite this, treatment of macrophages with a proteolytic fragment of fibronectin, which contained the major heparin binding sites, was unable to augment phagocytosis. This observation suggests that if fibronectin is a ligand for CD44 in this system, generation of a signal for augmented phagocytosis may require binding to additional receptors.

## 4. Structure and function of CD44

The CD44 gene in humans consists of 50-60 kilobase pairs located on the short arm of chromosome 11. The gene is comprised of 20 exons, 10 of which undergo variable splicing, giving rise to changes in the extracellular, membrane proximal region of the CD44 protein (Screaton, Bell et al., 1992; Cooper & Dougherty, 1995) (figure 1.2). The most prevalent isoform of CD44, termed CD44s ("standard")

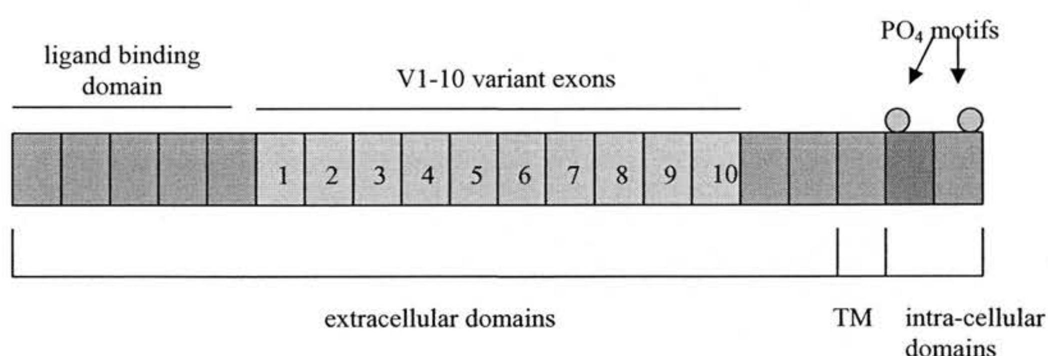



**FIGURE 1.2**
***Schematic representation of the CD44 gene.***
The CD44 gene in humans consists of 20 exons, including 10 variantly spliced exons. It is located on the short arm of chromosome 11.

TM – transmembrane domain.

contains no additionally spliced exons. CD44s consists of a single chain type I transmembrane protein containing 341 amino acids, which due to extensive post-translational glycosylation has a molecular weight of 80-100kD on SDS-PAGE (Camp, Kraus et al., 1991). The expression of these variant isoforms in combination with differential glycosylation alters the range of potential ligands that cells might respond to (Katoh, Zheng et al., 1995; Lesley, English et al., 1995).

The CD44 molecule was first discovered as an antigen that was recognised by a monoclonal antibody against human leukocytes (Picker, Nakache et al., 1989). Further studies indicated that antibodies which were thought to be against three separate distinct antigens were recognising epitopes common to a group of polymorphic proteins (Picker, Nakache, & Butcher, 1989). The Hermes lymphocyte adhesion antigen had previously been proposed to mediate lymphocyte adhesion to

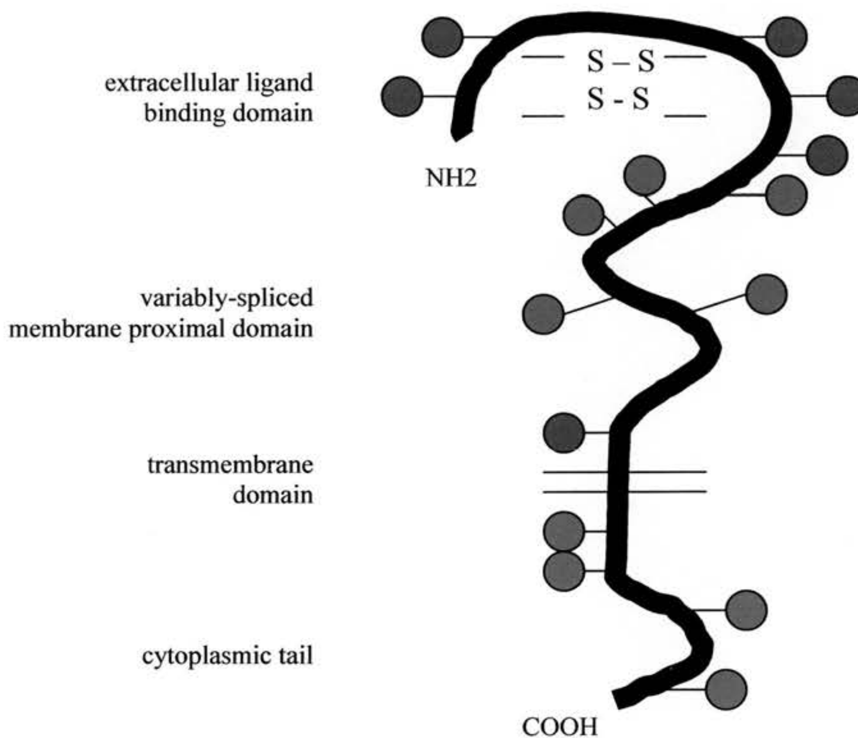



**FIGURE 1.3**
***Diagrammatic representation of CD44***
CD44 consists of a large extracellular ligand-binding domain, an alternatively spliced membrane proximal domain, a highly conserved transmembrane sequence, and a short cytoplasmic tail containing phosphorylation sites (green circles). CD44 undergoes extensive glycosylation indicated by the blue (N-glycosylation) and red (O-glycosylation) circles.

endothelial cells, which line post-capillary venules in lymphoid tissue (Jalkanen, Reichert et al., 1986). Expression of Pgp-1, a polymorphic murine glycoprotein, was shown to be up-regulated on prothymocytes and activated T lymphocytes. Antibodies against the CDw44 cluster recognised a widely distributed human glycoprotein related to the Lutherian blood group antigen that was a marker for medullary thymocytes. The discovery that these proteins were identical was followed by expression cloning of CD44, which revealed that the amino terminal domain of the protein shared homology with cartilage link proteins (Goldstein, Zhou et al., 1989; Stamenkovic, Amiot et al., 1989). The formation of the extracellular matrix as well as many other aspects of cell behaviour relies upon interactions between hyaluronan and hyaluronan-binding proteins (Knudson & Knudson, 1993). In cartilage, link proteins stabilise aggregates of proteoglycan core protein and the linear sugar hyaluronan by binding either the proteoglycan monomer or hyaluronan. These interactions are mediated by a common protein domain called the link module. The link module consists of approximately 100 amino acids and contains four disulphide-bonded cysteine residues. Link modules are present in matrix proteins such as aggrecan, versican and the arthritis-associated protein TNF-stimulated gene 6 (TSG-6) (Day & Prestwich, 2002). Structural analysis of the link domain has shown it to be approximately 100 residues in length, consisting of two alpha helices and two anti-parallel beta sheets, stabilised by two disulphide bridges and bearing structural similarity to C-type lectins(Blundell, Mahoney et al., 2003; Kohda, Morton et al., 1996).

Evidence has suggested that the link domain of CD44 is more complex, involving sequences from outwith the link domain. Constructs comprising of only the link domain of CD44 expressed in E.coli have been shown to fail to refold and are functionally inactive (Banerji, Day et al., 1998). Studies using site-directed mutagenesis suggest that basic amino acid residues flanking the link domain participate in binding of HA (Peach, Hollenbaugh et al., 1993). Recently, elucidation of the 3D crystal structure of the HA binding domain of CD44 by X-ray crystallography and NMR spectroscopy has provided new insights into the function and regulation of CD44 (Teriete, Banerji et al., 2004). The CD44 HA binding

domain was shown to contain a link domain similar to that in TSG-6, however, in the flanking regions an additional four beta strands contribute to the binding domain, forming an additional "structural lobe" in close contact with the link domain. Interestingly, this enlarged binding domain allows for regulation of ligand binding in response to proinflammatory cytokines and other factors which can produce changes in receptor N-glycosylation.

CD44 is expressed by many cell types, including leukocytes, fibroblasts, erythrocytes, endothelial and epithelial cells and a variety of tumour cells (Lesley, Hyman et al., 1993). CD44 has broad ligand specificity and has been proposed to bind the ECM molecules hyaluronan (Aruffo, Stamenkovic et al., 1990;Culty, Miyake et al., 1990), fibronectin (Jalkanen & Jalkanen, 1992), collagen (Faassen, Schrager et al., 1992), and fibrin (Henke, Roongta et al., 1996;Svee, White et al., 1996). In addition, CD44 has also been shown to bind to the extracellular phosphoprotein osteopontin (Weber, Ashkar et al., 1996), CD44 molecules on neighbouring cells (Droll, Dougherty et al., 1995), the proteoglycan serglycin present in lymphocyte and mast cell granules (Toyama-Sorimachi, Sorimachi et al., 1995) and metalloproteinases (Isacke & Yarwood, 2002). Interactions between CD44 and hyaluronan is thought to be important in a number of cell-adhesion related functions including cell adhesion and migration (DeGrendele, Estess et al., 1996;Culty, Miyake, Kincade, Sikorski, Butcher, Underhill, & Silorski, 1990;Milstone, Hough-Monroe et al., 1994), chemokine and cytokine production (McKee, Penno et al., 1996;Rameshwar, Chang et al., 1996;Zembala, Siedlar et al., 1994), lymphocyte proliferation (Pierres, Lipcey et al., 1992) and tumour formation and metastasis (Bartolazzi, Jackson et al., 1995;Price, Coombe et al., 1996).

The function of CD44 isoforms has become of interest due to their role in cell migration, especially in metastasis and invasion of cancer cells. CD44 is expressed as a number of splice variants and one splice variant CD44v4-7 is involved in the metastatic spread of tumour cells (Gunthert, Hofmann et al., 1991). The expression of these splice variants have been proposed as one way of diagnosing and indicating the prognosis of human tumours. However, the relationship between CD44 expression and metastatic potential may be more complex. The expression of variant

isoforms of CD44 in colorectal carcinomas indicates poor prognosis (Wielenga, Heider et al., 1993). In contrast, in neuroblastomas the absence of any CD44 expression signifies metastatic capacity and poor prognosis (Shtivelman & Bishop, 1991). In these cases, it is not the CD44 itself that is mutated but genes controlling the expression of CD44. Alternative splicing is controlled by the Ras-MAP kinase cascade, which are subject to mutation in many cancers (Hofmann, Rudy et al., 1993).

## *4.1 CD44 and hyaluronan binding*

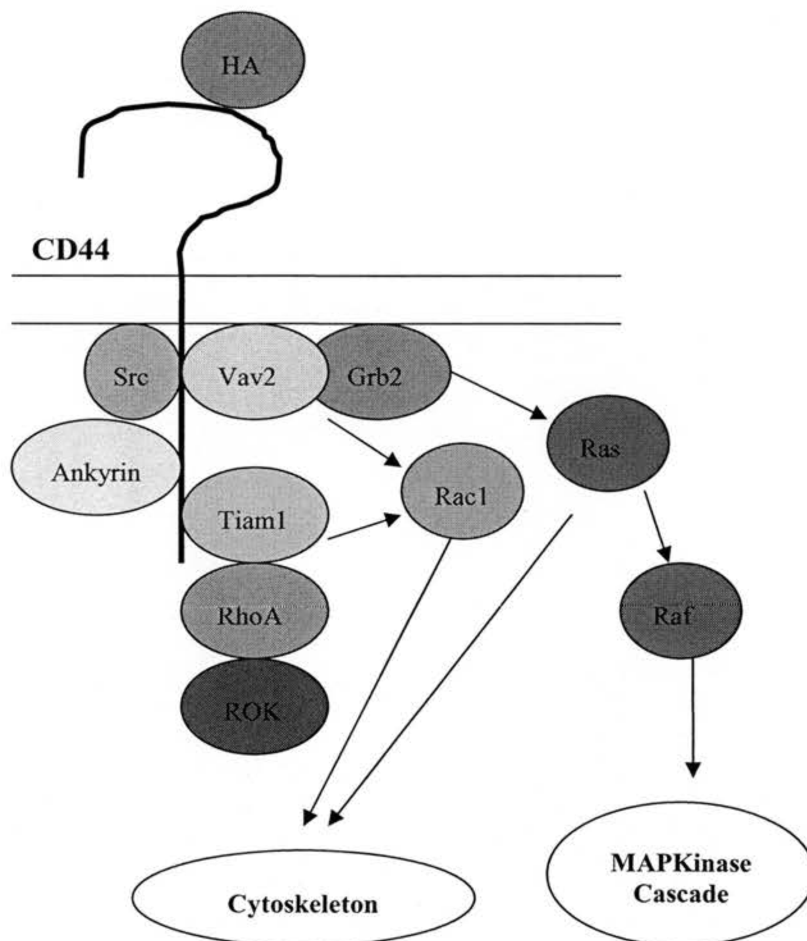



**FIGURE 1.4**
***A model for CD44 specific signalling pathways upon hyaluronan binding.***
CD44-Hyaluronan interactions promote Src phosphorylation leading to recruitment of Vav2 and Tiam1, which in turn activate RhoA and Rac1. Binding of hyaluronan also promotes interaction of CD44 with cytoskeletal proteins such as ankyrin and ERM proteins. Activation of these pathways can influence macrophage function in terms of adhesion, proliferation/growth, migration, phagocytosis and tumour cell migration.

Hyaluronic acid (HA) is an unbranched, non-sulphated glycosaminoglycan that consists of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid (Laurent & Hellstrom, 1992). It can be found as a high molecular weight (500-5000kD) polysaccharide at high concentrations in connective tissue, cartilage, synovial fluid, vitreous humor and umbilical cord (Laurent & Hellstrom, 1992;Toole, 2001). HA binds to a number of receptors, including CD44 (Aruffo, Stamenkovic, Melnick, Underhill, & Seed, 1990), receptor for HA-mediated motility (RHAMM) (Savani, Cao et al., 2001;Hardwick, Hoare et al., 1992) and lymphatic vessel endothelial receptor (LYVE-1) (Banerji, Ni et al., 1999).

Binding of HA to CD44 has been shown to result in altered tyrosine kinase activity within cells (figure 1.4). The cytoplasmic domain of CD44 binds to Src kinase at a single site with high affinity (Bourguignon, Zhu et al., 2001a). HA binding to CD44 stimulates Src kinase activity resulting in tyrosine phosphorylation of cortactin, a cytoskeletal protein which can cross-link filamentous actin (Bourguignon, Zhu, Shao, & Chen, 2001a). The Src kinases Lck and Fyn have been shown to interact with CD44 in lipid rafts present in the cell membrane of human peripheral blood lymphocytes (Bourguignon, Zhu et al., 1999). In tumour cells, binding of HA to CD44 has been shown to stimulate $p185^{HER2}$ tyrosine kinase activity leading to increased cell growth (Bourguignon, Zhu et al., 1997). Rho GTPases have been found to participate in the interaction between cytoskeletal proteins and CD44. Specifically, RhoA has been shown to be linked to a specific CD44 isoform in breast cancer cells (Oliferenko, Kaverina et al., 2000). When this isoform is complexed with RhoA, RhoA stimulates Rho kinase (ROK) leading to increased membrane-cytoskeleton interactions and tumour cell migration during the progression of breast cancers (Oliferenko, Kaverina, Small, & Huber, 2000). Binding of HA to CD44 also results in activation of Rac1 signalling, which can regulate actin assembly influencing membrane ruffling, formation of filopodia, cell motility and cell transformation (Bretscher, 1999;Bourguignon, Zhu et al., 2000). The cytoplasmic domain of CD44 has been proposed to bind guanine nucleotide exchange factors such as Tiam1 and Vav2, which can interact with Rac1 leading to hyaluronan

mediated tumour cell migration (Bourguignon, Zhu, Shao, & Chen, 2000;Bourguignon, Zhu et al., 2001b).

HA binding to CD44 may also result in the cytoplasmic domain interacting with the cytoskeleton membrane linker proteins, ezrin/radixin/moesin (ERM) (Bourguignon, Zhu, Shao, & Chen, 2000). Moreover, the cytoplasmic domain of CD44 can also interact with ankyrin, a membrane-associated cytoskeletal protein. Overexpression of ankyrin promotes hyaluronan-dependent and CD44-specific tumour cell migration, suggesting that CD44 – ankyrin interactions are required for cytoskeletal changes during hyaluronan signalling (Bourguignon, Zhu, Shao, & Chen, 2000).

Studies have also shown that a soluble form of CD44 (sCD44) is detectable in serum, lymph, arthritic synovial fluid and bronchoalveolar lavage (Katoh, McCarthy et al., 1994;Katoh, Taniguchi et al., 1999;Shi, Dennis et al., 2001). Increased levels of sCD44 are often present in malignant disease and inflammatory conditions. These findings suggest that CD44 levels may be a potential indicator for tumour growth, metastasis and inflammation. An alternatively spliced form of CD44, lacking the transmembrane and cytoplasmic domains has been described, providing a mechanism for *de novo* synthesis of sCD44 (Yu & Toole, 1996). However, the major mechanism for production of sCD44 is proteolytic cleavage of cell surface CD44 (Okamoto, Kawano et al., 1999). CD44 has been shown to be constitutively released from cells. Release can be promoted by phorbol esters, the calcium ionophore, ionomycin (DeGrendele, Kosfiszer et al., 1997), cytokines (Ristamaki, Joensuu et al., 1997) and various proteinases (Cichy, Bals et al., 2002;Lazaar, Plotnick et al., 2002). Cytoskeletal rearrangement occurs concurrently with CD44 shedding and pharmacological disruption of actin assembly reduces CD44 shedding. Activation of Rho family GTPases, which regulate actin filament assembly, enhances CD44 cleavage (Shi, Dennis, Peschon, Chandrasekaran, & Mikecz, 2001) and Ras has also been shown to induce shedding of CD44, suggesting that both Ras and Rho GTPases control CD44 shedding through their effects upon the actin cytoskeleton.

Results from pharmacological inhibition studies of endogenous metalloproteinases and serine proteinases have suggested that they can contribute to CD44 shedding

(Okamoto, Kawano, Tsuiki, Sasaki, Nakao, Matsumoto, Suga, Ando, Nakajima, & Saya, 1999). Although, the ADAM (a disintegrin and metalloprotease) family of enzymes may contribute to CD44 receptor shedding, TACE (TNFα converting enzyme) has been shown not to be involved (Shi, Dennis, Peschon, Chandrasekaran, & Mikecz, 2001). Membrane type 1 and membrane type 3 metalloproteinases (MT1-MMP and MT3-MMP) have been shown to play a role in CD44 processing and co-expression of MT1-MMP and MT3-MMP in human breast carcinoma cells was found to result in shedding of CD44 (Kajita, Itoh et al., 2001)

Release of CD44 from the cell surface can possibly have a number of effects. It is likely that sCD44 can compete with cell surface CD44 for ligand binding. sCD44 has been shown to disrupt CD44-HA interactions resulting in apoptosis and inhibition of tumour cell metastasis (Yu, Toole et al., 1997;Yu, Woessner, Jr. et al., 2002). Cleavage of CD44 may also assist in preventing cell - cell or cell - extracellular matrix adhesion. It could also regulate the migratory capacity of the cell, especially in tumour cells.

### *4.2 CD44 in inflammation and disease*

Several different observations have recently implicated CD44 in the regulation of the inflammatory response. Freshly isolated peripheral blood monocytes have been shown to express CD44S strongly (Mackay, Terpe et al., 1994), and differentiation of monocytes into macrophages during in vitro culture is associated with increased expression of variant isoforms while CD44S expression persists at high levels (Culty, O'Mara et al., 1994;Levesque & Haynes, 1996). Elevated local concentrations of CD44 ligands (such as hyaluronan and fibronectin) that follow tissue injury are likely to be important mediators of macrophage function as the inflammatory response progresses. Expression of the variant isoforms (CD44v6 and CD44v9) by monocyte-like THP-1 cells can be up-regulated following exposure to the inflammatory cytokines TNF-α or IFN-γ (Mackay, Terpe, Stauder, Marston, Stark, & Gunthert, 1994), and macrophage expression of some CD44 isoforms is increased at sites of

chronic inflammation (Levesque & Haynes, 1996). *In vivo*, systemic administration of CD44 monoclonal antibodies reduced the severity of joint inflammation in mice with experimental arthritis (Mikecz, Brennan et al., 1995;Verdrengh, Holmdahl et al., 1995;Verdrengh, Holmdahl, & Tarkowski, 1995).

Recent studies involving CD44 "knockout" mice have provided a more direct evidence for the involvement of CD44 in inflammation. Teder et al. demonstrated that CD44-deficient mice exhibited unremitting and often fatal inflammation following bleomycin-induced lung injury compared with wild type animals (Teder, Vandivier et al., 2002). The increased susceptibility to severe pneumonitis could be largely reversed by repopulating the bone marrow of the CD44-deficient mice with CD44+ wild type cells, demonstrating a critical role for CD44 expressed by macrophages or other haematopoietic cells in the resolution of lung inflammation.

### *4.3 CD44 and clearance of apoptotic neutrophils*

Ligation of CD44 on the surface of human macrophages has been shown to increase the capacity for phagocytosis of apoptotic neutrophils (Hart, Dougherty et al., 1997). Augmentation of phagocytosis required cross-linking of CD44 on the macrophage surface, as ligation of CD44 using Fab' fragments resulted in no increase in phagocytosis. Moreover, CD44 cross-linking has no effect upon phagocytosis of fresh neutrophils, apoptotic lymphocytes, IgG opsonised erythrocytes and zymosan, indicating that this augmentation is specific for apoptotic neutrophils. Moreover, the molecular mechanism of CD44 augmented phagocytosis has been shown to be distinct, being independent of integrin ligation, CD36, CD14, scavenger receptors or the PS receptor.

## AIMS

The aim of this thesis is to investigate potential molecular mechanisms for augmentated macrophage phagocytosis of apoptotic neutrophils, following cross-linking of CD44. Initially, CD44 augmented phagocytosis was characterised using flow cytometric phagocytosis assays, time-lapse microscopy and antibody/ligand inhibitor studies. Next, surface expression of CD44 on the macrophage was analysed using immunofluorescence and con-focal microscopy. Finally, the intracellular signalling pathways engaged following CD44 cross-linking would be defined.

# Chapter 2
# MATERIALS & METHODS

## Reagents and Antibodies

All cell culture materials were from Invitrogen (Paisley, UK) and other reagents were from Sigma (Poole, UK) unless otherwise stated. Antibodies used and their suppliers are detailed in table 2.1. All monoclonal antibodies (mAb) were used at saturating concentrations as determined by indirect immunofluorescence techniques and flow cytometric analysis (FACS Calibur, Becton-Dickinson, Oxford, UK).

## Preparation of CD44 F(ab')$_2$ antibody fragments.

CD44 mAb (5A4) was purified from hybridoma culture supernatant using Protein A affinity chromatography as described (Ey, Prowse et al., 1978). Hybridoma culture supernatant was passed through a column of protein A Sepharose microspheres, which was then washed with 0.1M phosphate buffer (pH8.0). To elute the antibody from the column, 0.1M citrate buffer pH4.0 was used. The optical density of each fraction was measured and the fractions with the highest protein content pooled for pepsin digest. Purified antibody underwent pepsin digestion (1% wt/wt) to generate F(ab')$_2$ fragments, for 5-13 hours depending on the antibody concentration. The digest was terminated by raising the pH of the sample to 8 with 1M Tris (pH9.0). Residual intact antibody was removed by three rounds of depletion using protein A agarose affinity resin (Sigma, Poole, UK) and the resulting F(ab')$_2$ fragments were analysed using SDS-PAGE under non-reducing conditions and visualised using GelCode Blue (Pierce), according to manufacturer's instructions, to confirm purity. Indirect immunofluorescence analysis together with flow cytometry was used to confirm that preparation of antibody fragments did not compromise binding and that antibody fragments were used at saturating concentrations in other assays.

| Antibody | Source |
| --- | --- |
| 5A4 (pan-CD44; supernatant) | Dr Dougherty, San Francisco, USA |
| C-11 (Rac2), rabbit polyclonal | Santa Cruz Biotechnology Inc |
| IV3 (CD32), mouse IgG2b | Medarex Inc, NJ, USA |
| DFT-1 (CD43), mouse IgG1 | Serotec, Oxford, UK |
| CD106 (VCAM), mouse IgG1 | Caltag Laboratories, CA, USA |
| panPKC, mouse IgG2a | BD Pharmingen |
| Goat anti-mouse IgG (Fc specific) | Sigma-Aldrich |
| Biotinylated goat anti-mouse Ig | Amersham Biosciences, St Albans, UK |
| ezrin, mouse IgG1 | BD Pharmingen |
| p44/42 MAPK, rabbit polyclonal | Cell Signalling Technology |
| Phospho-p44/42 MAPK, rabbit polyclonal | Cell Signalling Technology |
| IVC7 (CD36), mouse IgG1 | CLB, Amsterdam |
| 61D3 (CD14, ascites), mouse IgG1 | Dr Devitt, Edinburgh, UK |
| 52U (ascites control), mouse IgG1 | Dr Devitt, Edinburgh, UK |
| talin, mouse IgG1 | Serotec, Oxford, UK |
| vinculin, mouse IgG1 | Serotec, Oxford, UK |
| HRP-conjugated goat anti-rabbit Ig | Dako Cytomation |
| HRP-conjugated goat anti-mouse Ig | Dako Cytomation |

**TABLE 2.1**
*Antibodies used in this thesis*

## Cell Isolation

Mononuclear cells and neutrophils were isolated from human peripheral blood by dextran sedimentation and discontinuous Percoll™ (Amersham Pharmacia, St. Albans, UK) gradient centrifugation as described (Dransfield, Buckle et al., 1994). After removal of platelet-rich plasma by centrifugation at 220g, erythrocytes were sedimented using 6% Dextran in 0.9% NaCl (Amersham Pharmacia, 2.5ml/10ml cell pellet, made up to 50ml using saline) for 20min at room temperature. The leukocyte rich upper layer was then removed and centrifuged at 220g for 6 minutes. The resulting cell pellet was resuspended in 55% Percoll (90% in PBS without $Ca^{2+}/Mg^{2+}$) and layered gently onto a gradient of 68% and 81% Percoll. After centrifugation at 220g for 6 minutes, mononuclear cells and neutrophils were harvested (mononuclear cells from the 55/68 interface and neutrophils from the 68/81 interface), washed and counted. Mononuclear cells were suspended at 4 x $10^6$/ml in Iscove's Modified Dulbecco's Medium (Gibco Brl), supplemented with 50U/ml Penicillin and 50U/ml Streptomycin (Gibco), and allowed to adhere to cell culture plates (Costar, Corning Inc, NY, USA) during incubation at 37°C for 1 hour. Non-adherent cells (predominantly lymphocytes) were removed and adherent cells

washed three times in Hanks balanced salt solution without $Ca^{2+}/Mg^{2+}$ (HBSS). Adherent monocytes (approximately 90% CD14+) were then cultured in IMDM containing 10% autologous serum for 5 to 7 days. For microscopy based experiments, monocytes were cultured adherent to 13mm glass coverslips in 24 well plates.

For serum free experiments, monocytes were cultured in Macrophage SFM-Medium (Gibco Brl, supplemented with 50U/ml penicillin and 50U/ml streptomycin) supplemented with 250U/ml GM-CSF (R&D) for three days, washed with HBSS without $Ca^{2+}/Mg^{2+}$ and cultured for a further two days in IMDM.

Neutrophils were incubated at 2 x $10^7$/ml with 22μM CM-carboxyfluorescein diacetate (CFDA, Molecular Probes, Oregon, USA) at 37°C for 15 minutes. Labelled neutrophils were then cultured at 4 x $10^6$/ml in IMDM with 10% autologous serum for 24 hours during which time they underwent spontaneous apoptosis (Savill, Wyllie et al., 1989). Apoptosis was assessed by microscopic inspection of cytocentrifuge preparations that has been stained with Diff-Quik™ (Baxter Healthcare, Glasgow, UK) as per manufacturer's protocol.

### Preparation of human autologous serum

Platelet-rich plasma (PRP) was separated from human peripheral blood by centrifugation at 350g for 20 minutes. To prepare serum, 10ml PRP was incubated with 220μl of 1M $CaCl_2$ in glass tubes for 30min at 37°C.

### Assessment of macrophage phagocytosis by flow cytometry

Monocyte-derived macrophages were washed with HBSS without $Ca^{2+}/Mg^{2+}$ and pre-incubated with IMDM alone or CD44 antibody 5A4 (supernatant; diluted 1:5 in IMDM) for 15 minutes at 37°C. Excess mAb was removed and monocyte-derived macrophages washed with HBSS without $Ca^{2+}/Mg^{2+}$ prior to adding 2 x $10^6$ CM-CFDA-labelled apoptotic neutrophils in 0.5ml of either IMDM or (25mM and pH

7.0) Hepes Buffered Saline (divalent cation-free) to each well for 60 minutes at 37°C. At the end of the assay, a solution of 0.25%Trypsin/1mM EDTA was added to each well and the cells incubated at 37°C for 15 minutes to detach all monocyte-derived macrophages and remove non-internalised neutrophils. After 15 minutes incubation on ice, the proportion of macrophages that exhibited increased fluorescence (corresponding to phagocytosis of fluorescently labelled apoptotic neutrophils) was determined by flow cytometry using a FACSCaliber analyser with post-acquisition data analysis performed using CellQuest software (Becton-Dickinson).

In the cation depletion phagocytosis assay, 2 x $10^6$ CM-CFDA-labelled apoptotic neutrophils in 25mM Hepes-buffered Saline supplemented with 1 mM calcium chloride, 1mM magnesium chloride or 250μM manganese chloride were used.

In the CD32 inhibition studies, either macrophages or apoptotic neutrophils were incubated with 10μg/ml IV3 mAb for 15min at 37°C, prior to carrying out the phagocytosis assay as described above.

### Assessment of macrophage phagocytosis by plate assay

5 day cultured monocyte-derived macrophages were gently washed with HBSS without $Ca^{2+}/Mg^{2+}$. The media was then replaced with 1ml of unlabelled overnight "aged" neutrophils and the plate incubated at 37°C for 1 hour. The cells were then washed three times with PBS without $Ca^{2+}/Mg^{2+}$ to remove all bound, non-ingested neutrophils. The cells were then fixed in 2.5 % gluteraldehyde for 20 minutes at room temperature. The cells were then washed as before to remove the fixative. The neutrophils were then stained for myeloperoxidase activity with 0.1mg/ml dimethoxybenzidene and 0.03% (v/v) hydrogen peroxidase in PBS for 20 minutes.

### The effects of antibody/ligand inhibitors upon phagocytosis

Human monocyte-derived macrophages were washed with HBSS without $Ca^{2+}/Mg^{2+}$ and incubated with mAb 5A4 (1:5 in IMDM) for 15min at 37°C. Unbound antibody

was washed off and the cells treated either with 1mg/ml fucoidan (Sigma, Poole, UK), 50U/ml heparin (provided by Dr M. McElroy, Department of Pathology, University of Edinburgh, UK), 10μg/ml IV3 (CD32; Medarex, Annandale, NJ, USA), 1μg/ml IVC7 (anti-CD36; CLB, Amsterdam), 1mg/ml 61D3 (anti-CD14 provided by Dr A. Devitt, Centre for Inflammation Research, University of Edinburgh, UK), 1mg/ml 52U (IgG1 ascites control) or IMDM alone, for 15min at 37°C. The cells were then washed with HBSS without $Ca^{2+}/Mg^{2+}$ and 2 $x10^6$ aged neutrophils labelled with CM-Green in 0.5ml HBS were added to each well. After incubation for 60min at 37°C the medium was removed and 0.25%Trypsin/1mM EDTA added to each well. The cells were incubated for 15min at 37°C and then on ice for a further 15min. The percentage of macrophage phagocytosis was then assessed using the flow cytometric assay described above.

### Preparation of macrophage lysates

Freshly isolated human monocytes were cultured adherent to six well culture plates (Costar, Corning Inc) for the time indicated in individual experiments. Mature macrophages were washed with HBSS without $Ca^{2+}/Mg^{2+}$, incubated with either 5A4 or a control antibody diluted in IMDM at 37°C for 15min, and then washed again. For western blotting, macrophages were lysed with 200μl/well 140mM NaCl/20mM Tris pH 7.6 containing 1% Triton X-100, protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitors (1mM sodium orthovanadate, 20mM glycerophosphate). The lysates were incubated on ice for 20min, the macrophage monolayer scraped with a plastic cell scraper and the lysates aspirated into 1.5ml Eppendorf centrifuge tubes. To remove detergent-insoluble material, the samples underwent centrifugation at 10000g for 15min at 4°C, the supernatants were removed and stored at -80°C prior to use.

### Western blotting for p44/p42 MAPK (Erk1/2) activity

Macrophage lysates were run on a non-reducing 12% SDS-polyacrylamide gel. The proteins were blotted (80v for 1 hour) onto a nitrocellulose membrane (Amersham

Pharmacia), which was then blocked for 1 hour in PBS containing 0.1% Tween-20. The membrane was incubated overnight with either 1:1000 rabbit anti-human p44/42 MAPK in Tris buffered saline with 0.1% Tween-20 and 5% nonfat dry milk or 1:1000 goat anti-human phospho-p44/42 MAPK in TBS with 0.1% Tween-20. The membrane was then washed with TBS/Tween and probed for 1 hour with either 1:2500 HRP-conjugated goat anti-rabbit immunoglobulin (Dako) in TBS/Tween to detect whole ERK or 1:2500 HRP-conjugated goat anti-mouse immunoglobulin (Dako) in TBS/Tween to detect phosphorylated ERK. The blot was washed with TBS/Tween and developed using ECL+ (Amersham Biosciences) according to manufacturer's instructions (Kodak Biomax Light Film, Sigma-Aldrich).

### Inhibition of p44/42 MAPK (Erk1/2)

To inhibit activation of p44/42 MAPK, macrophages were washed and pre-treated with 50μM PD98059 (Calbiochem) in IMDM for 15min at 37°C prior to making lysates or using them in phagocytosis and migration assays.

### Time-lapse microscopy migration assay

$4x10^6$/ml freshly isolated human monocytes were adhered to slide flasks (Nunc) for 1 hour, washed with HBSS without $Ca^{2+}/Mg^{2+}$, and cultured in IMDM with 10% autologous serum for 5 days at 37°C. Macrophages were washed as before, incubated with either whole 5A4, 5A4 $F(ab')_2$ fragments or IMDM alone for 15min at 37°C, washed and incubated in IMDM with 10% autologous serum for the duration of the experiment. Macrophage monolayers were wounded using a pastette. The migration of macrophages back into the wound was examined by phase-contrast microscopy with an image captured every 5 minutes for 20 hours. The resulting images were played back at 6 frames/second and made into movies using QuickTime™ Player.

Data was analysed by overlaying the initial image at time 0 with an acetate sheet in order to define the "wound". Subsequent images were then examined and the number of cells within the wound recorded.

### Immunofluorescence microscopy of CD44 surface expression

Human monocyte-derived macrophages, grown adherent to 13 mmcoverslips, were washed gently with HBSS without $Ca^{2+}/Mg^{2+}$, and incubated with human heat-inactivated serum (heat-inactivated at 65 °C for 10min, 1:10 in IMDM,) for 10min at 37°C. After washing as before, macrophages were incubated with 5A4 (1:5 in IMDM) for 15min at 37°C. Macrophages were fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature, washed and permeabilised with 0.1% Triton X-100 in PBS without $Ca^{2+}/Mg^{2+}$ for 4min at room temperature. Macrophages were washed and stained with goat anti-mouse Alexa 488 (1:200; Molecular Probes, Oregon, USA) for 30min on ice in the dark. Coverslips were washed as before and mounted on microscope slides with Movial mounting medium.

For the CD44 expression time-course experiments, macrophages were incubated for differing lengths of time, after stimulation with 5A4 antibody, at both 4°C and 37°C before washing and fixing.

### Antibody biotinylation

5A4 $F(ab')_2$ fragments or goat anti-mouse immunoglobulin (Fc specific, Sigma-Aldrich) was dialysed into PBS without $Ca^{2+}/Mg^{2+}$ overnight. Biotinylation was carried out using EZ-Link™ Sulfo-NHS-Biotinylation Kit (Pierce) according to the manufacturer's instructions.

### Dot blotting

Dot blotting was used to assess if biotinylation of 5A4 F(ab)$_2$ fragments had been successful. 3µl dots of antibody at 1mg/ml or PBS were applied to a nitrocellulose membrane with a pipette tip and allowed to dry. The membrane was blocked for 30min in PBS/0.1% Tween-20 at room temperature and incubated with HRP-conjugated goat anti-mouse immunoglobulin (1:3000 in PBS/0.1% Tween-20) for 30min. The membrane was washed with PBS/0.1% Tween-20 and the blot developed using 0.1mg/ml 3,3-Diaminobenzidine (DAB; Sigma-Aldrich) and 0.05% $H_2O_2$.

### Preparation of antibody conjugated microspheres

Streptavidin-conjugated latex microspheres (6µm; Polysciences Inc, PA, USA) at 1x10$^8$/ml were incubated with either biotinylated 5A4 F(ab')$_2$ fragments, anti-CD106 (VCAM) F(ab')$_2$ fragments or IgG1 control (all at 1mg/ml) for 30min at 37°C with constant mixing. The antibody conjugated microspheres were washed, centrifuged and the resulting supernatant discarded twice to remove any residual, unbound antibody.

### Analysis of microsphere binding by flow cytometry

Monocyte-derived macrophages were washed, incubated with 5mM EDTA, 0.1% BSA in PBS without $Ca^{2+}/Mg^{2+}$ for 30min on ice. Macrophages were then removed from culture plates by vigorous washing and resuspended at 1x10$^7$/ml in flow buffer (0.2% BSA, 0.1% sodium azide in PBS without $Ca^{2+}/Mg^{2+}$). To block non-specific binding, macrophages were incubated with normal rabbit immunoglobulin (1:10, Dako) for 5min on ice. 6µm fluorescent microspheres conjugated to either 5A4 F(ab')$_2$ fragments or IgG1 control were incubated with macrophages at a ratio of 10 microspheres to every macrophage for 30min at 37°C. The level of microsphere binding to macrophages was assessed using an Epics Coulter analyser with Expo 32 software (Beckman Coulter).

EX BIBL. UNIV. EDINBURGH

### Analysis of microsphere binding by phase-contrast microscopy

Antibody conjugated microspheres were prepared as described above. Macrophages adherent to glass cover-slips were washed with PBS without $Ca^{2+}/Mg^{2+}$, and incubated with IMDM containing 1:10 human heat-inactivated serum to block non-specific microsphere binding. 500μl of microspheres either alone or conjugated to 5A4 $F(ab')_2$ fragments or IgG1 negative control were incubated with macrophages for 30min at 37°C. After washing to remove unbound microspheres, the cover-slips were mounted on microscope slides using Movial mounting medium. The slides were then analysed and photographed using a Zeiss Axiovert 5100 fluorescent microscope and OpenLab 3.0 software.

### Preparation of Fc specific goat anti-mouse immunoglobulin secondary antibody

100μl of 2.5mg/ml biotinylated goat-anti mouse immunoglobulin (Fc specific) and 100μl of 1mg/ml streptavidin-conjugated Alexa Fluor 488 were incubated for 15min at 37°C with constant mixing. The resulting conjugate was used at 1:150.

### Imaging cytoskeletal changes and signalling events after CD44 antibody binding

Macrophages grown adherent to cover-slips were washed with PBS without $Ca^{2+}/Mg^{2+}$ and incubated with IMDM containing human heat-inactivated serum (1:10). 50μl of microspheres conjugated to either VCAM-1 $F(ab')_2$ fragments or 5A4 $F(ab')_2$ fragments were incubated with the macrophages for 30min at room temperature. After washing, the macrophages were fixed in PBS without $Ca^{2+}/Mg^{2+}$ containing 0.1% paraformaldehyde for 20min at room temperature. The macrophages were then washed and used immediately or kept at 4°C for later use. Fixed macrophages were permeabilised with 0.1% Triton X-100 in PBS without $Ca^{2+}/Mg^{2+}$ for 4min at room temperature, washed and incubated with rhodamine

phalloidin (1:500) or phalloidin-alexa fluor 488 (1:200; Molecular Probes) or antibodies against either ezrin (1:50; mouse anti-human; BD Pharmingen, ), Rac2 (1:50; rabbit anti-human; Santa Cruz Biotechnology Inc), panPKC (1:50; mouse anti-human mAb), vinculin (1:50; Serotec), talin (1:50; Serotec) or mouse IgG1 negative control (1:50; Dako) for 30min on ice. Macrophages were washed and incubated with the appropriate secondary antibody for 30min on ice – goat anti-rabbit immunoglobulin Alexa fluor 488 (1:200; Molecular Probes) for Rac2 primary and goat anti-mouse immunoglobulin Fc specific Alexa fluor 488 (1:200; Molecular Probes) for all others. Macrophages were washed and the cover-slips mounted on microscope slides with Movial mounting medium. Macrophages stained for actin required no secondary and were just washed and mounted on microscope slides after primary antibody staining.

### Statistical Analysis

Statistical analysis of flow cytometry results was carried out using GraphPad InStat™ software. To compare samples the Student-Newman-Keuls Multiple Comparisons Test was used.

# Chapter 3
# CHARACTERISATION OF CD44-AUGMENTED PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS

## INTRODUCTION

Apoptosis of neutrophils and their subsequent phagocytic clearance by macrophages represents an essential process in the resolution of inflammation. However, if the rate of apoptosis within the inflamed tissue becomes so high that the capacity for phagocytic clearance becomes overwhelmed, apoptotic cells eventually undergo secondary necrosis, resulting in the release of cytotoxic cellular contents and damage to the surrounding tissue (Haslett, Savill et al., 1994). It has been shown that inadequate clearance of apoptotic cells and the resulting neutrophil-mediated tissue damage can contribute to exacerbation of the inflammatory response and lead to chronic inflammatory diseases. For example, mice exposed to anti-Fas antibodies have been shown to develop pulmonary fibrosis due to Fas - Fas ligand interactions leading to apoptosis of bronchial and alveolar epithelial cells (Hagimoto, Kuwano, Miyazaki, Kunitake, Fujita, Kawasaki, Kaneko, & Hara, 1997). Moreover, lung tissue from patients with fibrosing lung disease showed upregulation of Fas ligand expression in comparison to normal lung tissue (Kuwano, Hagimoto et al., 1999). Similar observations have been recorded from patients who have ulcerative colitis (Yukawa, Iizuka et al., 2002). Colonic mucosal specimens from patients with ulcerative colitis also had elevated levels of Fas ligand expression (Ueyama, Kiyohara et al., 1998). Moreover, neutrophils from patients with inflammatory bowel diseases have been found to undergo apoptosis more slowly than neutrophils from other patients (Brannigan, O'Connell et al., 2000) suggesting that the whole machinery of apoptosis and subsequent clearance is disrupted in these patients. The fibrosis and tissue injury observed in these experimental studies possibly resulted from the failure of tissue phagocytes to clear the level of apoptotic cells present within the tissues. Therefore, by understanding the physiological mechanisms that regulate phagocytic clearance capacity for apoptotic cells, it may be possible to

augment phagocyte capacity for clearance of apoptotic cells and prevent tissue load of apoptotic cells becoming excessive. Furthermore, induction of inflammatory cell apoptosis combined with enhancement of macrophage clearance of apoptotic cells may represent an effective therapeutic strategy for the treatment of human inflammatory disease.

The phagocytic capacity of a macrophage can be influenced by soluble mediators such as cytokines (Ren & Savill, 1995), prostaglandins (Rossi, McCutcheon, Roy, Chilvers, Haslett, & Dransfield, 1998), and steroid hormones (LIU, 1999 170 /id). Moreover, cellular interactions with the ECM can modulate many aspects of cell behaviour, through ligation of adhesion receptors and transduction of an intracellular signal (Brown, 1986). Adhesion of macrophages to the ECM component fibronectin has been shown to significantly augment their capacity for phagocytosis of apoptotic neutrophils (McCutcheon, Hart, Canning, Ross, Humphries, & Dransfield, 1998). In view of the finding that $\beta_1$ integrins only partially accounted for observed augmentation, we examined the role of CD44 was examined, as this receptor has also been reported to recognise fibronectin (Jalkanen & Jalkanen, 1992). Ligation of CD44 on the macrophage surface using antibodies was found to strongly promote the capacity for phagocytosis of apoptotic neutrophils (Hart, Dougherty, Haslett, & Dransfield, 1997).

CD44 augmented phagocytosis was found to be specific for apoptotic neutrophils as CD44 ligation has no effect upon phagocytosis of fresh neutrophils, apoptotic lymphocytes, IgG opsonised erythrocytes and zymosan. This observation suggests that CD44 augmented phagocytosis could potentially represent a specific mechanism for promotion of clearance of apoptotic neutrophils during inflammation. The molecular basis for CD44 augmented phagocytosis of apoptotic neutrophils was suggested to be distinct, being independent of integrin ligation, CD36, CD14, scavenger receptors or the PS receptor.

This chapter describes experimental approaches to further characterise the mechanism of antibody-mediated CD44 ligation on macrophage phagocytosis of apoptotic neutrophils.

## RESULTS & DISCUSSION

### Time-course of CD44 augmented phagocytosis.

I sought to determine whether CD44 antibody treatment of monocyte-derived macrophages initiated intracellular signals that resulted in augmented phagocytosis through indirect clustering of associated signalling molecules. Alternatively, CD44 antibody might cause membrane receptor redistribution, altered mobility of receptors in the plasma membrane, or "patching" of receptors to reveal pro-phagocytic receptors that are normally hidden by receptors such as CD44, which would be predicted to adopt an extended conformation.

By examining the duration of the increase in phagocytosis promoted by CD44 antibody, I hoped to gain insight to the nature of the mechanism of the response. A signalling response would generally be predicted to be very rapid. For example the activation of Rac2 in human neutrophils in response to f-Met-Leu-Phe (fMLP) and Leukotriene-B4 (LTB4) is both rapid and transient (Akasaki, Koga et al., 1999). However, signalling events may result in transcriptional activation that results in expression of new proteins, which result in acquisition of a more phagocytic phenotype. Responses which involve redistribution of molecules within the cell membrane might be expected to be slower and longer-lived. First, I investigated CD44 antibody mediated augmentation of phagocytosis of apoptotic neutrophils over time, monocyte-derived macrophages were treated with 5A4 for 15min and incubated with fluorescently labelled apoptotic neutrophils for 30, 60, 90 and 120 min. These experiments aimed to test whether CD44 mAb treatment simply acted to accelerate the phagocytic process. Phagocytosis was stopped with trypsin/EDTA and

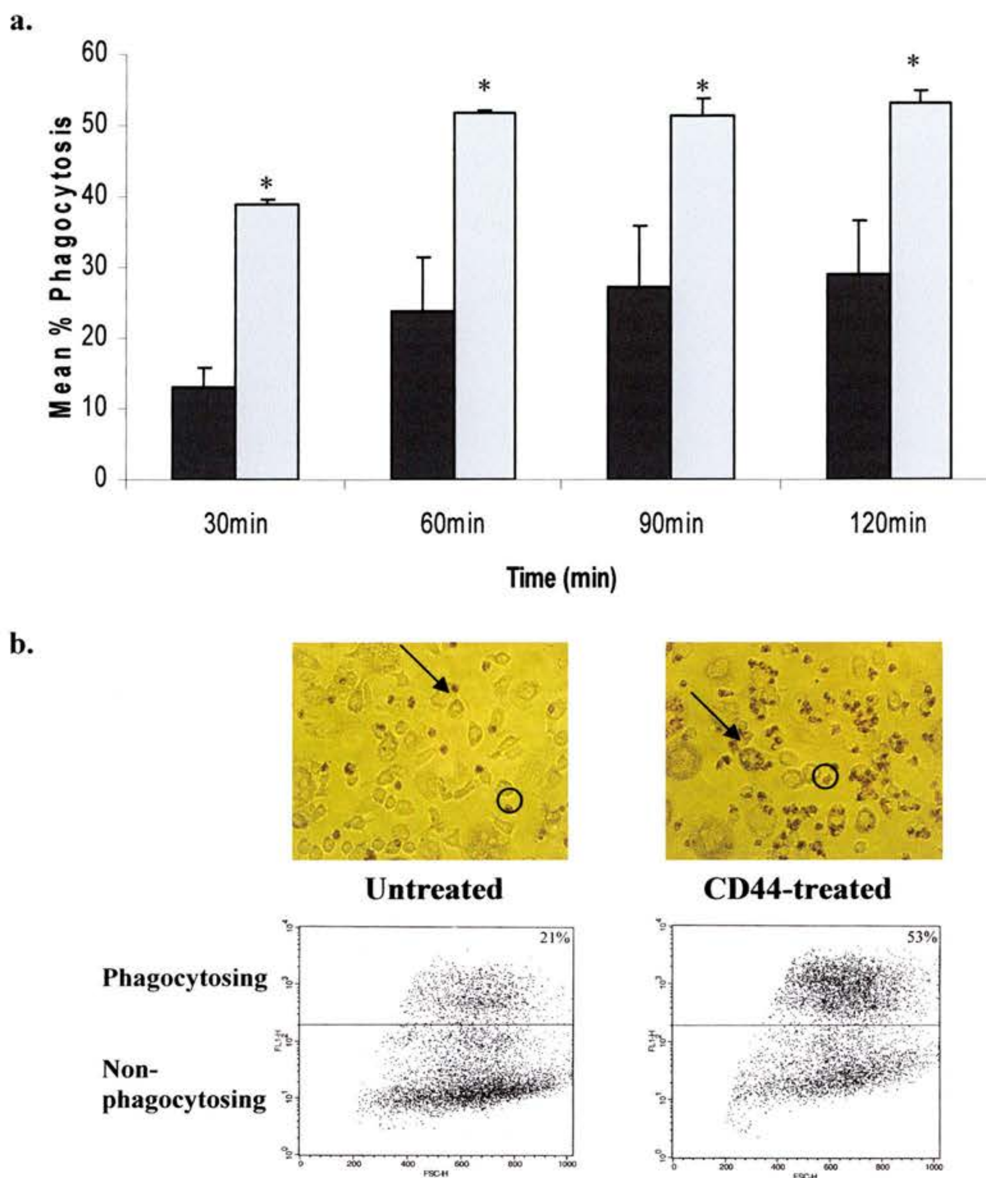



**FIGURE 3.1**

***The effect of CD44 stimulation on macrophage phagocytosis of apoptotic neutrophils over 120 minutes.***

a. Macrophages were incubated with IMDM alone (black bars) or 5A4 (anti-CD44, grey bars) for 15min, washed, and incubated with a suspension of aged neutrophils for the length of time indicated. After Trypsin/EDTA treatment, the percentage of macrophages that had undergone phagocytosis was analysed using a flow cytometric assay. ($n=3$, $P<0.05$)

b. Comparison of untreated and CD44 treated macrophages after 60 minutes incubation with apoptotic neutrophils using flow cytometric and microscopic analysis. Black circle indicates a non-injested neutrophil. Black arrow indicates phagocytosis.

the proportion of fluorescent (phagocytic) macrophages was analysed by flow cytometry (figure 3.1).

These experiments revealed that for untreated macrophages, phagocytosis was essentially maximal after 1 hour (figure 3.1). Although there was a trend to higher proportions of phagocytic macrophages at longer time points, these failed to reach significance. Interestingly, CD44 mAb treatment augmented phagocytosis at all time points examined, with greatest effect observed at 60 minutes. This observation suggested that CD44 mAb treatment "enabled" a previously unresponsive population of macrophages for apoptotic cell phagocytosis, since untreated macrophage phagocytosis failed to approach the levels seen following CD44 treatment even with prolonged assay times (120min still less than 30min CD44). To be certain that I was examining optimal effects of CD44 mAb treatment, I therefore studied 60 minute time points in subsequent experiments.

### Time-course of CD44 cross-linking effect

Previous studies had shown that cross-linking of CD44 was essential for augmented phagocytosis. Ligation of CD44 with whole antibody and $F(ab')_2$ fragments resulted in augmentation of macrophage phagocytosis of apoptotic neutrophils, whereas

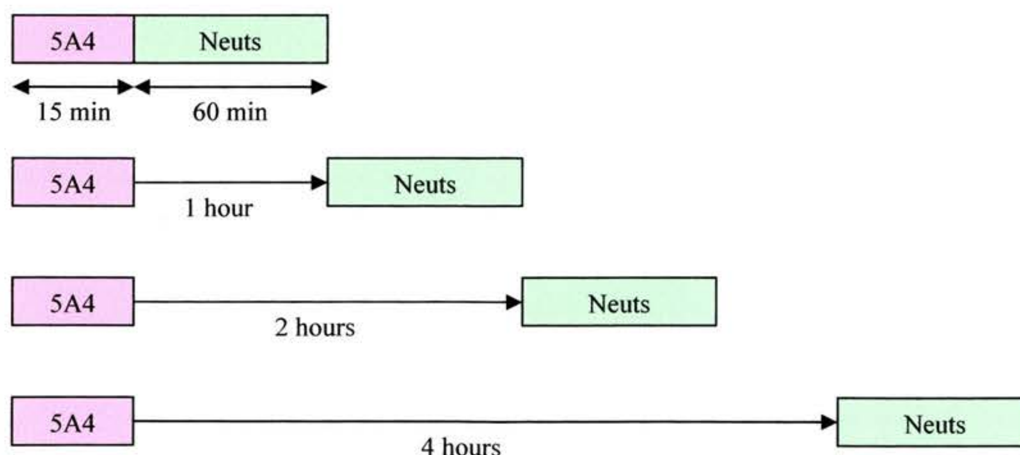



**FIGURE 3.2**
***Schematic representation of the CD44 cross-linking experiments***

stimulation with Fab' fragments failed to augment macrophage phagocytosis at all (Hart, Dougherty, Haslett, & Dransfield, 1997). I therefore decided to investigate how long this cross-linking effect persisted in terms of its effects on the levels of apoptotic cell phagocytosis. To determine how long the augmentation of macrophage phagocytosis lasts after CD44 mAb mediated cross linking, macrophages were incubated with CD44 mAb 5A4 for 15min, the antibody washed off and the macrophages incubated for a further 0hr, 1hr, 2hr and 4hr before addition of apoptotic neutrophils and assessment of phagocytosis using a 60 minute assay (figure 3.2).

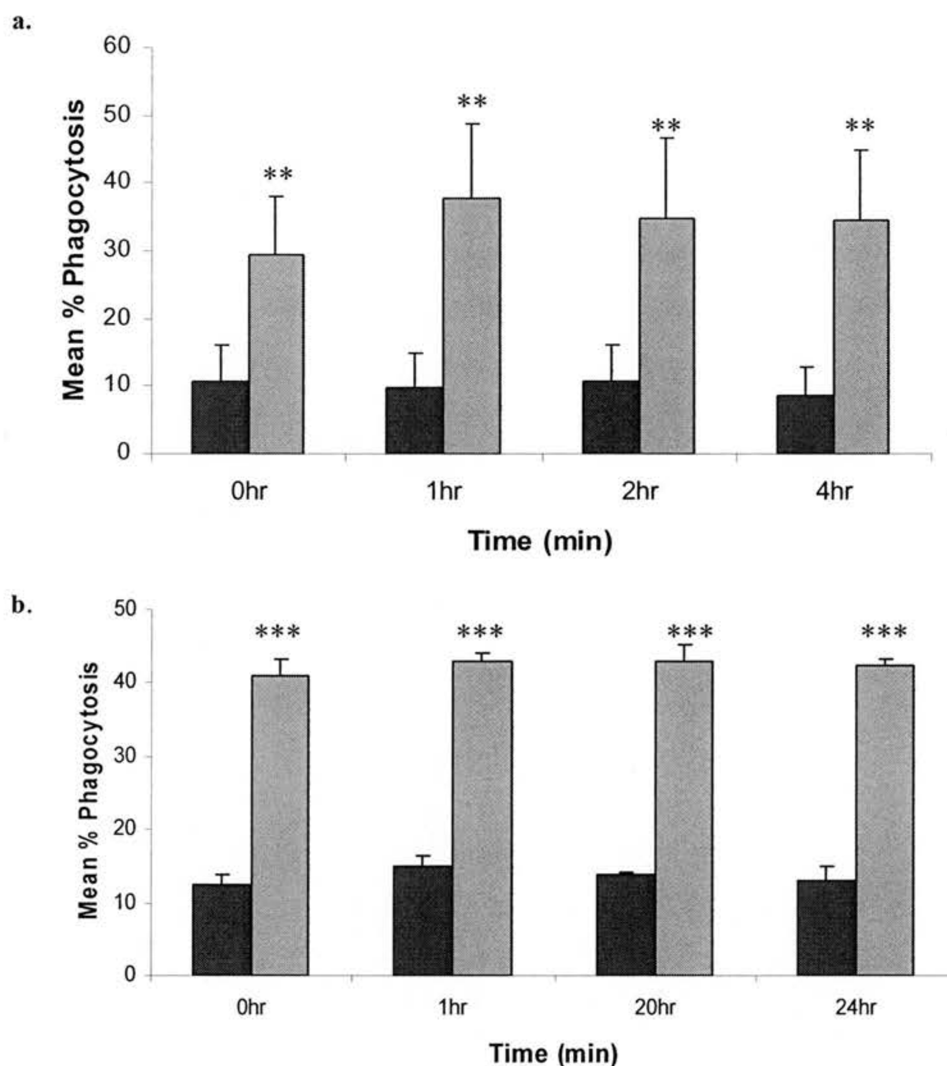



**FIGURE 3.3**

***The effect of cross-linking CD44 for varying lengths of time on macrophage phagocytosis of apoptotic neutrophils.***

Macrophages were incubated with either IMDM alone (black bars) or 5A4 (grey bars) for 15min, washed,and incubated for the length of time indicated above, before incubation with a suspension of aged neutrophils for 60min. After treatment with trypsin/EDTA, the percentage of macrophage phagocytosis was determined using a flow cytometric assay. ($n=3$, $P<0.01$)

Surprisingly, CD44 mAb treated monocyte derived macrophages exhibited augmented phagocytosis of apoptotic neutrophils even after several hours, suggesting that the effects of CD44 mAb cross-linking were not transient (figure 3.3a). In this regard, the effect of CD44 mAb contrasted chemokine induced activation of intercellular adhesion (e.g. neutrophil/endothelial cell interaction or T cell/antigen presenting cell), which has been shown to be transient (Chan Hyduk & Cybulsky,

2003). Thus, the effects of CD44 mAb may not be readily reversed and this contrasts with many other signalling events which exhibit reversibility.

To further explore this observation, I decided to investigate the cross-linking effect of CD44 over a longer period of time. Macrophages were treated with 5A4 for 15 min as before and incubated for 0hr, 1hr, 20hr and 24hr before assessing phagocytosis of apoptotic neutrophils. Cross-linking of CD44 resulted in augmented phagocytosis for up to 24 hours after mAb addition and thus potentially may be irreversible (figure 3.3b). Furthermore, it is notable that the percentage of phagocytosis in CD44 mAb treated macrophages does not significantly differ from that observed within 1 hour of phagocytosis, indicating that whatever the mechanism which underlies augmentation of phagocytosis by CD44 mAb it continues to influence the treated macrophages behaviour for a considerably longer time.

These results suggest that CD44 mAb binding to the macrophage leads to a long lasting change in macrophage phagocytic function. These data may indicate that a signalling event within the cell is unlikely to mediate the observed effect, unless CD44 cross-linking is associated with an essentially irreversible change in regulatory pathways.

### CD44-augmented phagocytosis in divalent cation deplete conditions

Previous experiments using blocking antibodies and competitive ligands had failed to identify a molecular basis for the observed augmentation of phagocytosis following CD44 mAb treatment of monocyte derived macrophages. Since a number a important cell-cell adhesion molecules are dependent upon divalent cations for function (including integrins and C-type lectin receptors), removal of these divalent cations provides a means to discriminate possible receptor pathways involved in CD44 augmentation of macrophage phagocytosis of apoptotic neutrophils. To investigate the effect of cation depletion upon CD44-augmented phagocytosis, macrophages were co-incubated with apoptotic neutrophils in Hepes buffered saline (HBS) or HBS supplemented with calcium, magnesium or manganese. The

percentage of macrophage phagocytosis for both unstimulated and CD44 stimulated macrophages were then compared using flow cytometry as before (figure 3.4).

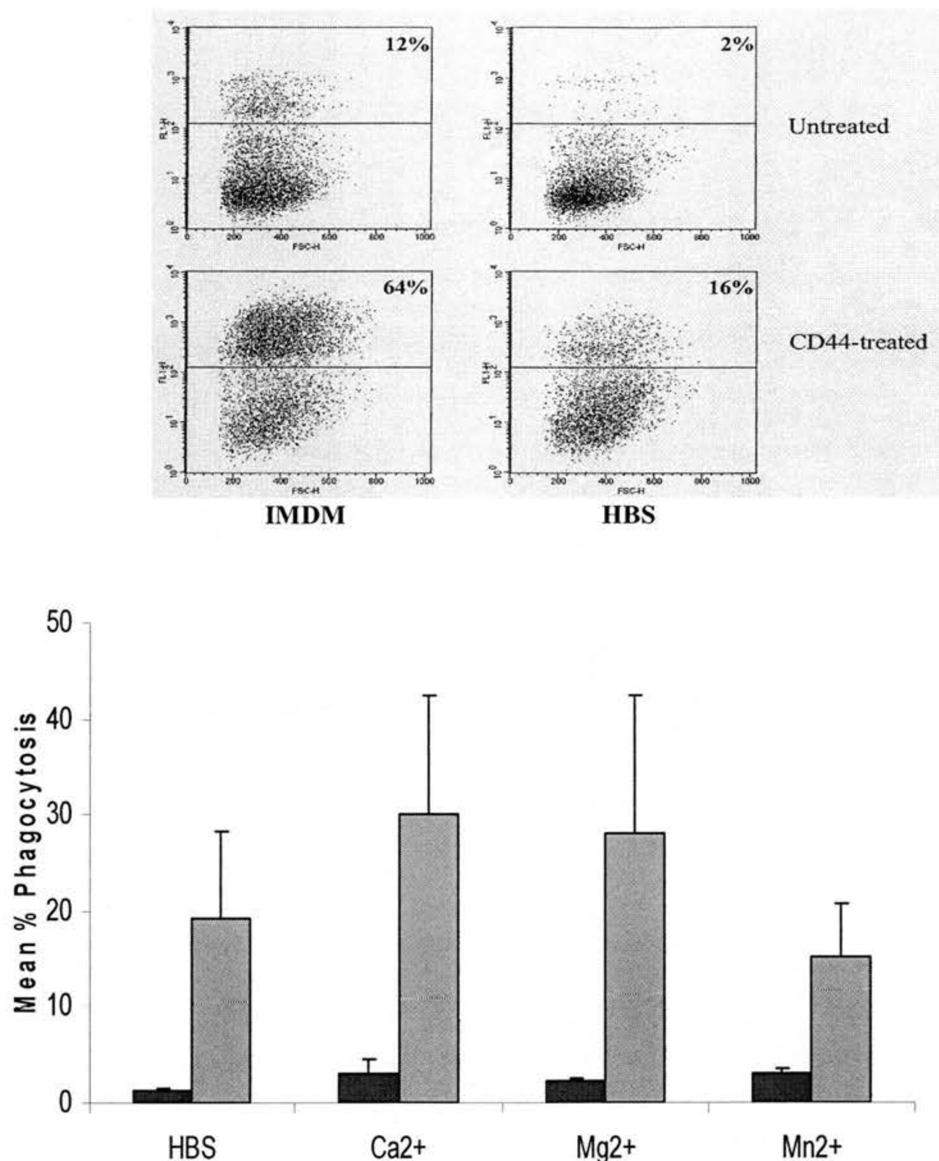



**FIGURE 3.4**
***The effect of cation depletion upon CD44 augmented macrophage phagocytosis of apoptotic neutrophils.***
Macrophages were incubated with either IMDM alone (black bars) or 5A4 (grey bars), washed and then incubated with a suspension of aged neutrophils in HBS alone or supplemented with the cation indicated above for 60min. After treatment with trypsin/EDTA, the percentage of macrophage phagocytosis was determined using a flow cytometric assay. (n=3)

For untreated macrophages, cation depletion almost eliminated baseline phagocytosis in comparison to phagocytosis in IMDM. Addition of different divalent cations failed to restore phagocytosis to control levels suggesting that multiple cations are required for phagocytosis. However, CD44 stimulation consistently resulted in augmentation of phagocytosis in a divalent cation deplete environment, although this augmentation was not as great as that observed in divalent cation replete conditions. Furthermore, supplementing HBS with either calcium or magnesium resulted in a higher level of augmentation. Thus, CD44-augmented phagocytosis of apoptotic neutrophils may be partially dependent upon divalent cations. The results from these experiments were surprising in several ways. First, the addition of either $Ca^{2+}$ or $Mg^{2+}$ alone failed to restore phagocytosis in untreated cells, raising the possibility that other media constituents in IMDM were necessary for phagocytosis, for example glucose. This possibility could be tested using media selectively depleted of divalent cations using special chelating resins. Second, the failure of $Mn^{2+}$ to restore phagocytosis is interesting since using microscopy based assays, 2 independent groups have observe augmentation by $Mn^{2+}$ (Dransfield and Savill, unpublished data). One possible explanation is that $Mn^{2+}$ promoted adhesion and phagocytosis. Since $Mn^{2+}$ strongly promotes integrin activation this observation makes it unlikely that integrins contribute to the phagocytic process. The data also strongly suggest that a major component of the effect of CD44 mAb treatment is distinct from that observed in untreated cells in that it is observed in HBS without divalent cations.

## Antibody/Ligand panel

Depletion of divalent cations dramatically reduces the effect of CD44 upon augmentation of phagocytosis (15-20% phagocytosis as opposed to 40-50% phagocytosis). It also reduces basal phagocytosis to around 2% giving an ideal system where the divalent cation independent component of CD44 augmentation can be investigated. In order to characterise the divalent cation independent component of CD44-augmentated phagocytosis, I treated macrophages with a number of known inhibitors of divalent cation independent cellular interactions. The polysaccharide

fucoidan is found in some species of Pheaeophycaea (brown seaweed), and has been shown to act as an antagonist of the macrophage scavenger receptor in macrophage cell lines THP-1, J774, RAW264.7 and murine peritoneal macrophages(Hsu, Hajjar et al., 1998; Kim, Ordija et al., 2003; Falcone, Rossi et al., 2001). Moreover, fucoidan can also to bind to CD14 resulting in signal transduction and cytokine release by the macrophage (Kim, Ordija, & Freeman, 2003). Heparin in high concentrations can influence the rate of macrophage phagocytosis (Savill, Wyllie, Henson, Walport, Henson, & Haslett, 1989; McCutcheon, Hart, Canning, Ross, Humphries, & Dransfield, 1998; Akbar, Savill et al., 1994). Anti-CD36 antibodies act as a blockade of the thrombospondin receptor, which along with integrins have been reported to assist in clearance of apoptotic cells (Savill, Hogg, Ren, & Haslett, 1992). Blockade of CD14 prevents it binding to ICAM3, which has also been shown to trigger uptake of apoptotic cells (Devitt, Moffatt, Raykundalia, Capra, Simmons, & Gregory, 1998; Moffatt, Devitt et al., 1999a). Anti-CD32 antibodies will block any FcγRII receptor interactions and were initially included as a negative control, since FcR interactions were thought to be unlikely to be involved in apoptotic cell clearance.

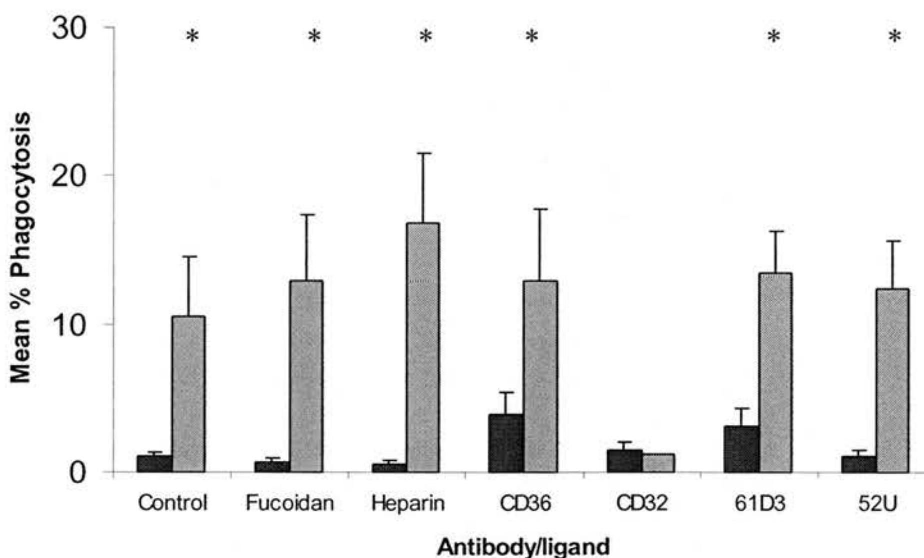



**FIGURE 3.5**
***The effect of a variety of antibodies & ligands on CD44 augmented macrophage phagocytosis of apoptotic neutrophils.***
Macrophages were incubated with either IMDM alone (black bars) or 5A4 (grey bars) for 15min, washed and then incubated with the antibody/ligand indicated above for a further 15min. After washing, macrophages were incubated with a suspension of aged neutrophils in HBS for 60min before trypsin/EDTA treatment. Analysis of macrophage phagocytosis was then carried out using a flow cytometric assay. (n=3, $P<0.01$)

Pre-treatment with fucoidan, heparin, anti-CD36 antibodies, anti-CD14 antibodies or an IgG1 control mAb prior to incubation with apoptotic neutrophils for 60 minutes at 37°C under divalent cation-free conditions failed to inhibit the CD44 augmented phagocytosis (figure 3.5). Surprisingly, treatment with an anti-CD32 antibody (IV3) resulted in inhibition of CD44-augmented phagocytosis in the absence of divalent cations. This finding suggests that CD32 (FcγRII) is responsible for the divalent cation independent component of CD44-augmented phagocytosis.

### Inhibition of CD32 on macrophages and neutrophils

CD32 is present upon both macrophages and neutrophils, therefore it was important to ascertain which was responsible for the observed inhibition of CD44-augmentation. Initially we thought that FcγR on macrophages were likely to be important since apoptotic neutrophils lose FcγRIII (CD16) and show a moderate down-regulation of FcγRII (Hart, Ross, Ross, Haslett, & Dransfield, 2000). Again, we were surprised to find that pre-incubation of macrophages with CD32 mAb IV3 had no inhibitory effect on CD44-augmented phagocytosis in divalent cation-free

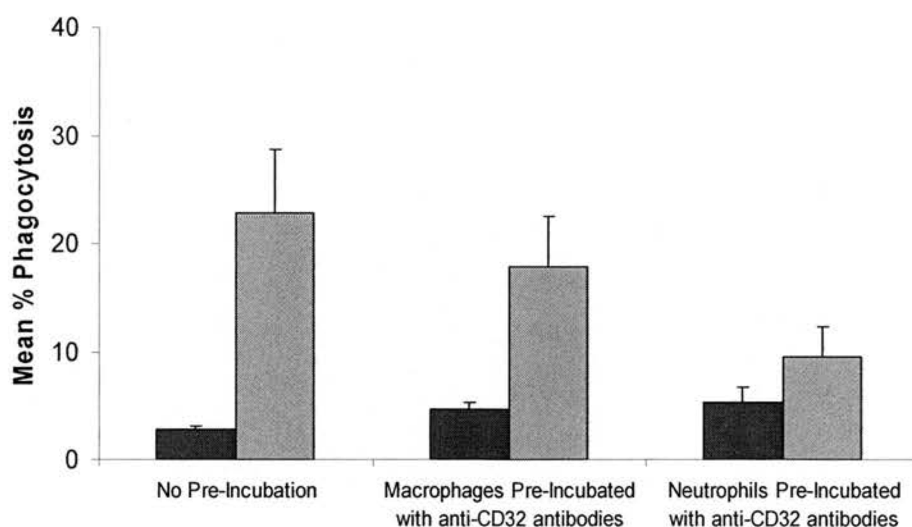



**FIGURE 3.6**
***The effect of pre-treating macrophages or neutrophils with anti-CD32 antibodies on CD44 augmented phagocytosis of apoptotic neutrophils.***
Macrophages or aged neutrophils were pre-incubated with anti-CD32 antibodies for 15min, washed and resuspended in IMDM. Macrophages were then incubated with IMDM alone (black bars) or 5A4 (grey bars) for 15min, washed and incubated with a suspension or aged neutrophils in HBS for 60min. Phagocytosis was stopped by addition of Trypsin/EDTA. The percentage of phagocytosis was determined using a flow cytometric assay. (n=3)

conditions (figure 3.6). This data suggests that macrophage FcγRII was not mediating the divalent cation-independent CD44-augmented phagocytosis.

In contrast, pre-incubation of apoptotic neutrophils with IV3, prior to use in phagocytosis assays, resulted in a major reduction of CD44-augmented phagocytosis. Thus, CD32 mAb blocks around 50% of the CD44-augmentation observed in the absence of divalent cations. Consequently, these data suggest that CD32 is the molecule responsible for the divalent cation independent component of CD44-augmented phagocytosis. One possible mechanism for this would be the binding of the 5A4 antibody to CD44 molecules on the macrophage surface and the Fc portion binding to CD32 expressed on the apoptotic neutrophil (figure 3.7). Thus, the CD32 mAb may act as a "bridge" between the macrophage and the apoptotic neutrophil and this tethering event then facilitates uptake of the neutrophil.

Neutrophils have been shown to express both CD16 (FcγRIIA) and CD32 (FcγRIIIB) upon their cell surface. As previously mentioned, during apoptosis the expression of these FcγRs is altered. CD16 expression is markedly downregulated (Dransfield, Buckle, Savill, McDowall, Haslett, & Hogg, 1994) and CD32 expression is down-regulated (Hart, Ross, Ross, Haslett, & Dransfield, 2000). It was believed that down-regulation of surface receptors associated with apoptosis resulted in functional down-regulation of neutrophil function, acting to "isolate" them from potentially pro-inflammatory stimuli e.g. antibody-immune complexes (Whyte et al, 1996).

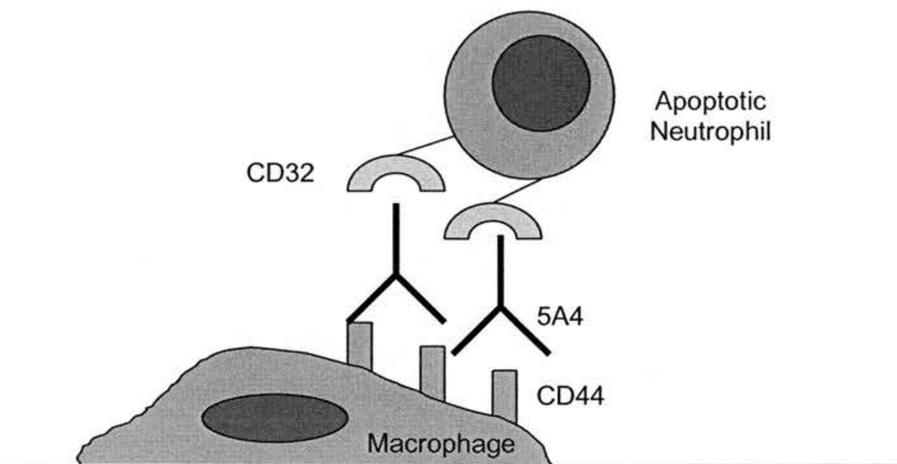



**FIGURE 3.7**

***Tethering the apoptotic neutrophil to the macrophage via CD44/5A4/CD32 interactions***

However, recent data suggests that apoptotic neutrophils may undergo an apoptosis-associated change in FcγR function resulting in the apoptotic neutrophil acquiring the ability to bind antibody-antigen complexes. (Hart et al, 2003). In this situation, the apoptotic neutrophil becomes opsonised by immune complexes and potentially flagged for clearance via alternative pathways.

Together these data support my hypothesis that neutrophil CD32 is responsible for the divalent cation-independent component of CD44 augmented phagocytosis.

## Generation of anti-CD44 F(ab')$_2$ fragments

To verify this hypothesis, I made anti-CD44 F(ab')$_2$ fragments to allow the role of CD32 in CD44-augmented phagocytosis to be confirmed. If my hypothesis was correct, then the anti-CD44 F(ab')$_2$ fragments would be a useful tool to explore the divalent cation dependent component of CD44-augmented phagocytosis. I therefore cultured the A4 hybridoma and purified the anti-CD44 mAb from supernatant using

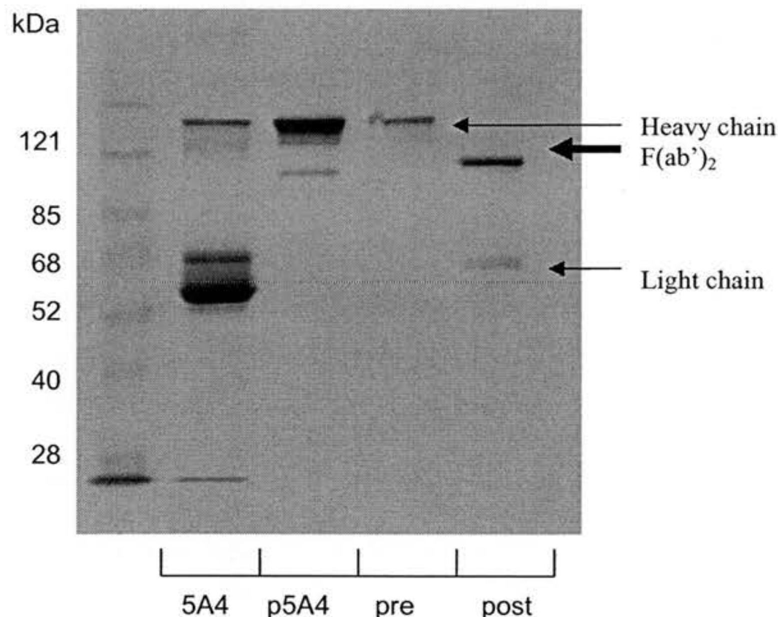



**FIGURE 3.8**
***SDS-PAGE analysis of pepsin digestion of 5A4 antibody***
Anti-CD44 antibody (5A4) was purified using protein A affinity chromatography (p5A4). Antibody was then eluted from the column and underwent pepsin digestion (pre – pre-digest, post – post-digest). Residual intact antibody was removed using protein A agarose affinity resin.

ammonium sulphate purification and protein A affinity chromatography. The isolated antibody was pepsin digested and any remaining whole antibody fragments removed by affinity chromatography on protein A sepharose. The resulting anti-CD44 $F(ab')_2$ fragments were analysed for purity (figure 3.8) and tested for their binding ability at varying concentrations. (figure 3.9)

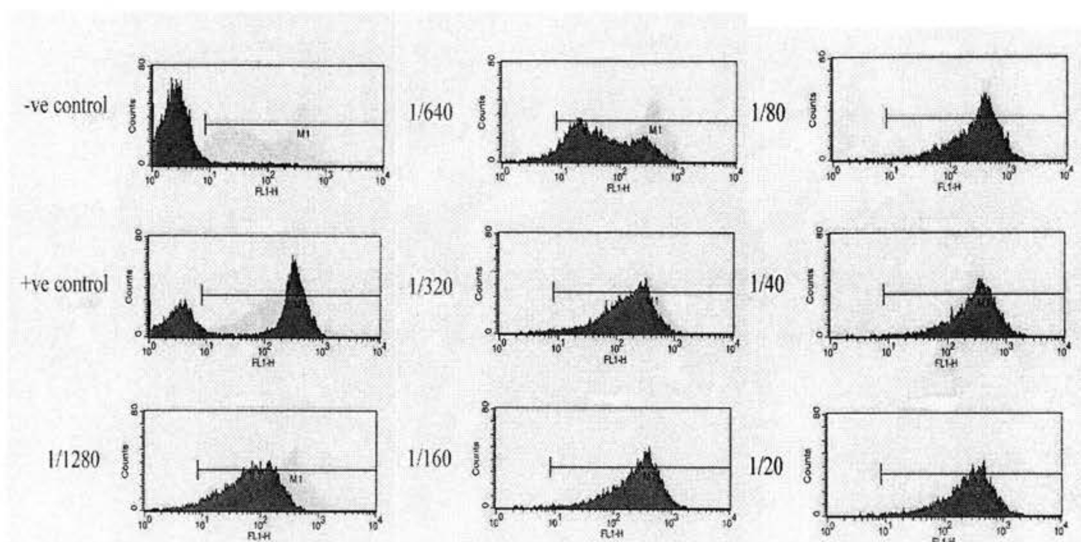



**FIGURE 3.9**
***Analysis of anti-CD44 F(ab')2 fragments for binding***
Macrophages were incubated with different concentrations of anti-CD44 $F(ab')_2$ fragments for 30min on ice. After thorough washing, the macrophages were incubated with goat anti-mouse FITC for 30 min on ice, washed thoroughly and antibody binding analysed using flow cytometry.

## Effect of anti-CD44 $F(ab')_2$ fragments on macrophage phagocytosis

To examine the role of CD32 in CD44-augmented macrophage phagocytosis, anti-CD44 $F(ab')_2$ fragments were used in phagocytosis assays in both divalent cation deplete and divalent cation replete conditions.

Pre-treatment of macrophages with anti-CD44 $F(ab')_2$ fragments prior to co-incubation with apoptotic neutrophils in IMDM (cations present) resulted in augmentation of phagocytosis, in comparison to untreated macrophages (figure 3.10a). The augmentation with $F(ab')_2$ was lower than that observed using whole antibody against CD44, supporting a contribution for the Fc portion of the antibody

in binding to CD32. Moreover, the observed augmentation by anti-CD44 $F(ab')_2$ fragments was very slight at 30min but had greatly increased by 60min, suggesting that CD32 mediated tethering may make a major contribution to apoptotic cell tethering, whereas CD44 cross-linking has a more delayed effect.

Macrophages pre-treated with anti-CD44 $F(ab')_2$ fragments before being allowed to interact with apoptotic neutrophils in HBS (cation deplete) had levels of phagocytosis similar to those of untreated macrophages (figure 3.10b). These data further suggest that my hypothesis that CD32 binding was responsible for the observed cation independent component of CD44-augmented phagocytosis.

Taken together, these data clearly indicate that CD32 assists in CD44-augmented phagocytosis through tethering the macrophage to the apoptotic neutrophil. However, CD44 antibody binding does augment phagocytosis independently of CD32 as indicated by the observed augmentation by CD44 $F(ab')_2$ in divalent cation replete conditions. It is this divalent cation dependent augmentation that likely represents the true effect of CD44 receptor ligation. Further examination of the molecular mechanism would therefore require studies with anti-CD44 $F(ab')_2$ antibody. Furthermore, these experiments also highlight the importance of taking the role of $Fc\gamma R$ into account when using whole antibodies in functional studies.

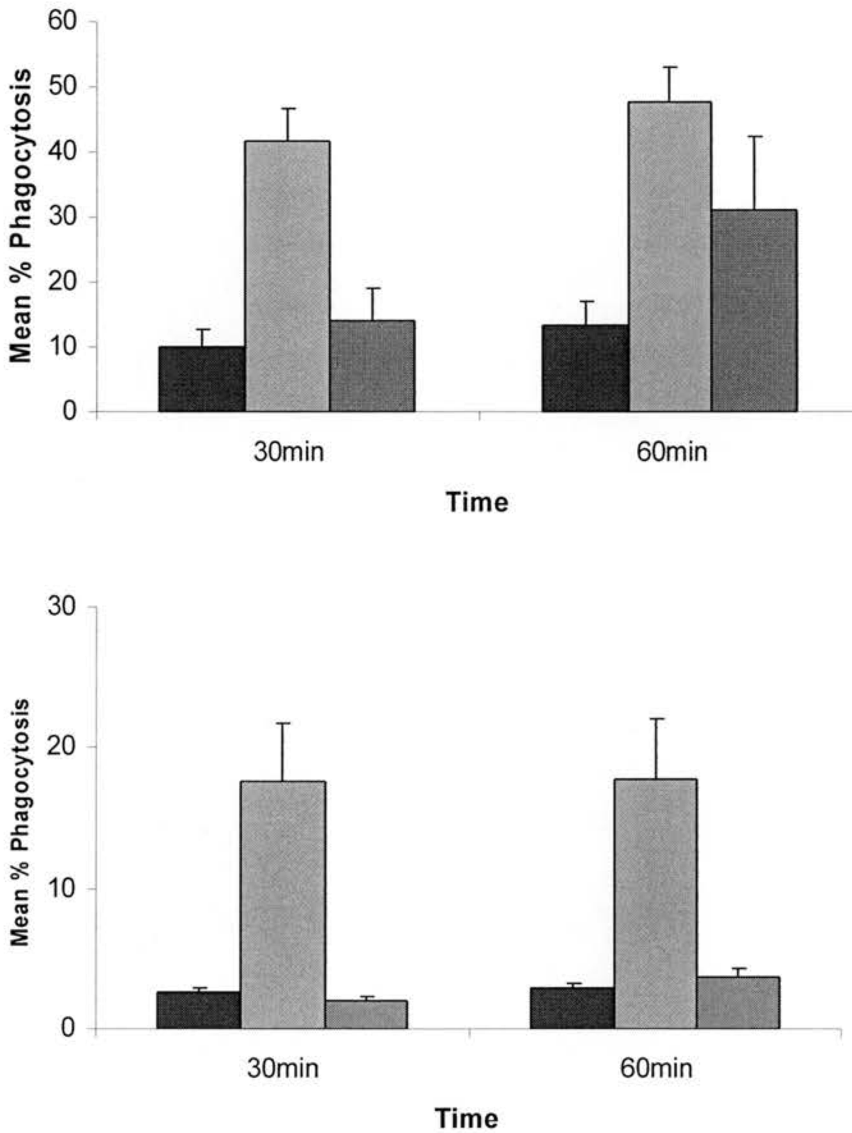



**FIGURE 3.10**

***Phagocytosis of apoptotic neutrophils by macrophages pre-incubated with anti-CD44 F(ab')2 fragments in either IMDM or HBS.***

Macrophages were incubated with either IMDM alone (black bars), whole 5A4 antibody (light grey bars) or anti-CD44 F(ab')2 fragments (dark grey bars) for 15 min, washed and then incubated with a suspension of aged neutrophils in either IMDM (a.) or HBS (b.) for a further 60 min. After treatment with trypsin/EDTA, the percentage of macrophage phagocytosis was determined using a flow cytometric assay. (n=3)

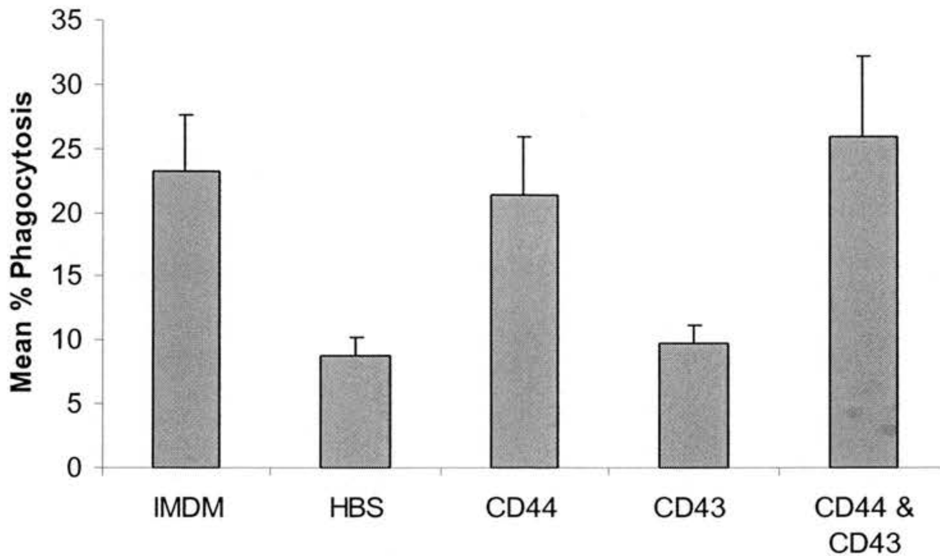



**FIGURE 3.11**
***Phagocytosis of apoptotic neutrophils by macrophages pre-treated with anti-CD43 antibody.*** Macrophages were pre-incubated with either CD44 mAb, CD43 mAb or a combination of both, or untreated control for 15 minutes, washed and then incubated with apoptotic neutrophils in the absence of divalent cations for 60 minutes. Macrophage phagocytosis of apoptotic neutrophils in duplicate samples was then determined by flow cytometry. Results are shown as mean ± SEM of six independent experiments.

## CD43 and macrophage phagocytosis

One important prediction from the above experiments is that any antibody, which binds to a molecule expressed at high levels on the macrophage surface should have the potential to augment phagocytosis in cation-depleted conditions. To investigate this possibility, I used an antibody against CD43, which has similar levels of expression to CD44, to see if phagocytosis could be augmented through binding to CD32.

Somewhat surprisingly, Pre-treatment of macrophages with an anti-CD43 antibody in divalent cation-free conditions failed to augment phagocytosis (figure 3.11). Moreover, there was no observed change in the effect of CD44 stimulation in the

presence of an anti-CD43 antibody. This observation contradicts my proposed mechanism for apoptotic cell tethering via CD32 and the reasons for this have not yet been clarified. It is possible that tethering of the apoptotic neutrophil to the macrophage does occur through CD43/CD32 interactions, when the anti-CD43 antibody is present, but that this does not result in phagocytosis. In contrast, with CD44 mAb binding to macrophages I have demonstrated that there is a pro-phagocytic signal via the $F(ab')_2$ portion of antibody. Thus, there is potentially a signal as well as tethering leading to phagocytosis, similar to the proposed "tether and tickle" model (figure 3.12). Tethering of an apoptotic cell to the macrophage may represent an independent process from phagocytosis and does not necessarily lead to phagocytosis. The flow cytometric assay used to measure levels of phagocytosis discriminates tethering from phagocytosis, as the majority of apoptotic neutrophils bound to the macrophages surface are removed by washing and trypsin/EDTA treatment and bound neutrophils were not observed in the phagocytic

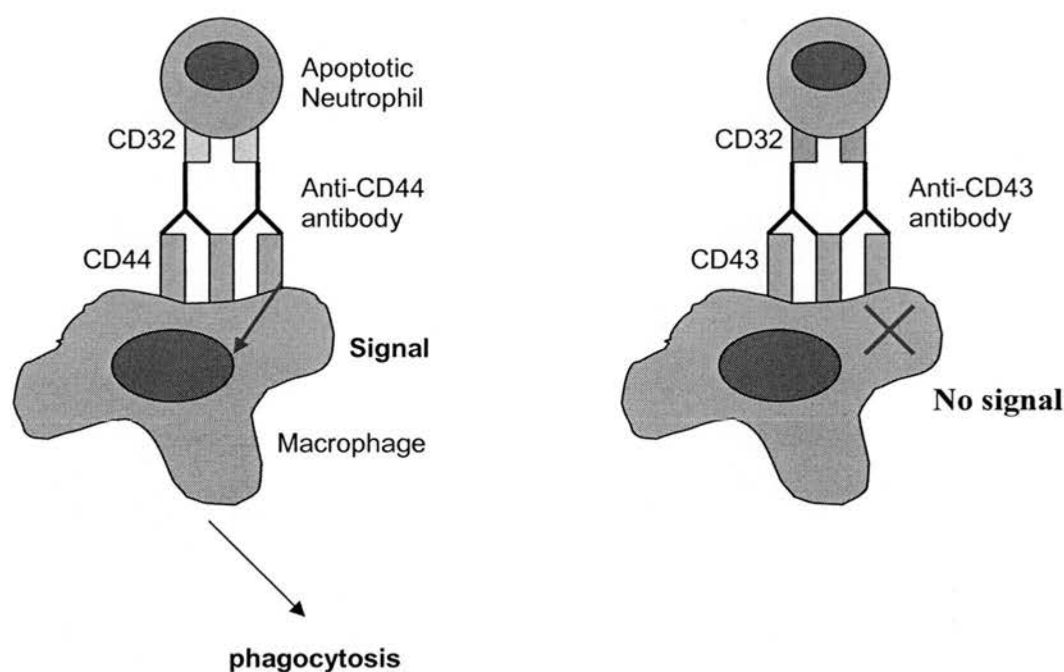



**FIGURE 3.12**
***Tethering of apoptotic neutrophil to macrophage via different cell surface molecules promotes different responses.***

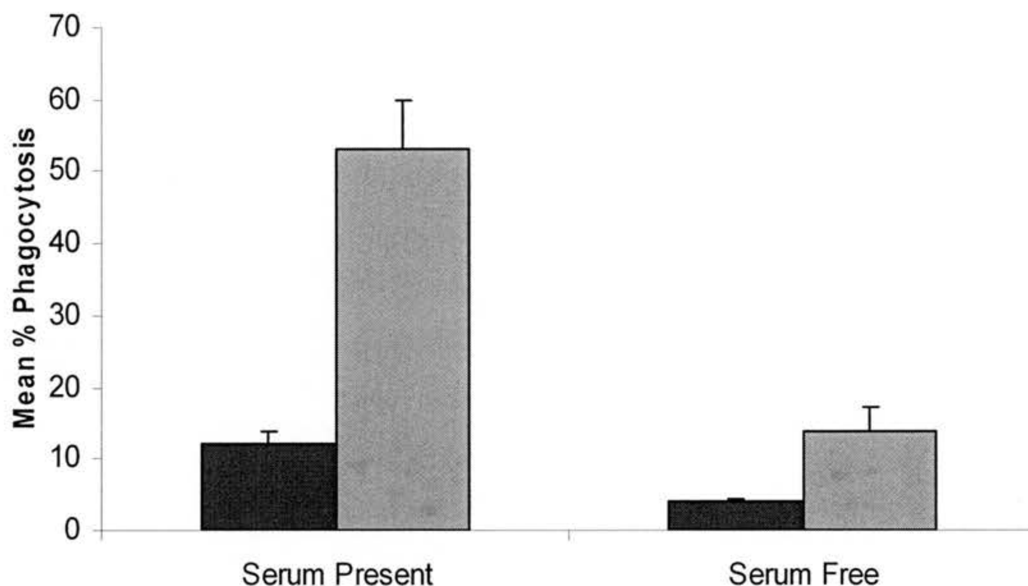



**FIGURE 3.13**
***Macrophage phagocytosis of apoptotic neutrophils in serum-free conditions.***
Macrophages, grown in serum-free conditions, were incubated with either IMDM (black bars) or 5A4 (grey bars) for 15min, washed and incubated with a suspension of aged neutrophils for 60min. After treatment with trypsin/EDTA, the percentage of macrophage phagocytosis

population when samples were sorted (Jersmann, Ross et al., 2003). Whilst, this is useful when a true indication of levels of phagocytosis is required, it is not useful when trying to quantify levels of tethering. Therefore, it would be necessary to develop a modified technique to assess the levels of tethering of apoptotic neutrophils to macrophages treated with either CD44 or CD43 mAb. It might also be useful to carry out a plate phagocytosis assay to allow visual quantification of bound in comparision to ingested apoptotic neutrophils. It may also be possible to not use trypsin/EDTA to detach neutrophils and instead quench fluorescence of surface bound neutrophils with trypan blue. Alternatively, binding of particles could be assessed by staining them with an anti-neutrophil antibody i.e. CD14. CD15 mAb would only stain neutrophils tethered to the macrophage and not those which have been ingested, allowing discrimination between tethering and phagocytosis.

**CD44-augmented phagocytosis in serum-free conditions**

Serum components have been shown to play a role in facilitating phagocytosis of apoptotic cells. One of the most prominent serum components implicated in the uptake of apoptotic cells are the proteins of the complement cascade. Mice deficient in the complement protein C1q have been shown to have a defect in clearance of apoptotic cells, which can then be observed in the kidney (Botto, 1998). This failure in clearance may contribute to the development of auto-antibodies associated with C1q deficiency.

It is possible that a variety of potential opsonins present in serum could assist in CD44-augmented phagocytosis by acting as a bridge between CD44 and a receptor on the apoptotic neutrophils surface, as this is the case for many phagocyte receptors. In fact, very few phagocyte receptors implicated in apoptotic cell clearance have been shown to interact directly with the apoptotic cell surface. Although, the flow cytometric phagocytosis assay is carried out in serum-free conditions both monocytes and neutrophils are cultured in the presence of 10% human autologous serum. Therefore, it is possible that both the macrophages and the apoptotic neutrophils will have been opsonised with serum components bound to their surfaces. To investigate the possible effect that serum may be having upon the macrophage response to CD44, macrophages were grown in serum-free conditions before being co-incubated with apoptotic neutrophils (also cultured using serum-free media).

Macrophages cultured in serum-free conditions had a lower level of control phagocytosis overall (figure 3.13). CD44 treatment of macrophages still increased phagocytosis of apoptotic neutrophils but not to as great an extent as those cultured in the presence of autologous serum. This raises the possibility that serum components are required for CD44-augmentation. These serum components are possibly essential for the maturation of monocytes into macrophages that are capable of phagocytosing. For example, GM-CSF is added to serum-free medium to assist in producing mature macrophages. However, GM-CSF may not be sufficient to allow the maturation pathway to be completed. It has been shown that it is macrophage

colony stimulating factor (M-CSF) that is essential to complete monocyte maturation into macrophages, and that addition of GM-CSF is not as effective (Brugger, Kreutz et al., 1991). Examination of the morphology of macrophages grown in serum-free conditions also seem to support this, as they were smaller and had a more spindly appearance than macrophages grown in the presence of autologous serum.

To investigate the effects of differentiation further I would assess the capacity for CD44 mAb to augment phagocytosis at different stages of macrophage culture, comparing monocytes and monocyte-derived macrophages cultured in serum-free media in the presence of different cytokines known to influence macrophage differentiation and induce different phenotypes e.g. IFN-$\gamma$, IL-4, IL-10 and GM-CSF.

### CD44 stimulation and macrophage migration

In view of the close relationship between adhesion, migration and phagocytosis in terms of regulatory mechanisms and proteins involved in cytoskeletal reorganisation (e.g. Rac, DOCK180 and crk), I sought to characterise whether CD44 influenced macrophage migration. In addition, it is possible that there may be an indirect association of cell motility with phagocytosis through the increased likelihood of macrophages making contact with apoptotic cells during the assay.

To investigate the association between phagocytic capacity and migration, macrophage monolayers were "wounded" with a pastette and the subsequent movement of macrophages into the "wound" was monitored using time lapse video microscopy over 20 hours. This system allowed the investigation of the effects of CD44 mAb binding to monolayers of cells under conditions similar to those used in the phagocytosis assay.

Surprisingly, pre-treatment of macrophages with CD44 mAb resulted in a reduced level of macrophage migration into the wound, in comparison with un-treated macrophages (figure 3.14). This observation suggests that stimulation of CD44 reduces macrophage capacity for migration. Alternatively, the anti-CD44 antibody may be blocking interactions between the macrophage CD44 and another molecule that is needed to facilitate migration. Macrophage migration is an incredibly complex and highly co-ordinated process. In order to investigate it further, it could be broken down into stages. The first stage involves extension of the plasma membrane forwards at the leading edge of the cell. This protrusion is stabilised by recruitment of signalling molecules and cytoskeletal proteins to form an adhesion. These adhesions are the attachments that drive the cell forward. The final stage consists of release of adhesions and retraction of the trailing edge of the cell.

Over 50 proteins have been found in adhesions (Zamir & Geiger, 2001). As it is these small adhesions that have been shown to drive migrating cells (Beningo, Dembo et al., 2001;Oliver, Dembo et al., 1999) then it would be useful to investigate

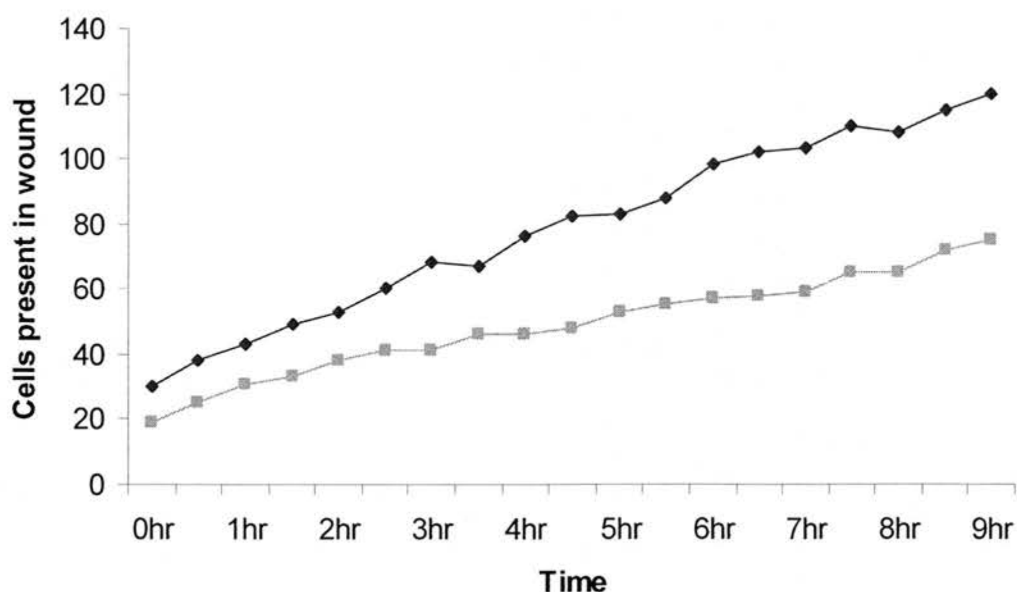



**FIGURE 3.14**
***The effect of CD44 stimulation on macrophage migration.***
Macrophages were incubated with either IMDM alone (black line) or 5A4 (grey line) for 15min, washed and the monolayer wounded using a pastette. Migration was recorded over a 20 hour period. (n=3)

differences in these structures between untreated and CD44 stimulated macrophages. An ideal strategy for this would be to carry out a protein array and protein expression profiling to highlight differences in protein expression between untreated and CD44 treated macrophages.

There are also a number of microscope-based techniques, which could be used to study molecular interactions during migration. Fluorescence Ratio Imaging (FRI) is one such technique, which can be used to examine co-localisation of signalling molecules and relative changes in protein concentration (Zamir, Katz et al., 1999; Kam & Boxer, 2001). The recent development of Traction Force Microscopy provides a mechanism to compare forces exerted by a moving cell (Dembo, Oliver et al., 1996). The technique involves plating cells onto a gel with marker microspheres embedded into it. The displacement of these microspheres, as the cell moves, can be translated into a traction stress map. The traction forces in both untreated and CD44 treated macrophages could be analysed for any differences between the cells.

Once these initial studies were carried out, they would provide an indication of potential molecules and their cellular locations, which were influencing migration in CD44 treated macrophages. Identification of these molecules would allow use of chemical inhibitors could be used to confirm their role in migration.

## SUMMARY

Augmentation of macrophage phagocytosis by apoptotic neutrophils as a result of CD44 mAb induced cross-linking may be a potential target for therapeutic intervention in chronic inflammatory conditions. Here it has been demonstrated that this increase in phagocytosis is long-lived and that cross-linking of CD44 can augment phagocytosis for up to 24 hours and possibly longer. Interestingly, it was observed that CD44 augmentation of macrophage phagocytosis is reduced in the absence of divalent cations. This suggests that the mechanism consists of both a cation-dependent and cation-independent component. Further investigation of the divalent cation independent component indicated that CD32 on the apoptotic neutrophil was playing a role in CD44 augmented phagocytosis. These observations

lead to the development of a hypothesis whereby the 5A4 antibody acts as a bridge between CD44 on the macrophage and CD32 on the apoptotic neutrophil, effectively tethering the two together.

# Chapter 4

# SIGNALLING IN RESPONSE TO CD44 CROSS-LINKING ON THE MACROPHAGE SURFACE

## INTRODUCTION

The previous chapter described how cross-linking of CD44 on the macrophage surface by bivalent antibodies resulted in a rapid and long-lasting augmentation of phagocytosis of apoptotic neutrophils. The observed effect consisted of two distinct components – divalent cation-independent and divalent cation-dependent. Using a number of inhibitors of known phagocytic receptors for apoptotic cells, it was possible to elucidate the mechanism of the divalent cation-independent component of CD44 augmented phagocytosis of apoptotic neutrophils. Data presented in the previous chapter demonstrated that CD32 on the apoptotic neutrophil interacted with the Fc region of anti-CD44 antibody, 5A4, which was bound to CD44 on the macrophage surface. The mechanism behind the divalent cation dependent component has proved more difficult to clarify. Two possible mechanisms have been suggested. Firstly, the rapid, yet prolonged change in macrophage behaviour suggested that generation of an intracellular signal might not be directly responsible for the augmentation of phagocytosis. An alternative that does not require a direct intracellular signal was that there might be a redistribution of CD44 molecules on the macrophage surface leading to exposure of "masked" receptors, which promote phagocytosis of apoptotic neutrophils. However, data from preliminary experiments investigating changes in macrophage surface membrane distribution of CD44 did not appear to support this hypothesis as no major alterations were observed. Secondly, there was a possibility that cross-linking of CD44 on the macrophage surface results in intracellular signalling cascades, which induce prolonged changes within the macrophage resulting in an increased capacity for phagocytosis of apoptotic neutrophils. Cross-linking of CD44 might initiate changes in cytoskeletal organisation or regulation, activation of phagocytic receptors for apoptotic cells or changes in gene expression that influence macrophage behaviour.

To investigate the role of signalling events occuring as a result of CD44 cross-linking, I used microspheres coated with anti-CD44 $F(ab')_2$ fragments to directly cross-link surface receptors. Providing that the antibody-coated microsphere was not internalised following binding to the macrophage surface and was not able to co-cross-link other membrane receptors or FcR, this system might represent an ideal method for investigating changes in localisation of signalling molecules within the macrophage. This approach has been used previously with great success, especially with regard to integrin function. Miyamoto and colleagues examined cytoskeletal responses to integrin ligation in fibroblasts using microspheres coated with integrin ligands (Miyamoto, Teramoto et al., 1995). They successfully elucidated signalling molecules and cytoskeletal proteins recruited at different stages of the adhesion response to integrin ligation. Furthermore, Green et al used different sizes of microspheres coated with anti-L-selectin antibodies to investigate levels of selectin ligation required for neutrophil activation (Green, Pearson et al., 2003). These studies provide precedence for use of antibody-coated microspheres to successfully investigate spatial and temporal changes during the signalling response to receptor ligation.

## Actin

In order to investigate signalling in response to CD44 cross-linking, I first had to select a number of signalling molecules known to be associated with CD44 or the phagocytic process. The cytoskeleton consists of actin filaments, which determine cell morphology, motility and endocytic capability (Yin & Hartwig, 1988). Phagocytosis of apoptotic cells is also an actin-dependent process; during phagocytosis the macrophage forms processes to surround an apoptotic cell, leading to actin-dependent internalisation of the apoptotic cell (Aderem & Underhill, 1999). Therefore, it was possible that the dynamics of actin remodelling were altered in response to CD44 ligation resulting in an increase in the macrophage's ability to phagocytose.

### Talin

Talin is a 270 kDa actin-binding protein that localizes to focal adhesions. Two talin molecules bind to form a homodimer with an antiparallel orientation. The N-terminal of talin can bind both phospholipids and focal adhesion kinase (FAK), while the C-terminal can bind actin and vinculin (Hemmings, Rees et al., 1996;Borowsky & Hynes, 1998). Talin can also bind the beta tails of integrin molecules, a step that is essential for integrin activation (Sampath, Gallagher et al., 1998). Moreover, talin is essential for formation of focal adhesions, as knocking out talin genes in embryonic stem cells abolishes focal adhesion and stress fibre formation (Priddle, Hemmings et al., 1998).

### Vinculin

Vinculin is a 117 kDa protein that also localizes to focal adhesions. The globular head region of vinculin (Vh) contains the binding sites for talin and alpha-actinin whereas its rod-like tail domain (Vt) contains the binding sites for F-actin and paxillin (Steimle, Hoffert et al., 1999). In its resting state, vinculin is held in a closed conformation through interactions between its head (Vh) and tail (Vt) domains. Vinculin is an essential regulator of both cell-cell (cadherin-mediated) and cell-matrix (integrin-talin-mediated focal adhesions) junctions, and it anchors these adhesion complexes to the actin cytoskeleton by binding to talin in integrin complexes or to actin in cadherin junctions (Goldmann, Kaufmann et al., 1992)

### Rac2

Rac2 belongs to the Rho family GTPases, which consists of small (approximately 21000 molecular weight) GTP-binding proteins. GTPases have intrinsic ability to catalyse the conversion of bound GTP to GDP. When bound to GTP, Rho proteins are active and can interact with their downstream targets. Once bound to GDP, they undergo conformational change resulting in a reduced affinity for their targets and they become inactive (figure 4.1). Cycling of GTPases between active and inactive

conformations is mediated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs promote exchange of GDP for GTP leading to activation of GTPases (Schmidt, Diriong et al., 2002). GAPs, in contrast, stimulate the intrinsic GTPase activity, resulting in inactivation (Moon & Zheng, 2003). Racl and Rac2 are 92% identical and have similar GTP binding and GTP hydrolysis motifs to other members of the Ras superfamily. Racl is expressed in a wide range of tissue types; however Rac2 is primarily expressed in myeloid cells (Didsbury, Weber et al., 1989).

One GEF in particular has been associated with CD44 signalling. Tiaml was first identified while using proviral tagging to identify genes that confer invasive properties upon tumour cells (Habets, Scholtes et al., 1994). Tiaml can activate Racl *in vitro* as well as *in vivo* and it is this property, which leads to induction of an oncogenic phenotype in NIH3T3 cells (Michiels, Habets et al., 1995;van Leeuwen, van der Kammen et al., 1995). Tiaml has also been shown to link CD44 to Racl signalling in breast cancer cells leading to increased motility of tumour cells (Bourguignon, Zhu, Shao, & Chen, 2000). However, preliminary experiments within our group have failed to show any link between Tiaml and CD44 augmented phagocytosis of apoptotic neutrophils (I. Dransfield and S. Hart, unpublished data).

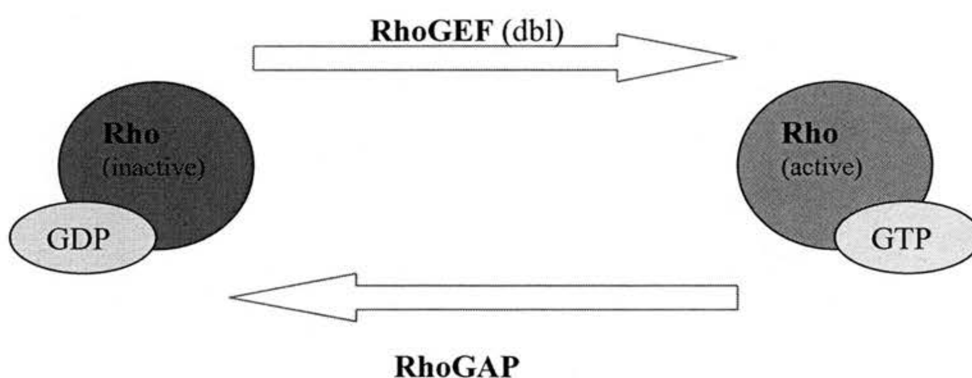



**FIGURE 4.1**

***Activation and inhibition of Rho family GTPases.***

Furthermore, GTPases have the ability to regulate ezrin, radixin, moesin family (ERM) proteins. Rac and ERM proteins are both required for membrane ruffling and lamellipodium extension. They are also important in cell migration and for the production of reactive oxygen species in phagocytes (Ridley, 2001;Bretscher, Edwards et al., 2002).

### PKC

Protein Kinase C (PKC) is a kinase that exists in a number of isoforms with different tissue specificity and is involved in a number of signalling pathways. PKC is activated by diacylglycerol, derived from cleavage of membrane lipids (Blumberg, 1988). Activation of ERM proteins requires phosphorylation of a threonine residue, which stabilises the proteins structure and prevents it folding into an inactive conformation (Gautreau, Louvard et al., 2000;Nakamura, Amieva et al., 1995). In the case of ezrin, several kinases have been found to have the ability to phosphorylate this threonine residue including PKCα, PKCθ, Cdk5 and MRCK (Ng, Parsons et al., 2001;Matsui, Maeda et al., 1998;Nakamura, Yamashita et al., 2002). In human melanoma cells, PKC has been shown to regulate the association of CD44 with ezrin. Once PKC is activated it dephosphorylates Ser325 in the cytoplasmic tail of CD44 and phosphorylates Ser291. This change in phosphorylation can alter the ability of the melanoma cell to migrate as dominant negative mutants (Ser291→ Ala291) lose the ability to undergo chemotaxis (Legg, Lewis et al., 2002).

### ERK 1/2

The extracellular signal-related protein kinases 1/2 (ERK1/2, p44/42) are one of the groups of MAP kinases found in mammalian cells. They are activated by dual phosphorylation of the tripeptide motif Thr-Glu-Tyr by MAP kinase kinases MKK1 and MKK2. MAP kinase kinases are activated by MAP kinase kinase kinases (MKKK), which can themselves be activated by a number of different upstream signals. One of these can be mediated through small G proteins – ERK can be activated by Ras through the Raf family of MKKK (summarised in figure 4.2).

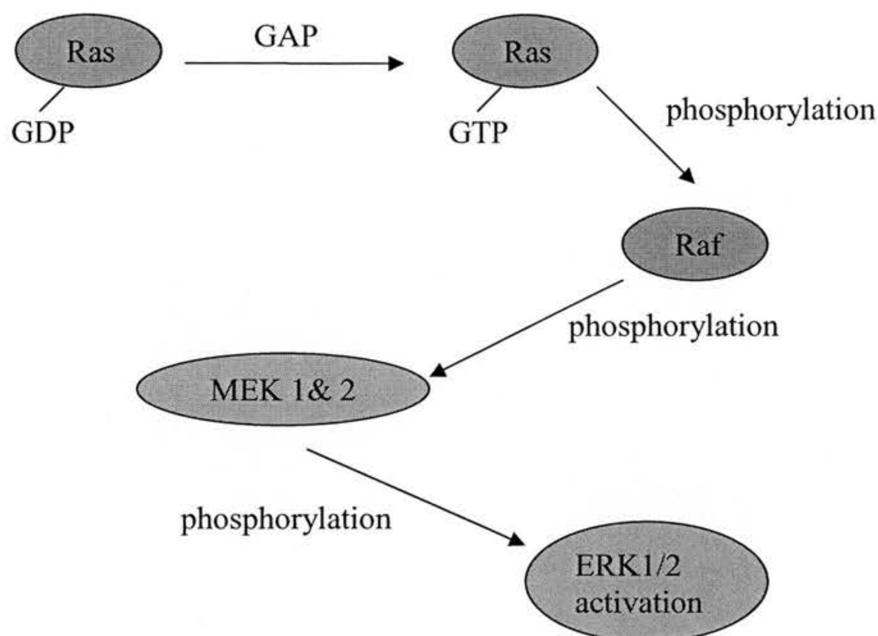



**FIGURE 4.2**
***A schematic diagram of the signalling pathway leading to ERK activation.***

ERK1/2 have been shown to play a large number of roles within the immune response. ERK can regulate production of TNFα, as murine macrophages treated with the ERK inhibitor PD98059 are deficient in TNFα production when exposed to LPS (Dumitru, Ceci et al., 2000). Interestingly, hyaluronan binding to CD44 has been shown to induce production of TNFα (Noble, Lake et al., 1993).

## Ezrin

Ezrin belongs to the ERM (ezrin, radixin, moesin) family of proteins, which are enriched in membrane structures such as microvilli, membrane ruffles and cell-cell junctions (Amieva & Furthmayr, 1995;Berryman, Franck et al., 1993). ERM family proteins have been shown to link integral membrane proteins to the cortical actin cytoskeleton (Tsukita & Yonemura, 1999). All three ERM proteins are known to bind CD44, providing a link between CD44 and the actin cytoskeleton in both BHK

cells and mouse fibroblasts (Tsukita, Oishi et al., 1994). In leukocytes, moesin has the highest level of expression, whereas there are little or no detectable levels of radixin. However, ezrin has been linked to potent phagocytic activity of melanoma cells, which makes it an ideal candidate for linking to CD44 in this system (Lugini, Lozupone et al., 2003).

## RESULTS & DISCUSSION

### CD44 surface expression

To investigate whether ligation of CD44 on the macrophages surface results in a major change in surface expression as determined by immunofluorescent microscopy, human monocyte-derived macrophages were treated at both 4°C and 37°C with CD44 mAb, 5A4 for 1 hour. The macrophages were then fixed, permeabilised and stained with goat anti-mouse Alexa Fluor 488. The distribution of CD44 on macrophages incubated at 37°C was then compared to that observed for those incubated at 4°C, which should not have undergone any changes in expression or distribution of receptors due to the inhibition of lateral membrane movement or receptor internalisation at low temperature.

Immunofluorescence microscopy analysis suggested that ligation of CD44 on the macrophage surface induces the membrane of the macrophage to undergo ruffling (figure 4.3). These ruffles appear rich in CD44 and stain very brightly. In contrast, at 4°C the macrophage surface is more uniformly stained with very little ruffling and the cells adopting a more rounded morphology.

However, these results were very difficult to quantify despite being representative of repeated observations. Moreover, using standard immunofluorescence microscopy there were limitations as to how accurately macrophages could be photographed at the same cross-section of the cell. To address this problem, con-focal microscopy was carried out using macrophages treated and fixed as previously described. This allowed cross-sections through macrophages to be photographed at 2μm optical

sections (supplementary figures provided on CD-ROM). This con-focal microscopy analysis revealed a similar pattern of staining as described above, even when comparing the same cross-sectional profile of both 4°C and 37°C macrophages.

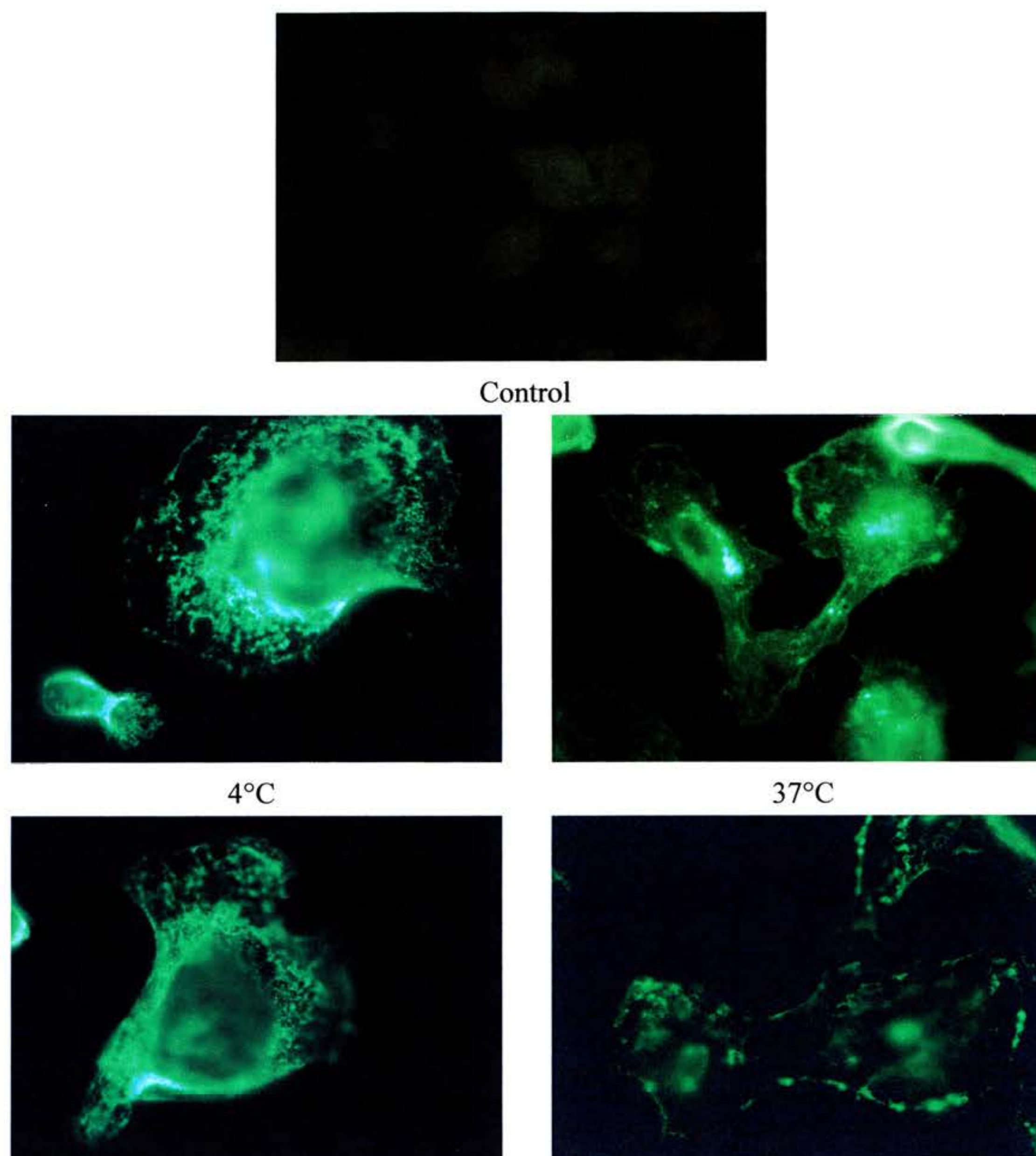



**FIGURE 4.3**

***Macrophage expression of CD44 after 60 min at 4°C or 37°C***

Macrophages were treated with either mouse IgG1 negative control (control) or 5A4 for 60min. Cells were incubated at 4°C or 37°C as indicated. After gentle washing, macrophages were fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature, washed and permeabilised with 0.1% Triton X-100 in PBS without $Ca^{2+}/Mg^{2+}$ for 4min at room temperature. After further washing, macrophages were stained with goat anti-mouse alexa fluor 488 for 30min on ice in the dark.

Photographs are representative of three experiments.

However, it would be necessary to undertake some level of quantification to confirm these alterations. This would involve using computational analysis to compare untreated and CD44 treated macrophages. There are a number of programmes available that would allow this to be carried out, for example the UTHSCSA Image Tool. In order to investigate changes between untreated and CD44 treated macrophages, it would be essential to define what a "normal" macrophage looked like. A number of untreated macrophages could be selected and compared by measuring their lengths, areas and diameters to give an idea of an average cell. The same could then be done for CD44 treated macrophages and the results compared. Areas of bright staining could also be quantified by setting a threshold to define normal staining from brightly stained areas, allowing areas of bright staining to be counted and compared. This threshold would have to be set at a high enough level to eliminate baseline staining and generate a manageable number of results. However, it would be important to maintain a fair comparison between untreated and CD44 treated macrophages. To do this, untreated and CD44 treated macrophages from each donor would have to be compared separately in terms of shape and staining. Moreover, a large number of macrophages from each donor would have to be isolated, possibly using MACS (magnetic cell sorting) to select a pure population of macrophages. These cells can then be analysed using immunofluorescence microscopy as before to give a clearer indication of any changes that occur in response to CD44 treatment.

### CD44 surface expression timecourse

To further characterise these changes in membrane expression of CD44, I examined the distribution of CD44 in a time-course experiment. In these experiments, macrophages were incubated with 5A4 at both 4°C and 37°C for 0, 15, 30, 45 and 60min. They were then fixed, permeabilised and stained as before to provide a temporal analysis of CD44 receptor distribution.

From these experiments, it was possible to observe that the macrophage begins to undergo membrane changes between 0 and 15 min with ruffles forming within 15

minutes. As noted above, these ruffles were found to be rich in CD44. However, there was expression of CD44 all over the surface of macrophages incubated at either

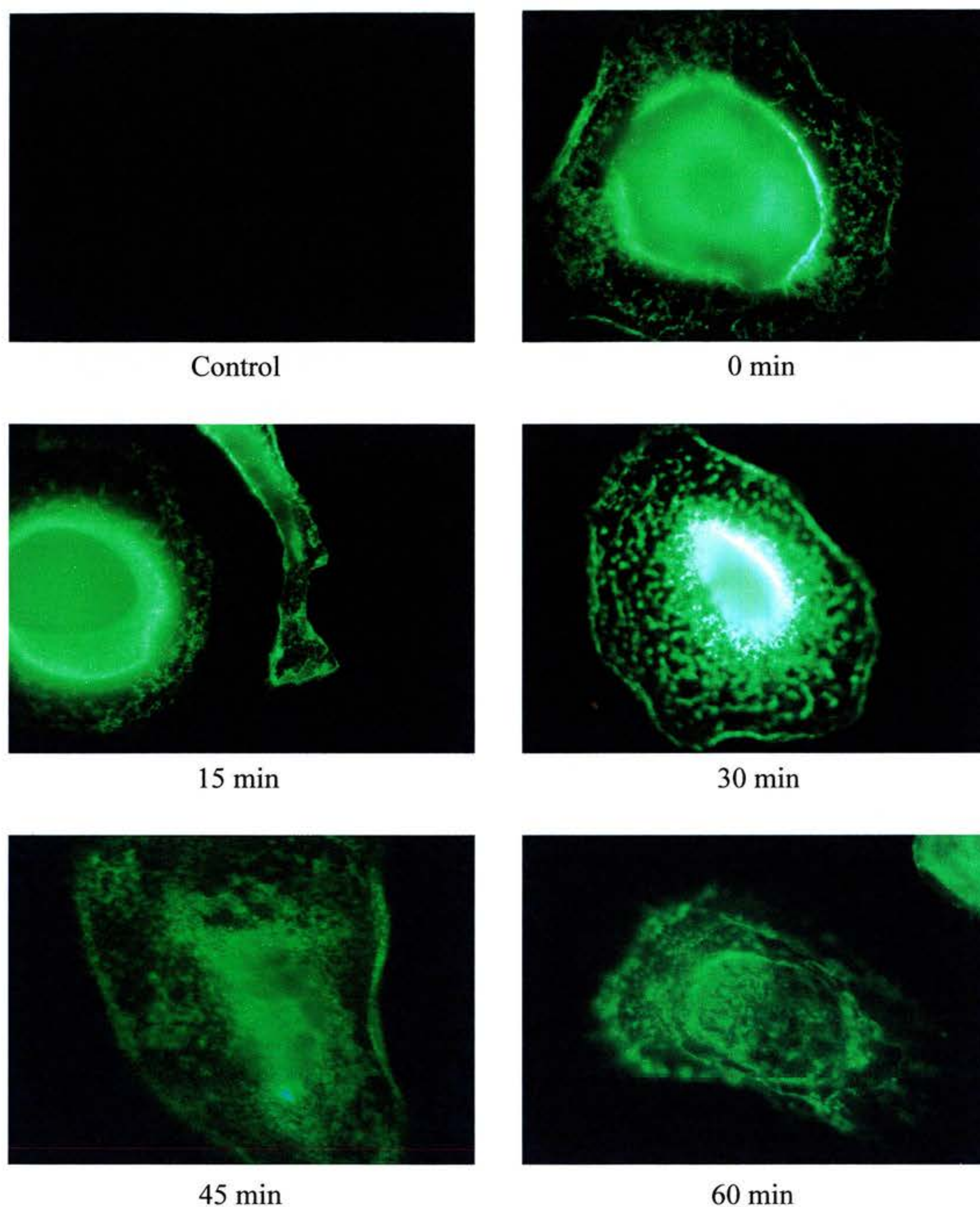



**FIGURE 4.4a**

***The surface expression of CD44 on macrophages at 4°C.***

Macrophages were incubated with either mouse IgG1 negative control or 5A4 for the length of time indicated at 4°C. After gentle washing, the macrophages were fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature, washed and permeabilised with 0.1% Triton X-100 in PBS without $Ca^{2+}/Mg^{2+}$ for 4min at room temperature. After further washing, macrophages were stained with goat anti-mouse alexa fluor 488 for 30min on ice in the dark.

Photographs are representative of three experiments.

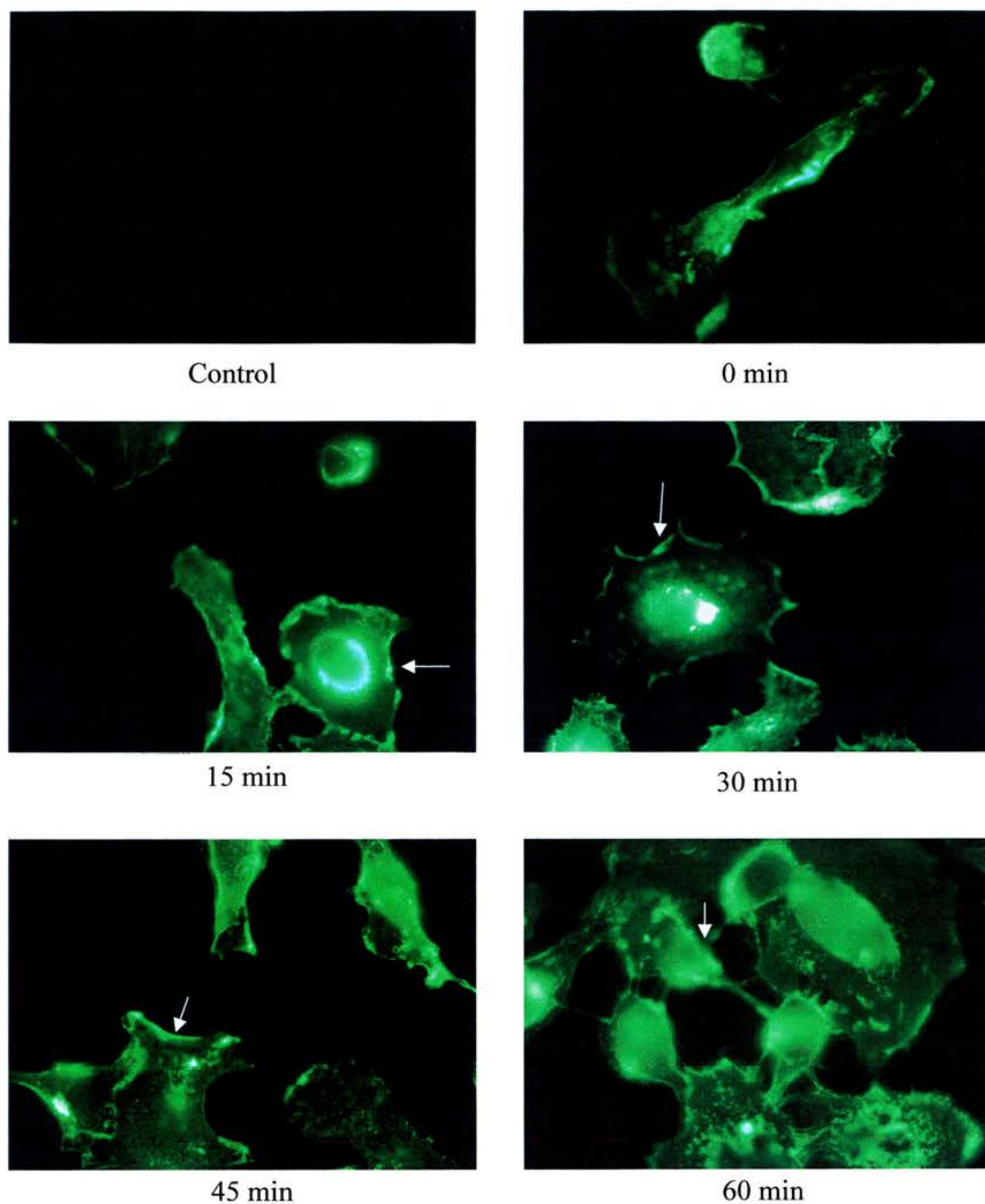



**FIGURE 4.4b**

***The surface expression of CD44 on macrophages at 37°C.***

Macrophages were incubated with either mouse Ig negative control or 5A4 for the length of time indicated at 37°C. After gentle washing, the macrophages were fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature, washed and permeabilised with 0.1% Triton X-100 in PBS without $Ca^{2+}/Mg^{2+}$ for 4min at room temperature. After further washing, macrophages were stained with goat anti-mouse alexa fluor 488 for 30min on ice in the dark.

Photographs are representative of three experiments.

37°C or 4°C (figure 4.4b), making it difficult to determine whether or not CD44 redistributes at 37°C or not. It is possible that any redistribution that occurs may be too subtle to be observed using the indirect immunofluorescence microscopy techniques used here and with the resolution of the microscope available.

During these studies, I observed that CD44-treated macrophages appeared to retract slightly in comparison to control macrophages (figures 4.4a and b) giving the cells a more "stellate" appearance. Again, although this change in morphology was repeatedly observed, quantification would be required to determine how representative the cellular retraction is within the CD44-treated macrophage populations.

### Migration of macrophages in response to treatment with $F(ab')_2$ fragments

As demonstrated in chapter 3, binding of intact CD44 mAb to macrophages resulted in an impaired ability to migrate into a mechanical "wound" created in a monolayer of macrophages. To examine whether this effect required only CD44 antibody

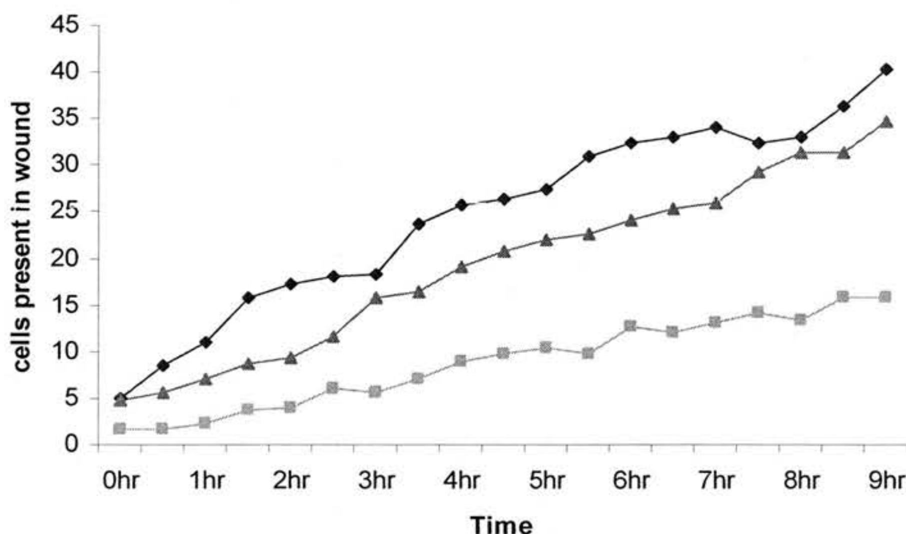



**FIGURE 4.5**
***Macrophage migration in response to anti-CD44 F(ab')2 fragments***
Macrophages were incubated with either IMDM alone (black line),5A4 (light grey line) or anti-CD44 $f(ab')_2$ fragments (dark grey line) for 15min, washed and the monolayer wounded using a pastette. Migration was recorded over a 20 hour period. (n=3)

binding interactions or whether an Fc receptor mediated component was involved, macrophages were treated with either IMDM alone, intact anti-CD44 (5A4) antibody or anti-CD44 $F(ab')_2$ fragments and their ability to migrate into a wound in the monolayer recorded using time-lapse microscopy.

Over the 9 hour time period analysed in these experiments, it was clear that preincubation of macrophages with intact CD44 antibody or $F(ab')_2$ fragments resulted in a reduction in migration of macrophages into a wound (figure 4.5 and movies provided on CD-ROM). However, the reduction in migration following binding of CD44 $F(ab')_2$ fragments was much smaller in comparison to the intact antibody, suggesting that antibody - Fc interactions with FcR may be responsible for the reduced migration of macrophages. These findings would need to be validated by careful repetition of these experiments. In particular, the rates of migration in individual experiments appeared to be dependent upon the extent of confluency of macrophage monolayers (data not shown) with more confluent monolayers resulting in increased migration.

### Microsphere assay to investigate macrophage signalling

In view of the fact that soluble antibody induced "cross-linking" did not noticeably alter receptor distribution, I next sought a method for inducing specific receptor engagement that would allow localisation of signalling molecules within the macrophage. I therefore decided to use antibody-coated microspheres as a method for inducing local "clustering" of receptors. I envisaged that the microspheres could be used to "cross-link" CD44 on the macrophage surface, without being internalised, giving an ideal system to visualise possible molecular mechanisms engaged at the site of microsphere binding. Initially, I used 6μm streptavidin-coated microspheres that had been pre-incubated with biotinylated anti-CD44 $F(ab')_2$ fragments. 6μm microspheres were chosen as they are roughly equivalent to the dimensions of an apoptotic neutrophil. Furthermore, other studies using ligand-coated microspheres to examine neutrophil function found that 6μm microspheres were able to induce functional responses whereas 1μm microspheres were not (Walker, Ruchaud-

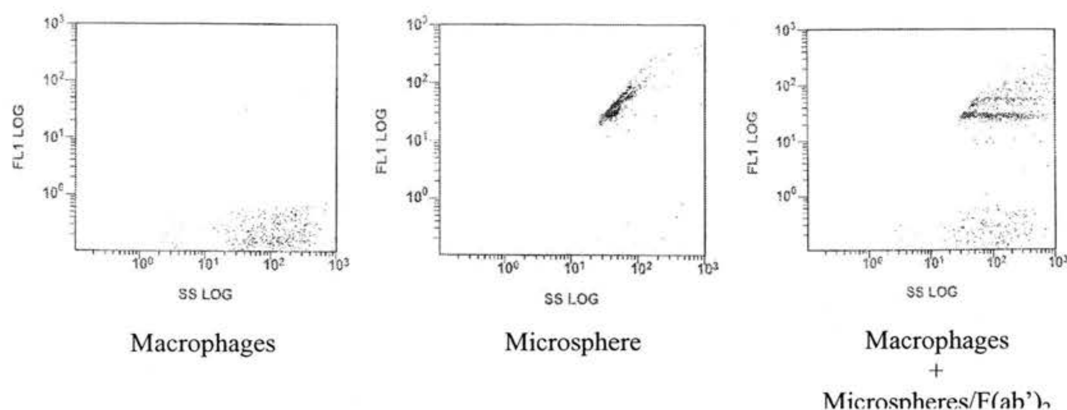



**FIGURE 4.6**
***Flow cytometric analysis of microsphere binding to macrophages***
Macrophages were incubated with anti-CD44 F(ab')2 coated microspheres for 30min at 37°C. After gentle washing, levels of microsphere binding to macrophages were assessed using flow cytometry.

Sparagano et al., 2004). In addition, microspheres of this size would be more readily visualised by light microscopy. I assessed the extent of binding of these microspheres to macrophages using both flow cytometry and microscopy. Association of fluorescent antibody-conjugated microspheres to macrophages was clearly demonstrated using flow cytometry (figure 4.6) and furthermore allowed for discrimination between macrophages that had different numbers of microspheres associated.

One problem with flow cytometry is that it is difficult to distinguish bound versus internalised particles. Using phase-contrast microscopy binding of the antibody-conjugated microspheres bound to macrophages was clearly seen (figure 4.7). Initially, I used IgG1-conjugated control microspheres to investigate non-specific association, which although there was some binding to the macrophage surface, the extent of binding was lower than that seen for the CD44-conjugated microsphere. However, it remained possible that binding of the IgG1 control was due to Fc receptors on the macrophage, I therefore decided to use anti-VCAM-1 $F(ab')_2$ fragments bound to streptavidin-coated microspheres. The lack of an Fc component on antibodies recognising either CD44 or VCAM-1 would eliminate any involvement of Fc receptor-mediated effects. Furthermore, since VCAM-1 is not

expressed upon macrophages, this represented a useful as a control for non-specific effects of $F(ab')_2$.

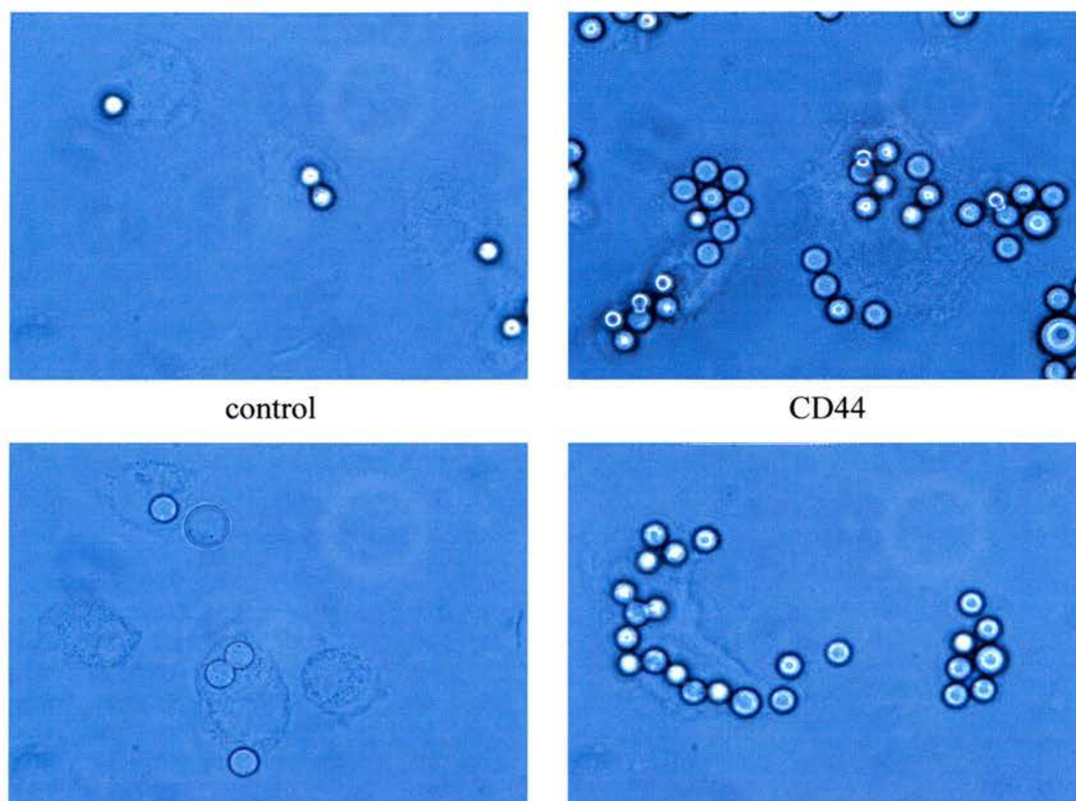



**FIGURE 4.7**
***Binding of antibody coated microspheres to macrophages***
Macrophages were incubated with microspheres conjugated to either a control antibody (mouse IgG1) or anti-CD44 $F(ab')_2$ fragments for 30min at 37°C, washed with PBS without $Ca^{2+}/Mg^{2+}$ and mounted on microscope slides.

Photographs are representative of three experiments.

### Microsphere internalisation

I considered it important that the antibody-coated microsphere used in these experiments cross-linked cell surface CD44. It was therefore essential to be certain that the microspheres were not internalised by macrophages. I incubated macrophages with either anti-CD44 or anti-VCAM $F(ab')_2$ coated microspheres for 30min, at a ratio of approximately 1 microsphere per macrophage, and then fixed them. The slides were then stained with goat anti-mouse-IgG-FITC to determine whether or not the microspheres had been internalised. Since internalised microspheres should not be stained, any visible FITC fluorescence would indicate that the microspheres remained on the surface of the macrophage and thus accessible for antibody binding. In contrast, if the microspheres were internalised then little or no staining would occur.

Using immunofluorescence microscopy, FITC stained microspheres were clearly observed with very little background staining (figure 4.8a). These data suggest that all of the microspheres are bound to the macrophage surface and not internalised. To confirm that the microspheres were not being internalised, con-focal microscopy was used, since this would allow serial optical sections (at 2 μM) to be scanned through the cell body. Using this combination of both phase-contrast and con-focal fluorescent microscopy, microspheres could be clearly observed to be bound to the surface of the macrophage.

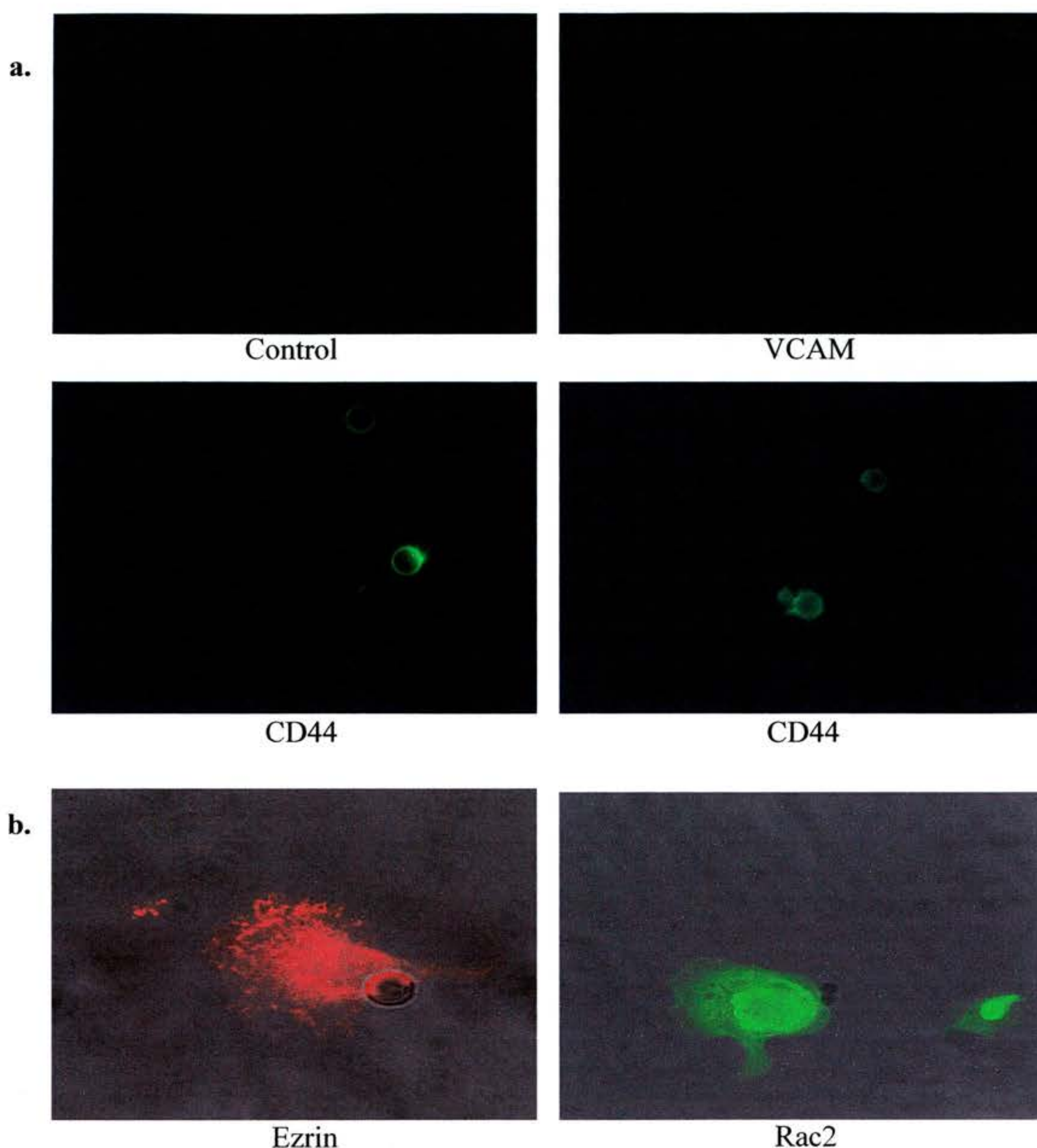



**FIGURE 4.8**

***Lack of internalisation of anti-CD44 F(ab')$_2$ coated microspheres by macrophages.*** Macrophages were incubated with either IMDM alone (control), anti-VCAM F(ab')$_2$ fragment conjugated microspheres (VCAM) or anti-CD44 F(ab')$_2$ fragment conjugated microspheres (CD44) for 30min at 37°C. After washing, macrophages were fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature and then incubated with GAM-FITC for 30min on ice, washed and mounted on microscope slides. Photographs are representative of three experiments.

The two lower figures are taken from con-focal microscopy analysis of microsphere binding. They were prepared and stained as described in figures 4.12 and 4.16.

Interestingly, although the microspheres appeared to be "cupped" by the surface membrane of the macrophage, there was no evidence to suggest that the microspheres were being internalised in anyway in con-focal analysis (fig 4.8b).

### Macrophage signalling in response to F(ab')$_2$ coated microspheres

Having demonstrated that the anti-CD44 F(ab')$_2$ coated microspheres had the ability to bind to CD44 without being internalised, it was possible to use them to investigate localisation of signalling molecules within the macrophage. Either CD44 or VCAM1-coated microspheres were bound to the macrophage surface and then the cells fixed and stained for a number of intracellular signalling molecules that the current literature had highlighted as being possibly involved in CD44 signalling.

### Actin

Given the critical role of actin in macrophage adhesion and phagocytosis, I decided to investigate the distribution of actin. Following binding of CD44 F(ab')$_2$ coated microspheres to macrophages, actin was found to have a "speckled" appearance (figure 4.9), probably representing bundles of actin filaments within the cytoskeleton. In unstimulated macrophages, these are present throughout the cell. However, in those macrophages incubated with CD44 F(ab')$_2$ coated microspheres, these actin structures appeared to be localised within the macrophage, often in proximity to a microsphere. Although this was generally the case, there were microspheres bound to some macrophages which had no actin bundles nearby. One possibility is that this may be a consequence of microspheres being bound for different lengths of time and that actin filament redistribution changes over time. In order to address this possibility, I could do a timecourse allowing the microspheres to bind for different periods of time, stain as normal and then compare differences in filament distribution at different timepoints.

### Talin & Vinculin

Given the changes in actin described above and that the principal CD44 ligand, hyaluronan, has been shown to promote protein phosphorylation and focal adhesion turnover (Hall, Wang et al., 1994) - changes that are associated with an increase in

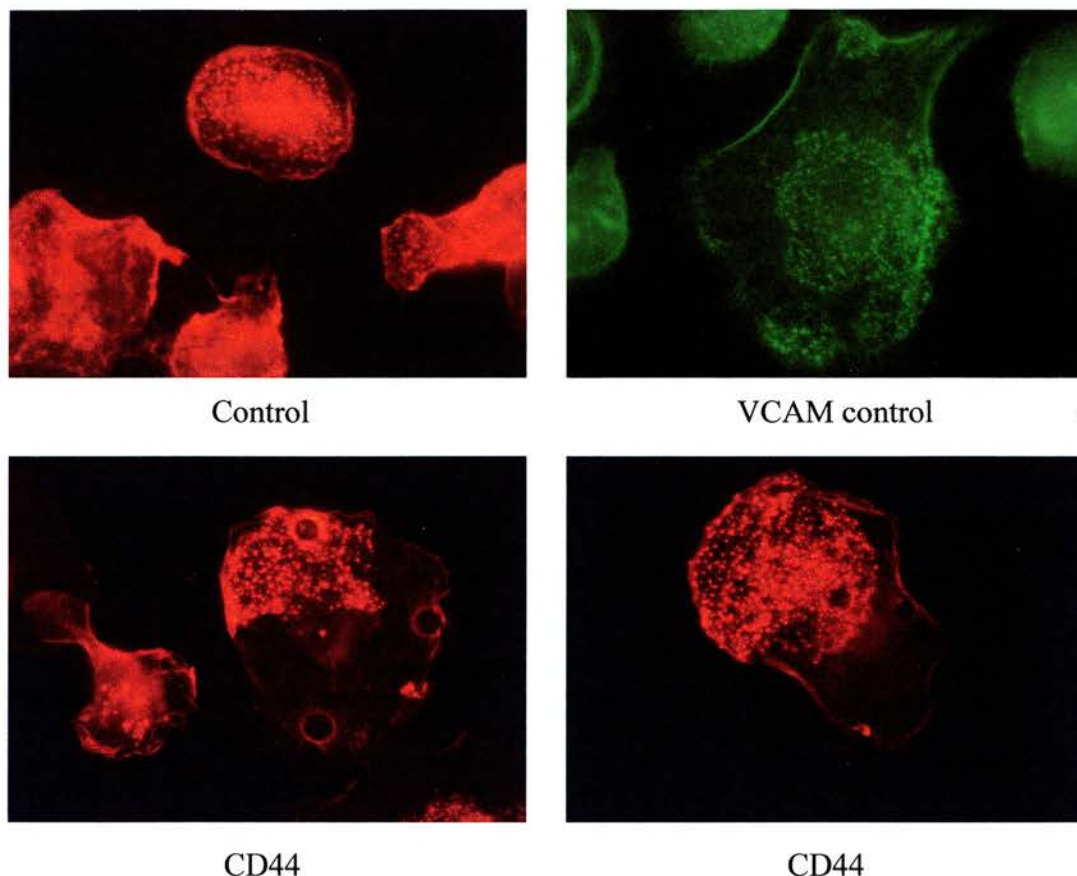



**FIGURE 4.9**

***Localisation of actin after cross-linking of CD44 with F(ab')$_2$ coated microspheres.***

Macrophages were incubated with either mouse Ig control, IMDM alone, anti-VCAM F(ab')2 fragment conjugated microspheres (VCAM) or anti-CD44 F(ab')2 fragment conjugated microspheres (CD44) for 30min at room temperature. Cells were then washed, fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature and permeabilised with 0.1% Triton X-100 in PBS without $Ca^{2+}/Mg^{2+}$ for 4min at room temperature. After further washing, macrophages were incubated with with rhodamine phalloidin 488 for 30min on ice. After washing, macrophages were mounted on slides and analysed using immunofluorescent microscopy.

These photographs are representative of three experiments.

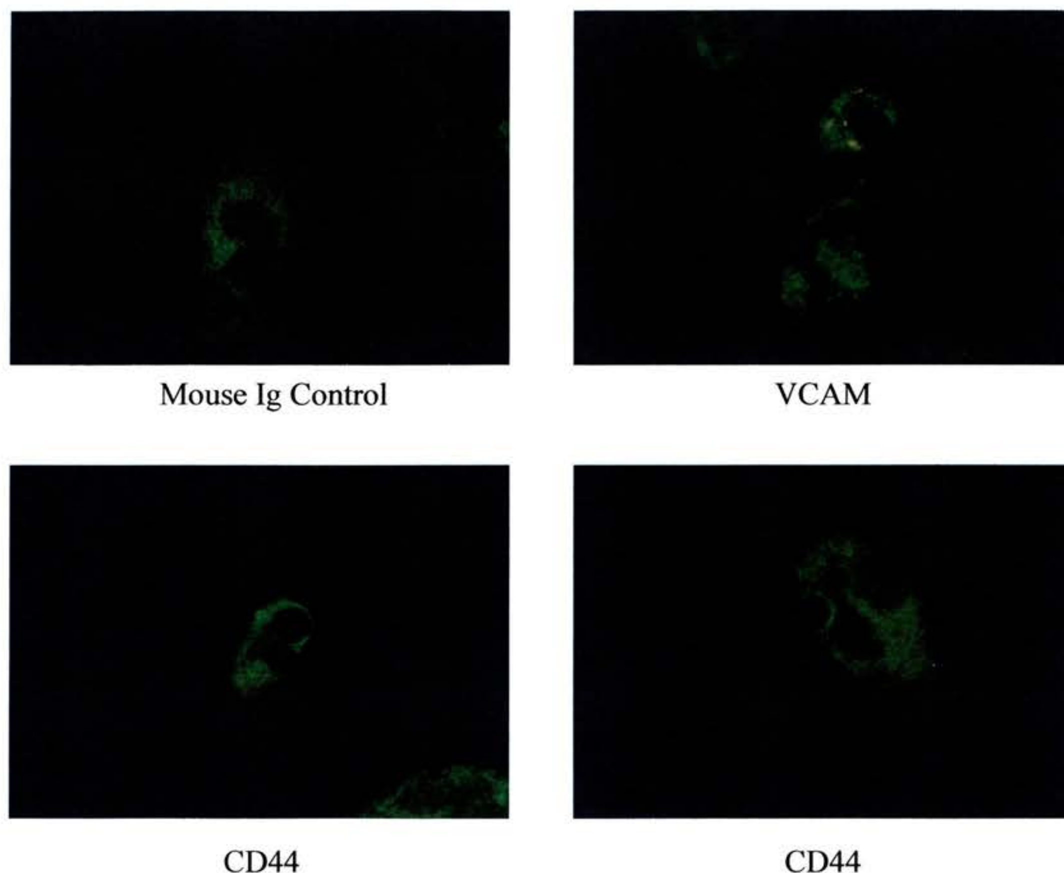



**FIGURE 4.10**

***Localisation of talin to the macrophage membrane in response to cross-linking of CD44 by F(ab')$_2$ coated microspheres.***

Macrophages were incubated with either mouse Ig control, IMDM alone, anti-VCAM F(ab')2 fragment conjugated microspheres (VCAM) or anti-CD44 F(ab')2 fragment conjugated microspheres (CD44) for 30min at room temperature. Cells were then washed, fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature, and permeabilised with 0.1% Triton X-100 in PBS without $Ca^{2+}/Mg^{2+}$ for 4min at room temperature before incubation with an anti-talin antibody for 30min on ice. After further washing, the macrophages were stained with a goat anti-mouse (Fc specific) secondary antibody for 30min on ice. After washing, macrophages were mounted on slides and analysed using immunofluorescent microscopy.

These photographs are representative of three experiments.

neutrophil motility (Hakansson L., Hallgren R. et al., 1980), I decided it would be interesting to investigate the possible involvement of talin and vinculin in signalling through CD44.

Microsphere induced cross-linking of CD44 on the macrophage surface was observed to lead to bright staining of talin at the region where the microsphere was bound to the macrophage (figure 4.10). This observation raises the possibility that talin may be specifically recruited following CD44 cross-linking. Although in this experiment background staining levels were quite high, it is still possible to observe definite changes in the staining present in macrophages with anti-CD44 microspheres bound to them. In contrast, it was not possible to see changes in vinculin distribution after cross-linking of CD44 (figure 4.11). The staining pattern for vinculin in untreated macrophages and macrophages treated with anti-VCAM microspheres was similar to that observed in macrophages treated with anti-CD44 microspheres.

These observations are interesting as talin has been shown to link to other molecules through vinculin. Our results suggest that this is not the case during the macrophage response to anti-CD44 microspheres. Talin can also link to actin directly, which my results suggest are involved in the macrophage response to anti-CD44 microspheres. However, one possibility is that recruitment and activation of vinculin occurs after recruitment of talin and thus, requires a longer period of time than that used in these assays. In order to investigate this further, it would be necessary to incubate the macrophages with anti-CD44 microspheres for differing lengths of time prior to examining talin and vinculin distribution. Another approach would be to investigate other signalling molecules known to interact with vinculin, such as paxillin, vasodilator-stimulated phosphoprotein (VASP) and actin-related protein 2/3 (Arp2/3). If these molecules were shown to be redistributed then this may support the likelihood that vinculin is being recruited also.

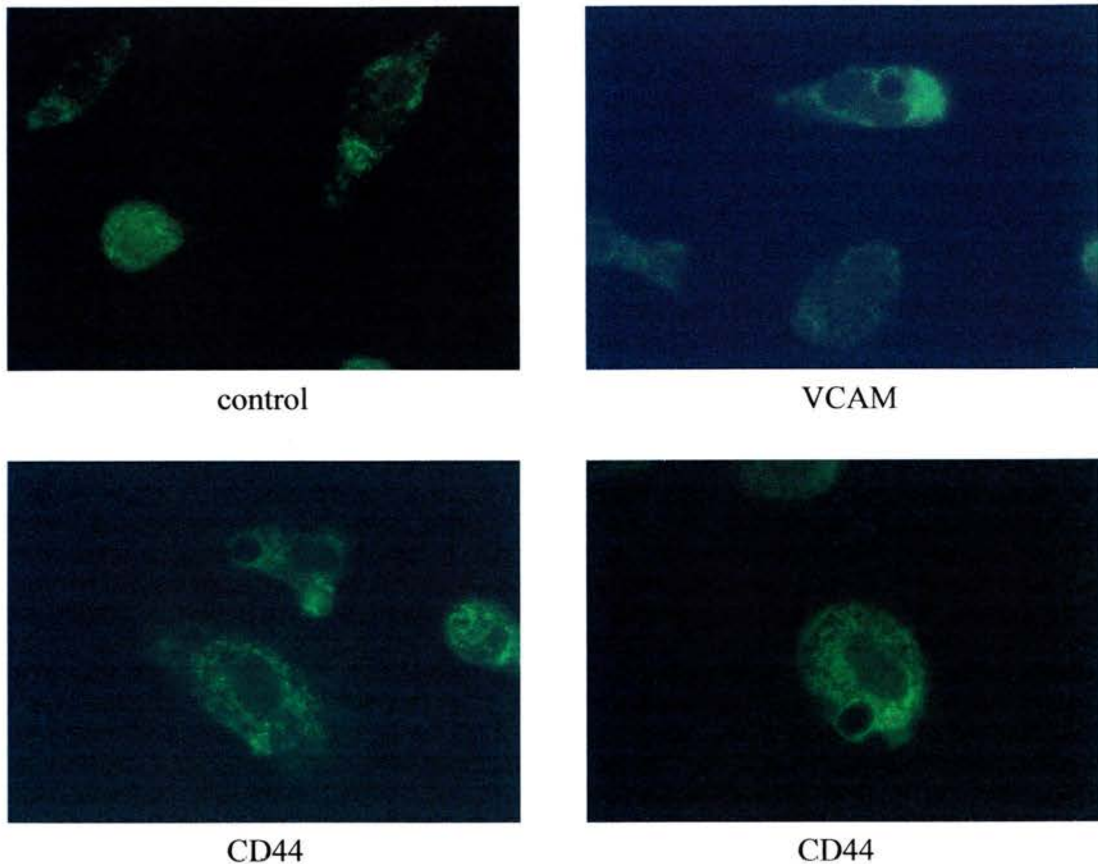



**FIGURE 4.11**

***Cross-linking of CD44 on macrophage surface does not result in changes in Vinculin distribution.***

Macrophages were incubated with either mouse Ig control, IMDM alone, anti-VCAM F(ab')2 fragment conjugated microspheres (VCAM) or anti-CD44 F(ab')2 fragment conjugated microspheres (CD44) for 30min at room temperature. Cells were then washed, fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature and permeabilised with 0.1% Triton X-100 in PBS without $Ca^{2+}/Mg^{2+}$ for 4min at room temperature, before incubation with an anti-vinculin antibody for 30min on ice. After further washing, the macrophages were stained with a goat anti-mouse (Fc specific) secondary antibody for 30min on ice. After washing, macrophages were mounted on slides and analysed using immunofluorescent microscopy.

These photographs are representative of three experiments.

### Rac2

Previous work within our group has suggested that Rac2 might have a role in CD44 augmented phagocytosis, therefore I decided to investigate the involvement of Rac2 further using the CD44 F(ab')$_2$ coated microspheres. Staining of CD44 stimulated macrophages for Rac2 showed FITC localisation in the membrane under and surrounding the microsphere (figure 4.12). This effect was not observed with either VCAM F(ab')$_2$ coated microspheres or in untreated macrophages. These data strongly support the suggestion that CD44 cross-linking leads to Rac2 activation and redistribution to the region of CD44 cross-linking.

### Protein Kinase C

Following crosslinking of CD44 on the macrophage surface, PKC relocation to the area surrounding the microsphere was observed (figure 4.13). In control macrophages, PKC staining was observed throughout the cytoplasm, in comparison to macrophages incubated with the anti-CD44 microspheres, which have much brighter staining near to the microsphere. These observations suggest that PKC is co-localising with CD44 in response to microsphere binding.

It would have been interesting to develop these studies using other approaches. One alternative would have been to look at specific phosphorylation events in macrophages following cross-linking of CD44. To do this, phospho-specific e.g phospho-tyrosine, phospho-threonine or phospho-serine specific antibodies could have been used to identify localisation of phosphorylated proteins in macrophages with microspheres bound. However, although these antibodies are available, experience within the laboratory suggests that they exhibit high levels of non-specific binding, which could cause difficulties in interpretation of staining patterns.

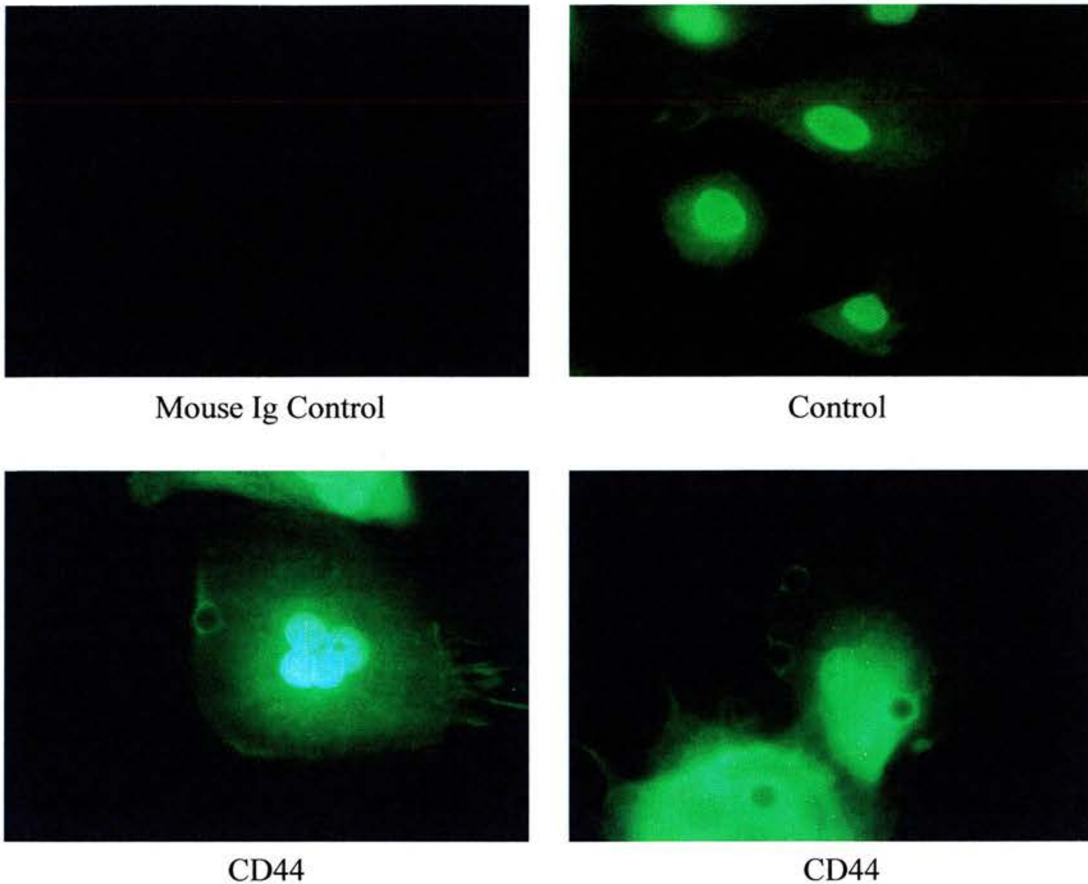



**FIGURE 4.12**

***Rac2 localisation to the macrophage surface upon cross-linking of CD44***

Macrophages were incubated with either mouse Ig control, IMDM alone, anti-VCAM F(ab')2 fragment conjugated microspheres (VCAM) or anti-CD44 F(ab')2 fragment conjugated microspheres (CD44) for 30min at room temperature. Cells were then washed, fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature and permeabilised with 0.1% Triton X-100 in PBS without $Ca^{2+}/Mg^{2+}$ for 4min at room temperature before incubation with an anti-Rac2 antibody for 30min on ice. After further washing, the macrophages were stained with goat anti-rabbit Alexa Fluor 488 for 30min on ice. After washing, macrophages were mounted on slides and analysed using immunofluorescent microscopy. These photographs are representative of three experiments.

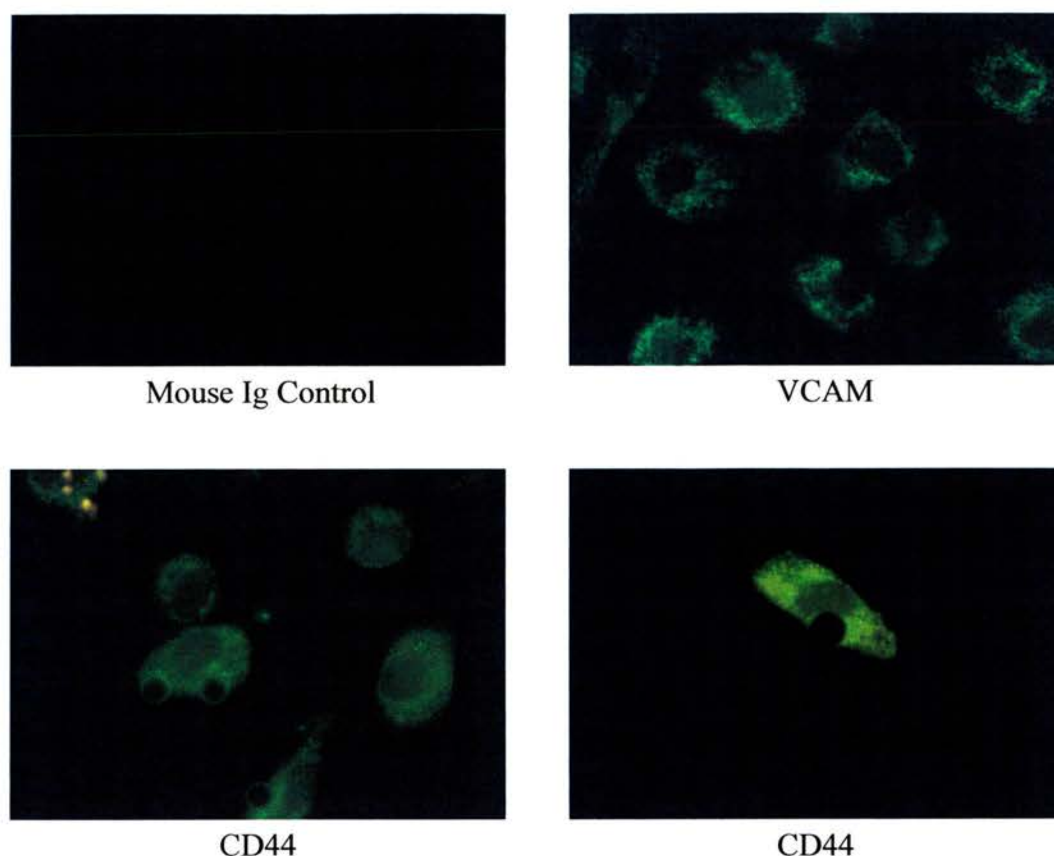



**FIGURE 4.13**

***Changes in PKC distribution in response to CD44 cross-linking on the macrophage surface.***
Macrophages were incubated with either mouse Ig control, IMDM alone, anti-VCAM F(ab')2 fragment conjugated microspheres (VCAM) or anti-CD44 F(ab')2 fragment conjugated microspheres (CD44) for 30min at room temperature. Cells were then washed, fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature and permeabilised before incubation with an anti-PKC antibody for 30min on ice. After further washing, the macrophages were stained with a goat anti-mouse (Fc specific) secondary antibody for 30min on ice. After washing, macrophages were mounted on slides and analysed using immunofluorescent microscopy. These photographs are representative of three experiments.

### ERK

Next, to examine the link between the changes described above and downstream signalling events, macrophage lysates were analysed for ERK1/2 expression and activation. Lysates were made from control and CD44 stimulated macrophages in the presence and absence of PD98059, which inhibits activation of ERK. The lysates were run on a gel and western blotting for both total and phosphorylated (activated) ERK carried out.

The level of total ERK, both activated and unactivated in the samples was of a similar level indicating that protein loading was the same in the different samples (figure 4.14a). Although, the levels of phosphorylated ERK were clearly reduced in response to PD treatment, there were no observable differences between control and CD44-stimulated macrophages in terms of ERK phosphorylation. To investigate the possibility that early or late activation of ERK had been missed, macrophage lysates were made at differing time-points after treatment with anti-CD44 antibody. These lysates were separated by SDS-PAGE, and western blotting used to determine levels of total and phosphorylated ERK. The level of total ERK observed at each time-point was similar as was the level of phosphorylated ERK, suggesting that ERK activation does not occur up to 60 minutes after stimulation of macrophages through CD44 (figure 4.14b). Therefore, it seems very unlikely that ERK activation plays a major role in CD44 augmented phagocytosis.

As an alternative approach, the effect of ERK inhibition upon CD44 augmented phagocytosis was investigated. As before, control and CD44 treated macrophages were treated with PD98059 for 15min prior to assessment of phagocytosis of apoptotic neutrophils. As predicted from the biochemical analysis described above, PD98059 treatment of macrophages had no effect upon either control or CD44 augmented phagocytosis of apoptotic neutrophils (figure 4.15a). This data supports my observation that CD44 stimulated macrophages have similar levels of activated ERK to control macrophages and that ERK does not contribute to the phagocytic process.

In view of my earlier data relating to the effects of CD44 upon migration and the clear role of ERK in some cellular migration events, I decided to examine the role or ERK activation in macrophage migration. Time-lapse microscopy was carried out as previously described. Control and CD44-treated macrophage migration was compared to that in the presence of PD98059 (figure 4.15b). Migration of CD44-

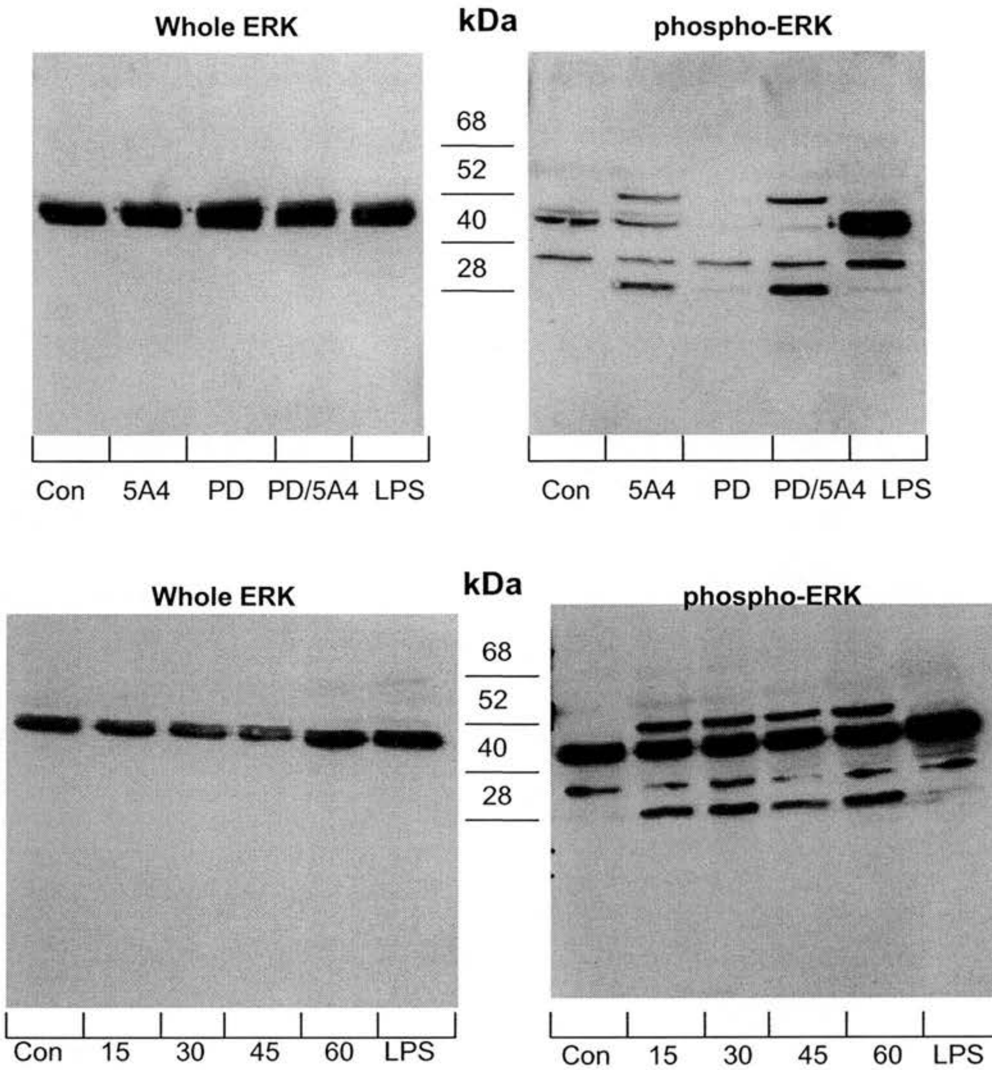



**FIGURE 4.14**

***ERK1/2 expression and activation in response to CD44 stimulation***

a. Macrophages lysates were made from macrophages incubated with IMDM alone or anti-CD44 antibody in the presence and absence of PD98059. The lysates were separated using SDS-PAGE and western blotted for levels of total and phosphorylated ERK.

b. Lysates were made from macrophages incubated with either IMDM alone or CD44-antibody for differing lengths of time. The lysates were then separated by SDS-PAGE and western blotted for levels of total and phosphorylated ERK.

These gels are representative of three experiments.

treated macrophages was once again reduced when compared to untreated macrophages. However, addition of PD98059 reduced the rate of migration in these macrophages even further, suggesting that ERK activity may contribute to migration of CD44-treated macrophages. These results imply that although ERK has a role in the migration of macrophages, it is clearly not required for phagocytosis of apoptotic neutrophils.

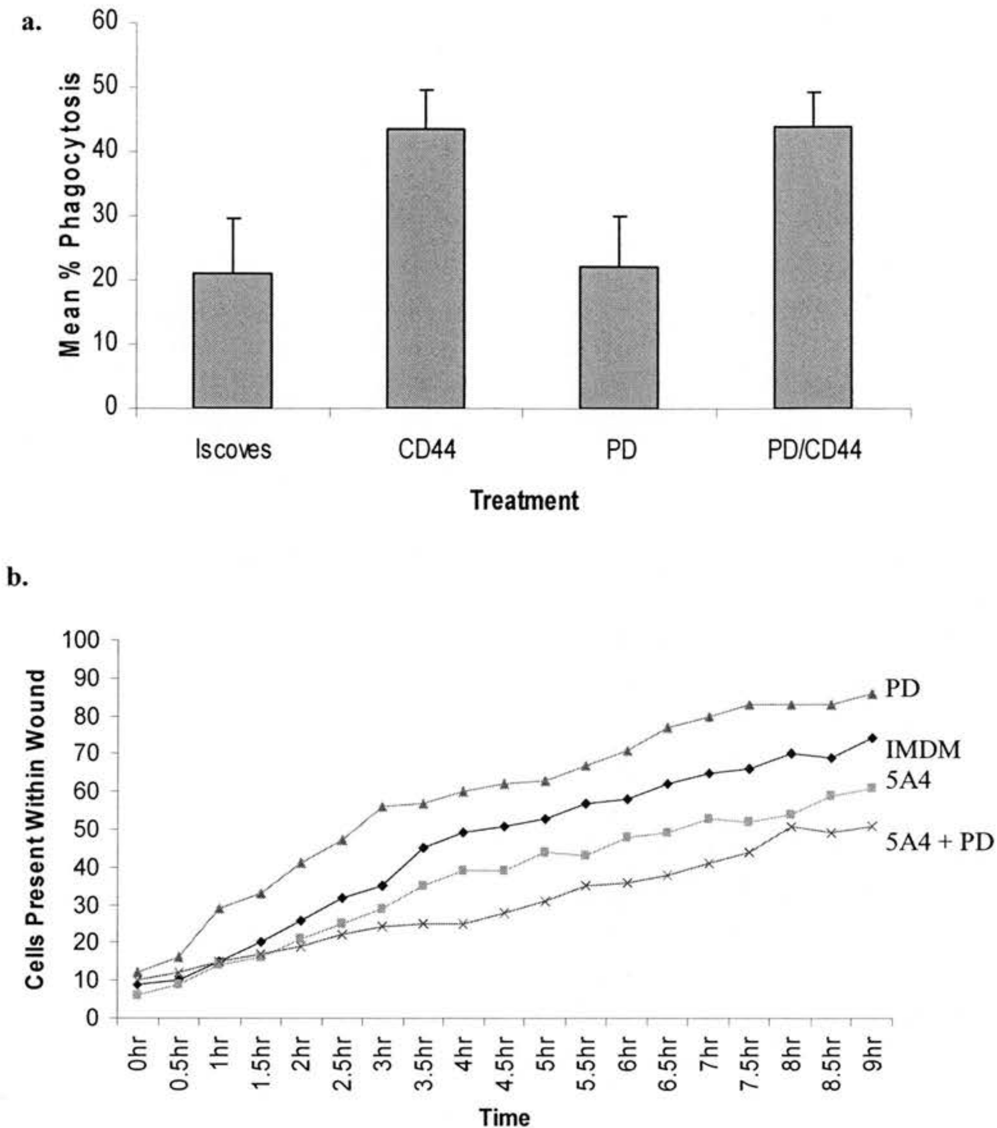



**FIGURE 4.15**

***Analysis of the role of ERK in signalling in response to CD44 cross-linking.***

a. Macrophages were incubated with IMDM alone or anti-CD44 antibody in the presence or absence of PD98059 for 15 min, washed,and incubated with a suspension of aged neutrophils for 60min. After treatment with trypsin/EDTA, the percentage of macrophage phagocytosis was determined using a flow cytometric assay. (n=3)

b. Macrophages were incubated with either IMDM alone (black diamonds), IMDM with PD98059 (grey triangles), anti-CD44 antibody (grey squares) or anti-CD44 antibody with PD98059 (grey crosses) for 15min, washed and the monolayer wounded using a pastette. Migration was recorded over a 20 hour period. (n=3)

### Ezrin

All the changes described above led me to consider whether ezrin was recruited to sites of CD44 cross-linking. I observed that binding of CD44 $F(ab')_2$ coated microspheres to a macrophage resulted in recruitment of ezrin to the area of the macrophage surface where the microsphere was bound (figure 4.16). This was indicated by intense staining for ezrin in the membrane both under and near the microsphere. This contrasted with the staining pattern for ezrin within control macrophages, which was more uniform in appearance. For untreated controls, ezrin was localised under the plasma membrane. Together, these observations suggest that these changes may drive ezrin redistribution and that these alterations play a role in the increase in the macrophage capacity for phagocytosis following CD44 cross-linking.

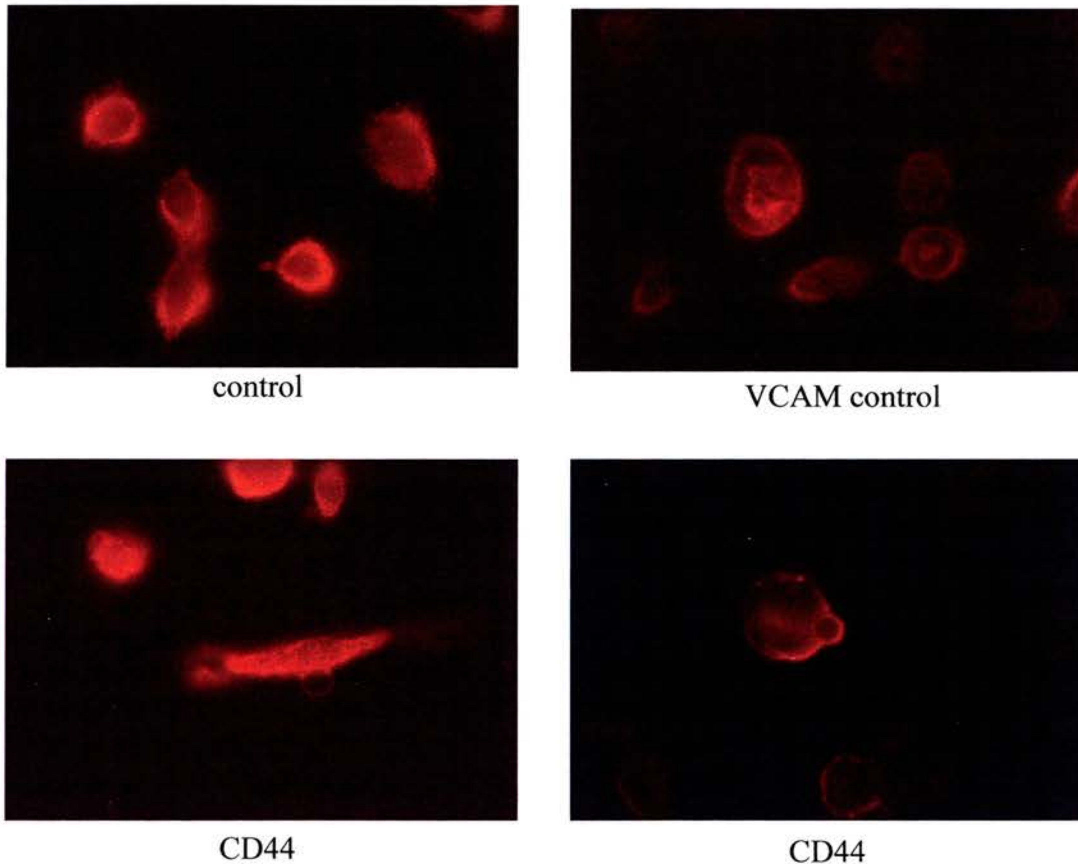



**FIGURE 4.16**

***Localisation of ezrin to areas of cross-linking of CD44 on the macrophage surface***

Macrophages were incubated with either mouse Ig control, IMDM alone, anti-VCAM F(ab')2 fragment conjugated microspheres (VCAM) or anti-CD44 F(ab')2 fragment conjugated microspheres (CD44) for 30min at room temperature. Cells were then washed, fixed with PBS without $Ca^{2+}/Mg^{2+}$ containing 3% paraformaldehyde for 20min at room temperature and permeabilised before incubation with an anti-ezrin antibody for 30min on ice. After further washing, the macrophages were stained with a goat anti-mouse (Fc specific) secondary antibody for 30min on ice. After washing, macrophages were mounted on slides and analysed using immunofluorescent microscopy.

These photographs are representative of three experiments.

## SUMMARY

In early sections of this chapter, it was shown that augmentation of macrophage phagocytosis of apoptotic neutrophils in response to CD44 cross-linking was not a result of redistribution of CD44 molecules. These observations suggested that signalling within the macrophage might contribute to the observed change in phagocytic capacity. To investigate this possibility an assay was developed using anti-CD44 $F(ab')_2$ fragments conjugated to a 6μm microsphere. The microsphere was used as a cross-linker of CD44, which was not internalised by the macrophage. Imaging macrophage responses to cross-linking of CD44 by immunofluorescent microscopy, allowed redistribution of potential components of a CD44-mediated signalling cascade to be investigated. The data presented in this chapter supports a role for actin, ezrin, PKC, Rac2 and talin as potential intermediates involved in augmentation of macrophage phagocytic capacity for apoptotic neutrophils. In contrast, these data suggest that vinculin may not be involved. Moreover, analysis of ERK expression and its function in phagocytosis and migration suggested that although it may have a role in migration, it is not involved in CD44 augmented phagocytosis.

# Chapter 5
# CONCLUSIONS & FUTURE DIRECTIONS

Inadequate clearance of apoptotic cells by macrophages has been shown to contribute to tissue damage and prevent resolution of the inflammatory response, resulting in a variety of chronic diseases. The macrophages ability to phagocytose apoptotic neutrophils can be influenced in a wide range of ways including interactions with soluble mediators or extracellular matrix molecules. One such way is ligation of the hyaluronan receptor, CD44, on the surface of monocyte-derived macrophages. Crosslinking of CD44 by bivalent antibody on the macrophage surface results in an increased capacity for phagocytosis of apoptotic neutrophils. This augmentation was specific for apoptotic neutrophils, suggesting that CD44 has the potential to regulate clearance of apoptotic neutrophils during the resolution of an inflammatory response.

The data presented here suggests that macrophage CD44 ligation by antibody results in augmentation of macrophage phagocytosis, which peaks at 60 minutes. The antibody-induced crosslinking of macrophage CD44 resulted in a long-lived change in macrophage behaviour, which lasted up to 24 hours and possibly longer. CD44 antibody augmentation of phagocytosis was reduced in the absence of divalent cations but not inhibited to as great an extent as baseline phagocytosis. This suggested that the observed CD44/antibody effect consisted of two components – a cation-independent component and a cation-dependent component. Further investigation of the cation-independent component was carried out using a panel of antibodies and ligands known to exert their effects in the absence of cations. Interestingly, an antibody against CD32 (FcγRII) was shown to reduce CD44 augmented phagocytosis entirely in the absence of divalent cations. Experiments blocking CD32 on macrophages or apoptotic neutrophils indicated that it was CD32 on the apoptotic neutrophil that was playing a role in CD44-antibody mediated augmented phagocytosis. These observations suggest that the CD32 on the apoptotic neutrophil was binding the anti-CD44 antibody, 5A4, allowing it to act as a bridge

between the apoptotic neutrophil and the macrophage. It also highlighted the need to use $F(ab')_2$ fragments when carrying out studies of the effects of receptor cross-linking.

The long-lived nature of CD44 antibody-mediated augmentation of phagocytosis would be consistent with the augmentation being the result of changes in surface distribution of CD44, as opposed to generation of an intracellular signal. However, analysis of surface expression and distribution of CD44 on untreated and CD44-treated macrophages by immunofluorescence microscopy did not appear to support this hypothesis. Therefore, I decided to investigate the possibility of a intracellular signal using anti-CD44 $F(ab')_2$ conjugated microspheres. This method may more closely approximate CD44 cross-linking induced by interaction with ligand. Data presented in this thesis demonstrates that binding of these microspheres to the macrophage provided one method for investigating changes in distribution of cytoskeletal and signalling molecules within the macrophage. In response to CD44 cross-linking, changes in actin, ezrin, talin, PKC and Rac2 distribution were observed, highlighting the possibility that they were contributing to CD44 augmented phagocytosis.

These studies provide an insight into possible signalling mechanisms that might contribute to CD44 augmented macrophage phagocytosis of apoptotic neutrophils. Moreover, they form a basis for further work to clarify the signalling mechanisms further and determine their role in augmented phagocytosis. It would be useful to examine further the molecules that my results have shown to localise with ligated CD44. One possible way to investigate this would be using fluorescence resonance energy transfer (FRET), which can be used to show molecules in close proximity to each other. In FRET, a donor fluorophore can transfer energy to an acceptor providing that they are in close enough proximity. This transfer of energy results in changes in fluorescence of the fluorophores that can be detected using fluorescent microscopy. In the absence of receptor associations, uniform staining would be observed. In contract, when FRET occurs there would be patches of different coloured areas where the two molecules of interest are interacting. FRET has

already been used successfully to examine the interaction between ezrin and CD44 (Legg 2002). Therefore, I would use this system further to investigate the potential interaction between CD44 and the molecules studied using the antibody coated microspheres. FRET does have limitations, however, and these would have to taken into account when using this system. One consideration is controlling the concentration of donor and acceptor fluorophores, as FRET occurs most easily when there are several acceptor fluorophores surrounding a donor fluorophore thus surface receptor density has an effect. Moreover, the size and structure of the molecules being investigated would also have to be taken into consideration as the donor and acceptor molecules have to be in close enough proximity to allow energy transfer to occur. Therefore, it would be essential to know where the fluorophores are binding on the molecules under investigation and to understand how this relates to their possible interactions.

Another approach would be to use the "yeast two-hybrid" system. In this system, fusion proteins are constructed containing regions of the molecules of interest, such as Rac2, talin and PKC, and either the binding domain or the activatory domain of the Gal4 gene. The generated fusion proteins can then be transfected into yeast cells using sonication or chemical means. If these proteins interact then the β–Galactosidase gene becomes transcriptionally activated resulting in a white to blue colour change on X-gal medium. This technique could allow identification of signalling molecules co-localising with the cytoplasmic tail of CD44. It would then be important to examine their contribution to the regulation of phagocytosis of apoptotic neutrophils. In order to investigate this, macrophages could be treated with specific inhibitors of signalling molecules to determine whether inhibition has an effect upon CD44-mediated augmented phagocytosis of apoptotic neutrophils. For example, Cytochalasin B could be used to disrupt the actin cytoskeleton allowing investigation of its effects upon CD44-augmented phagocytosis. It is likely that this approach would result in loss of phagocytic ability but it would be interesting to compare both control and CD44-treated macrophages under these conditions. An alternative approach would be to use *Clostridium difficile* toxin B, which selectively inactivates Rac activity. Research has shown Rac2 deficient murine macrophages

have defects in phagocytosis (Yamauchi 2004); therefore it seems highly possible that inhibition of Rac2 would reduce phagocytosis in both control and CD44-treated macrophages.

Another useful approach would be to compare the expression of signalling proteins in both control and CD44-treated macrophages. This would allow any differences in expression between control and CD44-treated macrophages to be highlighted and investigated further. Lysates from control and CD44-treated macrophages could be used in a proteomics based protein expression array. Comparison of protein expression profiles from the two samples would allow changes in protein expression to be identified. These types of arrays can generate a large number of results in a reasonably short period of time, however, they are also very expensive. The results obtained would allow a more targeted approach to investigating the changes stimulated in macrophages in response to CD44-treatment. As an alternative to a protein array, western blotting could be used to compare expression of proteins in macrophage lysates.

In addition, subsequent studies examining the mechanisms and roles of CD44-augmented phagocytosis might use chimeric CD4/CD44 molecules, which could be transfected into macrophages or use of the HIV-TAT protein transduction system to introduce different domains of the CD44 cytoplasmic tail. There are already constructs for two chimeric molecules available, both of which have CD4 extracellular domains with a CD44 intracellular domain. The difference is in the transmembrane domain which is CD4 in one and CD44 in the other. This transmembrane domain in CD44 contains cysteine residues which are known to be important in membrane receptor association (Liu 1996). Transfection of macrophages with these chimeric molecules would allow the role of this intracellular region to be investigated. Moreover, tranfection of CD44 negative cells with CD44 mutants lacking phosphorylation sites, the transmembrane cysteine residues or ezrin binding sites could be compared to wild type CD44 in a "gain of function" approach.

Macrophages from CD44 knockout mice could also be studied in terms of migration and function in inflammatory conditions. However, there may be compensatory mechanisms which function in place of CD44 augmented phagocytosis. An alternative approach would be to use siRNAs (small interfering RNAs) to "knockdown" the expression of the CD44 gene. However, one disadvantage with this approach would be that CD44 is expressed at a high level, meaning that a good level of knockdown would be difficult to achieve.

Another consideration is the difficulties of using a primary cell line. Macrophages are a very heterogeneous population meaning that there is considerable variation in responses when comparing cells derived from different donors. Moreover, it can be difficult getting enough cells for biochemical analysis from human donors and isolation of macrophages can often go wrong. Therefore, it would be worth considering use of a macrophage cell line such as J774 or THP1. However, these have problems in themselves as they don't exhibit true macrophage behaviour.

It would also be beneficial to study the response of macrophages to binding Hyaluronan (HA) instead of anti-CD44 antibody. One approach would be to allow macrophages to adhere to a tissue culture dish coated with HA. These macrophages could then be examined to see if augmented phagocytosis of apoptotic neutrophils takes place in response to HA binding. In addition other potential ligands that bind to CD44 such as Osteopontin could also be examined in this way. This approach would assist in identifying the CD44 ligand that triggers augmented phagocytosis of apoptotic neutrophils by binding to macrophage CD44. Expression of CD44 enhances the ability of fibroblasts to bind to HA and migrate (Peck 1996). Moreover, the binding of HA is regulated during development of macrophages, as monocytes are negative for HA binding whereas macrophages are positive (Culty 1994). As monocytes differentiate they change expression of CD44 isoforms, which may explain this alteration in ability to migrate and bind HA. Therefore, it would been of interest to investigate the effect of different of isoforms on CD44 migration and phagocytosis.

In summary, work presented in this thesis has investigated the role of CD44 in regulating macrophage phagocytosis of apoptotic cells. I have developed a novel microsphere-based system for examining the consequences of CD44 ligation in intact macrophages that could be used to define the underlying mechanisms.

# REFERENCES

Aderem, A. (2003). Phagocytosis and the inflammatory response. J.Infect.Dis. *187 Suppl 2*, S340-S344.

Aderem, A. and Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. Annu.Rev.Immunol. *17*, 593-623.

Akasaki, T., Koga, H., and Sumimoto, H. (1999). Phosphoinositide 3-kinase-dependent and -independent activation of the small GTPase Rac2 in human neutrophils. J.Biol.Chem. *274*, 18055-18059.

Akbar, A. N., Savill, J., Gombert, W., Bofill, M., Borthwick, N. J., Whitelaw, F., Grundy, J., Janossy, G., and Salmon, M. (1994). The specific recognition by macrophages of CD8+,CD45RO+ T cells undergoing apoptosis: a mechanism for T cell clearance during resolution of viral infections. J.Exp.Med. *180*, 1943-1947.

Albelda, S. M., Smith, C. W., and Ward, P. A. (1994). Adhesion molecules and inflammatory injury. FASEB J. *8*, 504-512.

Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. *413*, 732-738.

Allen, L. A. and Aderem, A. (1996). Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. J.Exp.Med. *184*, 627-637.

Amieva, M. R. and Furthmayr, H. (1995). Subcellular localization of moesin in dynamic filopodia, retraction fibers, and other structures involved in substrate exploration, attachment, and cell-cell contacts. Exp.Cell Res. *219*, 180-196.

Anderson, H. A., Maylock, C. A., Williams, J. A., Paweletz, C. P., Shu, H., and Shacter, E. (2003). Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nat.Immunol. *4*, 87-91.

Annema, A., Sluiter, W., and van Furth, R. (1992). Effect of interleukin 1, macrophage colony-stimulating factor, and factor increasing monocytopoiesis on the production of leukocytes in mice. Exp.Hematol. *20*, 69-74.

Arnold, R. R., Russell, J. E., Champion, W. J., Brewer, M., and Gauthier, J. J. (1982). Bactericidal activity of human lactoferrin: differentiation from the stasis of iron deprivation. Infect.Immun. *35*, 792-799.

Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., and Seed, B. (1990). CD44 is the principal cell surface receptor for hyaluronate. Cell. *61*, 1303-1313.

Austyn, J. M. and Gordon, S. (1981). F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur.J.Immunol. *11*, 805-814.

Bainton, D. F. (1980). The cells of inflammation: a general view. In "The cell biology of inflammation, volume 2". G. Weissman, Ed., pp. 1-24. Elsevier, New York.

Bainton, D. F., Ullyot, J. L., and Farquhar, M. G. (1971). The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. J.Exp.Med. *134*, 907-934.

Balasubramanian, K., Chandra, J., and Schroit, A. J. (1997). Immune clearance of phosphatidylserine-expressing cells by phagocytes. The role of beta2-glycoprotein I in macrophage recognition. J.Biol.Chem. *272*, 31113-31117.

Banerji, S., Day, A. J., Kahmann, J. D., and Jackson, D. G. (1998). Characterization of a functional hyaluronan-binding domain from the human CD44 molecule expressed in Escherichia coli. Protein Expr.Purif. *14*, 371-381.

Banerji, S., Ni, J., Wang, S. X., Clasper, S., Su, J., Tammi, R., Jones, M., and Jackson, D. G. (1999). LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J.Cell Biol. *144*, 789-801.

Bartolazzi, A., Jackson, D., Bennett, K., Aruffo, A., Dickinson, R., Shields, J., Whittle, N., and Stamenkovic, I. (1995). Regulation of growth and dissemination of a human lymphoma by CD44 splice variants. J.Cell Sci. *108 ( Pt 4)*, 1723-1733.

Beningo, K. A., Dembo, M., Kaverina, I., Small, J. V., and Wang, Y. L. (2001). Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. J.Cell Biol. *153*, 881-888.

Berger, M., O'Shea, J., Cross, A. S., Folks, T. M., Chused, T. M., Brown, E. J., and Frank, M. M. (1984). Human neutrophils increase expression of C3bi as well as C3b receptors upon activation. J.Clin.Invest. *74*, 1566-1571.

Berryman, M., Franck, Z., and Bretscher, A. (1993). Ezrin is concentrated in the apical microvilli of a wide variety of epithelial cells whereas moesin is found primarily in endothelial cells. J.Cell Sci. *105 ( Pt 4)*, 1025-1043.

Bevers, E. M., Comfurius, P., Dekkers, D. W., and Zwaal, R. F. (1999). Lipid translocation across the plasma membrane of mammalian cells. Biochim.Biophys.Acta. *1439*, 317-330.

Blumberg, P. M. (1988). Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture. Cancer Res. *48*, 1-8.

Blundell, C. D., Mahoney, D. J., Almond, A., DeAngelis, P. L., Kahmann, J. D., Teriete, P., Pickford, A. R., Campbell, I. D., and Day, A. J. (2003). The link module from ovulation- and inflammation-associated protein TSG-6 changes conformation on hyaluronan binding. J.Biol.Chem. *278*, 49261-49270.

Bonilla, F. A., Fujita, R. M., Pivniouk, V. I., Chan, A. C., and Geha, R. S. (2000). Adapter proteins SLP-76 and BLNK both are expressed by murine macrophages and are linked to signaling via Fcgamma receptors I and II/III. Proc.Natl.Acad.Sci.U.S.A. *97*, 1725-1730.

Borowsky, M. L. and Hynes, R. O. (1998). Layilin, a novel talin-binding transmembrane protein homologous with C-type lectins, is localized in membrane ruffles. J.Cell Biol. *143*, 429-442.

Bose, J., Gruber, A. D., Helming, L., Schiebe, S., Wegener, I., Hafner, M., Beales, M., Kontgen, F., and Lengeling, A. (2004). The phosphatidylserine receptor has essential functions during embryogenesis but not in apoptotic cell removal. J.Biol. *3*, 14.

Botto, M. (1998). C1q knock-out mice for the study of complement deficiency in autoimmune disease. Exp.Clin.Immunogenet. *15*, 231-234.

Bourguignon, L. Y., Zhu, H., Chu, A., Iida, N., Zhang, L., and Hung, M. C. (1997). Interaction between the adhesion receptor, CD44, and the oncogene product, p185HER2, promotes human ovarian tumor cell activation. J.Biol.Chem. *272*, 27913-27918.

Bourguignon, L. Y., Zhu, H., Shao, L., and Chen, Y. W. (2000). CD44 interaction with tiam1 promotes Rac1 signaling and hyaluronic acid- mediated breast tumor cell migration. J.Biol.Chem. *275*, 1829-1838.

Bourguignon, L. Y., Zhu, H., Shao, L., and Chen, Y. W. (2001a). CD44 interaction with c-Src kinase promotes cortactin-mediated cytoskeleton function and hyaluronic acid-dependent ovarian tumor cell migration. J.Biol.Chem. *276*, 7327-7336.

Bourguignon, L. Y., Zhu, H., Shao, L., Zhu, D., and Chen, Y. W. (1999). Rho-kinase (ROK) promotes CD44v(3,8-10)-ankyrin interaction and tumor cell migration in metastatic breast cancer cells. Cell Motil.Cytoskeleton. *43*, 269-287.

Bourguignon, L. Y., Zhu, H., Zhou, B., Diedrich, F., Singleton, P. A., and Hung, M. C. (2001b). Hyaluronan promotes CD44v3-Vav2 interaction with Grb2-p185(HER2) and induces Rac1 and Ras signaling during ovarian tumor cell migration and growth. J.Biol.Chem. *276*, 48679-48692.

Brannigan, A. E., O'Connell, P. R., Hurley, H., O'Neill, A., Brady, H. R., Fitzpatrick, J. M., and Watson, R. W. (2000). Neutrophil apoptosis is delayed in patients with inflammatory bowel disease. Shock. *13*, 361-366.

Bretscher, A. (1999). Regulation of cortical structure by the ezrin-radixin-moesin protein family. Curr.Opin.Cell Biol. *11*, 109-116.

Bretscher, A., Edwards, K., and Fehon, R. G. (2002). ERM proteins and merlin: integrators at the cell cortex. Nat.Rev.Mol.Cell Biol. *3*, 586-599.

Brown, E. J. (1986). The role of extracellular matrix proteins in the control of phagocytosis. J.Leukoc.Biol. *39*, 579-591.

Brown, S. B. and Savill, J. (1999). Phagocytosis triggers macrophage release of Fas ligand and induces apoptosis of bystander leukocytes. J.Immunol. *162*, 480-484.

Brugger, W., Kreutz, M., and Andreesen, R. (1991). Macrophage colony-stimulating factor is required for human monocyte survival and acts as a cofactor for their terminal differentiation to macrophages in vitro. J.Leukoc.Biol. *49*, 483-488.

Camp, R. L., Kraus, T. A., and Pure, E. (1991). Variations in the cytoskeletal interaction and posttranslational modification of the CD44 homing receptor in macrophages. J.Cell Biol. *115*, 1283-1292.

Carroll, M. C. (1998). The role of complement and complement receptors in induction and regulation of immunity. Annu.Rev.Immunol. *16*, 545-568.

Chan, J.R., Hyduk, S.J, and Cybulsky, M.I. (2003). Detecting rapid and transient upregulation of leukocyte integrin affinity induced by chemokines and chemoattractants. J. Immunol Methods. *273*. 43-52

Chu, W. M., Ostertag, D., Li, Z. W., Chang, L., Chen, Y., Hu, Y., Williams, B., Perrault, J., and Karin, M. (1999). JNK2 and IKKbeta are required for activating the innate response to viral infection. Immunity. *11*, 721-731.

Cichy, J., Bals, R., Potempa, J., Mani, A., and Pure, E. (2002). Proteinase-mediated release of epithelial cell-associated CD44. Extracellular CD44 complexes with components of cellular matrices. J.Biol.Chem. *277*, 44440-44447.

Cikala, M., Alexandrova, O., David, C. N., Proschel, M., Stiening, B., Cramer, P., and Bottger, A. (2004). The phosphatidylserine receptor from Hydra is a nuclear protein with potential Fe(II) dependent oxygenase activity. BMC.Cell Biol. *5*, 26.

Cooper, D. L. and Dougherty, G. J. (1995). To metastasize or not? Selection of CD44 splice sites. Nat.Med. *1*, 635-637.

Cotran R.S., Kumar V., and Robbins S.L. (1989). Inflammation and Repair. In "Robbins Pathologic Basis of Disease". Cotran R.S., Kumar V., and Robbins S.L., Eds., pp. 39-86. W.B.Saunders Company, Philadelphia.

Couto, J. R., Taylor, M. R., Godwin, S. G., Ceriani, R. L., and Peterson, J. A. (1996). Cloning and sequence analysis of human breast epithelial antigen BA46 reveals an RGD cell adhesion sequence presented on an epidermal growth factor-like domain. DNA Cell Biol. *15*, 281-286.

Crofton, R. W., Diesselhoff-den Dulk, M. M., and van Furth, R. (1978). The origin, kinetics, and characteristics of the Kupffer cells in the normal steady state. J.Exp.Med. *148*, 1-17.

Crowley, M. T., Costello, P. S., Fitzer-Attas, C. J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V. L., and DeFranco, A. L. (1997). A critical role for Syk in signal transduction and phagocytosis mediated by Fcgamma receptors on macrophages. J.Exp.Med. *186*, 1027-1039.

Cui, P., Qin, B., Liu, N., Pan, G., and Pei, D. (2004). Nuclear localization of the phosphatidylserine receptor protein via multiple nuclear localization signals. Exp.Cell Res. *293*, 154-163.

Culty, M., Miyake, K., Kincade, P. W., Sikorski, E., Butcher, E. C., Underhill, C., and Silorski, E. (1990). The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins. J.Cell Biol. *111*, 2765-2774.

Culty, M., O'Mara, T. E., Underhill, C. B., Yeager, H., Jr., and Swartz, R. P. (1994). Hyaluronan receptor (CD44) expression and function in human peripheral blood monocytes and alveolar macrophages. J.Leukoc.Biol. *56*, 605-611.

Daeron, M. (1997). Fc receptor biology. Annu.Rev.Immunol. *15*, 203-234.

Davie, E. W., Fujikawa, K., Kurachi, K., and Kisiel, W. (1979). The role of serine proteases in the blood coagulation cascade. Adv.Enzymol.Relat Areas Mol.Biol. *48*, 277-318.

Day, A. J. and Prestwich, G. D. (2002). Hyaluronan-binding proteins: tying up the giant. J.Biol.Chem. *277*, 4585-4588.

DeGrendele, H. C., Estess, P., Picker, L. J., and Siegelman, M. H. (1996). CD44 and its ligand hyaluronate mediate rolling under physiologic flow: a novel lymphocyte-endothelial cell primary adhesion pathway. J.Exp.Med. *183*, 1119-1130.

DeGrendele, H. C., Kosfiszer, M., Estess, P., and Siegelman, M. H. (1997). CD44 activation and associated primary adhesion is inducible via T cell receptor stimulation. J.Immunol. *159*, 2549-2553.

DeLeo, F. R., Allen, L. A., Apicella, M., and Nauseef, W. M. (1999). NADPH oxidase activation and assembly during phagocytosis. J.Immunol. *163*, 6732-6740.

Dembo, M., Oliver, T., Ishihara, A., and Jacobson, K. (1996). Imaging the traction stresses exerted by locomoting cells with the elastic substratum method. Biophys.J. *70*, 2008-2022.

Devitt, A., Moffatt, O. D., Raykundalia, C., Capra, J. D., Simmons, D. L., and Gregory, C. D. (1998). Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature. *392*, 505-509.

Devitt, A., Parker, K. G., Ogden, C. A., Oldreive, C., Clay, M. F., Melville, L. A., Bellamy, C. O., Lacy-Hulbert, A., Gangloff, S. C., Goyert, S. M., and Gregory, C. D. (2004). Persistence of apoptotic cells without autoimmune disease or inflammation in CD14-/- mice. J.Cell Biol. *167*, 1161-1170.

Didsbury, J., Weber, R. F., Bokoch, G. M., Evans, T., and Snyderman, R. (1989). rac, a novel ras-related family of proteins that are botulinum toxin substrates. J.Biol.Chem. *264*, 16378-16382.

Dini, L., Autuori, F., Lentini, A., Oliverio, S., and Piacentini, M. (1992). The clearance of apoptotic cells in the liver is mediated by the asialoglycoprotein receptor. FEBS Lett. *296*, 174-178.

Dransfield, I., Buckle, A. M., and Hogg, N. (1990). Early events of the immune response mediated by leukocyte integrins. Immunol.Rev. *114*, 29-44.

Dransfield, I., Buckle, A. M., Savill, J. S., McDowall, A., Haslett, C., and Hogg, N. (1994). Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. J.Immunol. *153*, 1254-1263.

Droll, A., Dougherty, S. T., Chiu, R. K., Dirks, J. F., McBride, W. H., Cooper, D. L., and Dougherty, G. J. (1995). Adhesive interactions between alternatively spliced CD44 isoforms. J.Biol.Chem. *270*, 11567-11573.

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. *102*, 33-42.

Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J. H., Patriotis, C., Jenkins, N. A., Copeland, N. G., Kollias, G., and Tsichlis, P. N. (2000). TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell. *103*, 1071-1083.

Duvall, E., Wyllie, A. H., and Morris, R. G. (1985). Macrophage recognition of cells undergoing programmed cell death (apoptosis). Immunology. *56*, 351-358.

Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu.Rev.Biochem. *68*, 383-424.

Ehlers, M. R. (2000). CR3: a general purpose adhesion-recognition receptor essential for innate immunity. Microbes.Infect. *2*, 289-294.

Ellison, R. T., III and Giehl, T. J. (1991). Killing of gram-negative bacteria by lactoferrin and lysozyme. J.Clin.Invest. *88*, 1080-1091.

Ey, P. L., Prowse, S. J., and Jenkin, C. R. (1978). Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. Immunochemistry. *15*, 429-436.

Ezekowitz, R. A. and Stahl, P. D. (1988). The structure and function of vertebrate mannose lectin-like proteins. J.Cell Sci.Suppl. *9*, 121-133.

Faassen, A. E., Schrager, J. A., Klein, D. J., Oegema, T. R., Couchman, J. R., and McCarthy, J. B. (1992). A cell surface chondroitin sulfate proteoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. J.Cell Biol. *116*, 521-531.

Fadok, V. A., Bratton, D. L., Rose, D. M., Pearson, A., Ezekewitz, R. A., and Henson, P. M. (2000). A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature. *405*, 85-90.

Fadok, V. A., de Cathelineau, A., Daleke, D. L., Henson, P. M., and Bratton, D. L. (2001). Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. J.Biol.Chem. *276*, 1071-1077.

Fadok, V. A., McDonald, P. P., Bratton, D. L., and Henson, P. M. (1998). Regulation of macrophage cytokine production by phagocytosis of apoptotic and post-apoptotic cells. Biochem.Soc.Trans. *26*, 653-656.

Fadok, V. A., Warner, M. L., Bratton, D. L., and Henson, P. M. (1998). CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). J.Immunol. *161*, 6250-6257.

Falcone, F. H., Rossi, A. G., Sharkey, R., Brown, A. P., Pritchard, D. I., and Maizels, R. M. (2001). Ascaris suum-derived products induce human neutrophil activation via a G protein-coupled receptor that interacts with the interleukin-8 receptor pathway. Infect.Immun. *69*, 4007-4018.

Familian, A., Zwart, B., Huisman, H. G., Rensink, I., Roem, D., Hordijk, P. L., Aarden, L. A., and Hack, C. E. (2001). Chromatin-independent binding of serum amyloid P component to apoptotic cells. J.Immunol. *167*, 647-654.

Fenton, M. J. and Golenbock, D. T. (1998). LPS-binding proteins and receptors. J.Leukoc.Biol. *64*, 25-32.

Fitzer-Attas, C. J., Lowry, M., Crowley, M. T., Finn, A. J., Meng, F., DeFranco, A. L., and Lowell, C. A. (2000). Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. J.Exp.Med. *191*, 669-682.

Forman, H. J. and Torres, M. (2002). Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. Am.J.Respir.Crit Care Med. *166*, S4-S8.

Franc, N. C., Heitzler, P., Ezekowitz, R. A., and White, K. (1999). Requirement for croquemort in phagocytosis of apoptotic cells in Drosophila. Science. *284*, 1991-1994.

Gabay, C. and Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation. N.Engl.J.Med. *340*, 448-454.

Gabbiani, G. (2003). The myofibroblast in wound healing and fibrocontractive diseases. J.Pathol. *200*, 500-503.

Gautreau, A., Louvard, D., and Arpin, M. (2000). Morphogenic effects of ezrin require a phosphorylation-induced transition from oligomers to monomers at the plasma membrane. J.Cell Biol. *150*, 193-203.

Gershov, D., Kim, S., Brot, N., and Elkon, K. B. (2000). C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. J.Exp.Med. *192*, 1353-1364.

Ghiran, I., Barbashov, S. F., Klickstein, L. B., Tas, S. W., Jensenius, J. C., and Nicholson-Weller, A. (2000). Complement receptor 1/CD35 is a receptor for mannan-binding lectin. J.Exp.Med. *192*, 1797-1808.

Giles, K. M., Hart, S. P., Haslett, C., Rossi, A. G., and Dransfield, I. (2000). An appetite for apoptotic cells? Controversies and challenges. Br.J.Haematol. *109*, 1-12.

Giles, K. M., Ross, K., Rossi, A. G., Hotchin, N. A., Haslett, C., and Dransfield, I. (2001). Glucocorticoid augmentation of macrophage capacity for phagocytosis of apoptotic cells is associated with reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation, and high levels of active Rac. J.Immunol. *167*, 976-986.

Goldmann, W. H., Kaufmann, S., and Isenberg, G. (1992). Thermodynamics of talin and talin-vinculin binding to G-actin. Biochem.Soc.Trans. *20*, 50S.

Goldstein, I. M., Kaplan, H. B., Radin, A., and Frosch, M. (1976). Independent effects of IgG and complement upon human polymorphonuclear leukocyte function. J.Immunol. *117*, 1282-1287.

Goldstein, L. A., Zhou, D. F., Picker, L. J., Minty, C. N., Bargatze, R. F., Ding, J. F., and Butcher, E. C. (1989). A human lymphocyte homing receptor, the hermes antigen, is related to cartilage proteoglycan core and link proteins. Cell. *56*, 1063-1072.

Gordon, S. (1995). The macrophage. Bioessays. *17*, 977-986.

Gordon, S., Crocker, P. R., Morris, L., Lee, S. H., Perry, V. H., and Hume, D. A. (1986). Localization and function of tissue macrophages. Ciba Found.Symp. *118*, 54-67.

Green, C. E., Pearson, D. N., Christensen, N. B., and Simon, S. I. (2003). Topographic requirements and dynamics of signaling via L-selectin on neutrophils. Am.J.Physiol Cell Physiol. *284*, C705-C717.

Griffin, F. M., Jr., Bianco, C., and Silverstein, S. C. (1975). Characterization of the macrophage receptro for complement and demonstration of its functional independence from the receptor for the Fc portion of immunoglobulin G. J.Exp.Med. *141*, 1269-1277.

Griffin, J. H. (1978). Role of surface in surface-dependent activation of Hageman factor (blood coagulation factor XII). Proc.Natl.Acad.Sci.U.S.A. *75*, 1998-2002.

Gunthert, U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Haussmann, I., Matzku, S., Wenzel, A., Ponta, H., and Herrlich, P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell. *65*, 13-24.

Habets, G. G., Scholtes, E. H., Zuydgeest, D., van der Kammen, R. A., Stam, J. C., Berns, A., and Collard, J. G. (1994). Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. Cell. *77*, 537-549.

Hagimoto, N., Kuwano, K., Miyazaki, H., Kunitake, R., Fujita, M., Kawasaki, M., Kaneko, Y., and Hara, N. (1997). Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen. Am.J.Respir.Cell Mol.Biol. *17*, 272-278.

Hakansson L., Hallgren R., and Venge P. (1980). Regulation of granulocyte function by hyaluronic acid. In vitro and in vivo effects on phagocytosis, locomotion, and metabolism. J Clin Invest. *66*, 298-304.

Hall, C. L., Wang, C., Lange, L. A., and Turley, E. A. (1994). Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity. J.Cell Biol. *126*, 575-588.

Hardwick, C., Hoare, K., Owens, R., Hohn, H. P., Hook, M., Moore, D., Cripps, V., Austen, L., Nance, D. M., and Turley, E. A. (1992). Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. J.Cell Biol. *117*, 1343-1350.

Hart, S. P., Dougherty, G. J., Haslett, C., and Dransfield, I. (1997). CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages. J.Immunol. *159*, 919-924.

Hart, S. P., Jackson, C., Kremmel, L. M., McNeill, M. S., Jersmann, H., Alexander, K. M., Ross, J. A., and Dransfield, I. (2003). Specific binding of an antigen-antibody complex to apoptotic human neutrophils. Am.J.Pathol. *162*, 1011-1018.

Hart, S. P., Ross, J. A., Ross, K., Haslett, C., and Dransfield, I. (2000). Molecular characterization of the surface of apoptotic neutrophils: implications for functional downregulation and recognition by phagocytes. Cell Death.Differ. *7*, 493-503.

Haslett, C. (1992). Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. Clin.Sci.(Lond). *83*, 639-648.

Haslett, C., Savill, J. S., Whyte, M. K., Stern, M., Dransfield, I., and Meagher, L. C. (1994). Granulocyte apoptosis and the control of inflammation. Philos.Trans.R.Soc.Lond B Biol.Sci. *345*, 327-333.

Hemmings, L., Rees, D. J., Ohanian, V., Bolton, S. J., Gilmore, A. P., Patel, B., Priddle, H., Trevithick, J. E., Hynes, R. O., and Critchley, D. R. (1996). Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. J.Cell Sci. *109 (Pt 11)*, 2715-2726.

Henke, C. A., Roongta, U., Mickelson, D. J., Knutson, J. R., and McCarthy, J. B. (1996). CD44-related chondroitin sulfate proteoglycan, a cell surface receptor implicated with tumor cell invasion, mediates endothelial cell migration on fibrinogen and invasion into a fibrin matrix. J.Clin.Invest. *97*, 2541-2552.

Henson, P. M., Bratton, D. L., and Fadok, V. A. (2001). Apoptotic cell removal. Curr.Biol. *11*, R795-R804.

Hoebe, K., Georgel, P., Rutschmann, S., Du, X., Mudd, S., Crozat, K., Sovath, S., Shamel, L., Hartung, T., Zahringer, U., and Beutler, B. (2005). CD36 is a sensor of diacylglycerides. Nature. *433*, 523-527.

Hoefsmit, E.C., Duijvestijn, A.M., and Kamperdijk, E.W. (1982). Relation between langerhans cells, veiled cells, and interdigitating cells. Immunobiology. *161*, 255-265

Hofmann, M., Rudy, W., Gunthert, U., Zimmer, S. G., Zawadzki, V., Zoller, M., Lichtner, R. B., Herrlich, P., and Ponta, H. (1993). A link between ras and metastatic behavior of tumor cells: ras induces CD44 promoter activity and leads to low-level expression of metastasis-specific variants of CD44 in CREF cells. Cancer Res. *53*, 1516-1521.

Hsu, H. Y., Hajjar, D. P., Khan, K. M., and Falcone, D. J. (1998). Ligand binding to macrophage scavenger receptor-A induces urokinase-type plasminogen activator expression by a protein kinase-dependent signaling pathway. J.Biol.Chem. *273*, 1240-1246.

Hume, D. A., Ross, I. L., Himes, S. R., Sasmono, R. T., Wells, C. A., and Ravasi, T. (2002). The mononuclear phagocyte system revisited. J.Leukoc.Biol. *72*, 621-627.

Hurley, J. V. (1983). Terminations of acute inflammation I. Resolution. In "Acute Inflammation". J. V. Hurley, Ed., pp. 109-117. Churchill Livingstone, London.

Ideda, K., Sannoh, T., Kawasaki, N., and Yamashina, I. (1987). Serum lectin with known structure activates complement through the classical pathway. J.Biol.Chem. *262*, 7451.

Ii, M., Kurata, H., Itoh, N., Yamashina, I., and Kawasaki, T. (1990). Molecular cloning and sequence analysis of cDNA encoding the macrophage lectin specific for galactose and N-acetylgalactosamine. J.Biol.Chem. *265*, 11295-11298.

Isacke, C. M. and Yarwood, H. (2002). The hyaluronan receptor, CD44. Int.J.Biochem.Cell Biol. *34*, 718-721.

Iyoda, T. and Kobayashi, Y. (2004). Involvement of MIP-2 and CXCR2 in neutrophil infiltration following injection of late apoptotic cells into the peritoneal cavity. Apoptosis. *9*, 485-493.

Jalkanen, S. and Jalkanen, M. (1992). Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. J.Cell Biol. *116*, 817-824.

Jalkanen, S., Reichert, R. A., Gallatin, W. M., Bargatze, R. F., Weissman, I. L., and Butcher, E. C. (1986). Homing receptors and the control of lymphocyte migration. Immunol.Rev. *91*, 39-60.

Janeway, C. A., Jr. (1992). The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol.Today. *13*, 11-16.

Jersmann, H. P., Ross, K. A., Vivers, S., Brown, S. B., Haslett, C., and Dransfield, I. (2003). Phagocytosis of apoptotic cells by human macrophages: Analysis by multiparameter flow cytometry. Cytometry. *51A*, 7-14.

Jones, H. A., Schofield, J. B., Krausz, T., Boobis, A. R., and Haslett, C. (1998). Pulmonary fibrosis correlates with duration of tissue neutrophil activation. Am.J.Respir.Crit Care Med. *158*, 620-628.

Kagan, V. E., Fabisiak, J. P., Shvedova, A. A., Tyurina, Y. Y., Tyurin, V. A., Schor, N. F., and Kawai, K. (2000). Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. FEBS Lett. *477*, 1-7.

Kajita, M., Itoh, Y., Chiba, T., Mori, H., Okada, A., Kinoh, H., and Seiki, M. (2001). Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. J.Cell Biol. *153*, 893-904.

Kam, L. and Boxer, S. G. (2001). Cell adhesion to protein-micropatterned-supported lipid bilayer membranes. J.Biomed.Mater.Res. *55*, 487-494.

Kaplan, A. P. and Austen, K. F. (1971). A prealbumin activator of prekallikrein. II. Derivation of activators of prekallikrein from active Hageman factor by digestion with plasmin. J.Exp.Med. *133*, 696-712.

Kaplan, G. (1977). Differences in the mode of phagocytosis with Fc and C3 receptors in macrophages. Scand.J.Immunol. *6*, 797-807.

Kato, T., Takeda, Y., Nakada, T., and Sendo, F. (1995). Inhibition by dexamethasone of human neutrophil apoptosis in vitro. Nat.Immun. *14*, 198-208.

Katoh, S., McCarthy, J. B., and Kincade, P. W. (1994). Characterization of soluble CD44 in the circulation of mice. Levels are affected by immune activity and tumor growth. J.Immunol. *153*, 3440-3449.

Katoh, S., Taniguchi, H., Matsubara, Y., Matsumoto, N., Fukushima, K., Kadota, J., Matsukura, S., and Kohno, S. (1999). Overexpression of CD44 on alveolar eosinophils with high concentrations of soluble CD44 in bronchoalveolar lavage fluid in patients with eosinophilic pneumonia. Allergy. *54*, 1286-1292.

Katoh, S., Zheng, Z., Oritani, K., Shimozato, T., and Kincade, P. W. (1995). Glycosylation of CD44 negatively regulates its recognition of hyaluronan. J.Exp.Med. *182*, 419-429.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br.J.Cancer. *26*, 239-257.

Kiefer, F., Brumell, J., Al Alawi, N., Latour, S., Cheng, A., Veillette, A., Grinstein, S., and Pawson, T. (1998). The Syk protein tyrosine kinase is essential for Fcgamma receptor signaling in macrophages and neutrophils. Mol.Cell Biol. *18*, 4209-4220.

Kim, W. S., Ordija, C. M., and Freeman, M. W. (2003). Activation of signaling pathways by putative scavenger receptor class A (SR-A) ligands requires CD14 but not SR-A. Biochem.Biophys.Res.Commun. *310*, 542-549.

Knudson, C. B. and Knudson, W. (1993). Hyaluronan-binding proteins in development, tissue homeostasis, and disease. FASEB J. *7*, 1233-1241.

Kohda, D., Morton, C. J., Parkar, A. A., Hatanaka, H., Inagaki, F. M., Campbell, I. D., and Day, A. J. (1996). Solution structure of the link module: a hyaluronan-

binding domain involved in extracellular matrix stability and cell migration. Cell. *86*, 767-774.

Kreutzberg, G. W. (1996). Microglia: a sensor for pathological events in the CNS. Trends Neurosci. *19*, 312-318.

Kuhlman, M., Joiner, K., and Ezekowitz, R. A. (1989). The human mannose-binding protein functions as an opsonin. J.Exp.Med. *169*, 1733-1744.

Kunisaki, Y., Masuko, S., Noda, M., Inayoshi, A., Sanui, T., Harada, M., Sasazuki, T., and Fukui, Y. (2004). Defective fetal liver erythropoiesis and T lymphopoiesis in mice lacking the phosphatidylserine receptor. Blood. *103*, 3362-3364.

Kurosaka, K., Takahashi, M., Watanabe, N., and Kobayashi, Y. (2003). Silent cleanup of very early apoptotic cells by macrophages. J.Immunol. *171*, 4672-4679.

Kusner, D. J., Hall, C. F., and Jackson, S. (1999). Fc gamma receptor-mediated activation of phospholipase D regulates macrophage phagocytosis of IgG-opsonized particles. J.Immunol. *162*, 2266-2274.

Kuwano, K., Hagimoto, N., Kawasaki, M., Yatomi, T., Nakamura, N., Nagata, S., Suda, T., Kunitake, R., Maeyama, T., Miyazaki, H., and Hara, N. (1999). Essential roles of the Fas-Fas ligand pathway in the development of pulmonary fibrosis. J.Clin.Invest. *104*, 13-19.

Lachmann, P. J. and Hughes-Jones, N. C. (1984). Initiation of complement activation. Springer Semin.Immunopathol. *7*, 143-162.

Laible, N. J. and Germaine, G. R. (1985). Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against Streptococcus sanguis and Streptococcus faecalis: inhibition by chitin oligosaccharides. Infect.Immun. *48*, 720-728.

Lapinet, J. A., Scapini, P., Calzetti, F., Perez, O., and Cassatella, M. A. (2000). Gene expression and production of tumor necrosis factor alpha, interleukin-1beta (IL-1beta), IL-8, macrophage inflammatory protein 1alpha (MIP-1alpha), MIP-1beta, and gamma interferon-inducible protein 10 by human neutrophils stimulated with group B meningococcal outer membrane vesicles. Infect.Immun. *68*, 6917-6923.

Laurent, C. and Hellstrom, S. (1992). Fetal cleft lip repair in rabbits: histology and role of hyaluronic acid. J.Oral Maxillofac.Surg. *50*, 1252-1253.

Law, S. K. (1988). C3 receptors on macrophages. J.Cell Sci.Suppl. *9*, 67-97.

Lazaar, A. L., Plotnick, M. I., Kucich, U., Crichton, I., Lotfi, S., Das, S. K., Kane, S., Rosenbloom, J., Panettieri, R. A., Jr., Schechter, N. M., and Pure, E. (2002). Mast cell chymase modifies cell-matrix interactions and inhibits mitogen-induced proliferation of human airway smooth muscle cells. J.Immunol. *169*, 1014-1020.

Legg, J. W., Lewis, C. A., Parsons, M., Ng, T., and Isacke, C. M. (2002). A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility. Nat.Cell Biol. *4*, 399-407.

Lennartz, M. R., Yuen, A. F., Masi, S. M., Russell, D. G., Buttle, K. F., and Smith, J. J. (1997). Phospholipase A2 inhibition results in sequestration of plasma membrane into electronlucent vesicles during IgG-mediated phagocytosis. J.Cell Sci. *110 ( Pt 17)*, 2041-2052.

Lesley, J., English, N., Perschl, A., Gregoroff, J., and Hyman, R. (1995). Variant cell lines selected for alterations in the function of the hyaluronan receptor CD44 show differences in glycosylation. J.Exp.Med. *182*, 431-437.

Lesley, J., Hyman, R., and Kincade, P. W. (1993). CD44 and its interaction with extracellular matrix. Adv.Immunol. *54*, 271-334.

Levesque, M. C. and Haynes, B. F. (1996). In vitro culture of human peripheral blood monocytes induces hyaluronan binding and up-regulates monocyte variant CD44 isoform expression. J.Immunol. *156*, 1557-1564.

Li, M., Carpio, D. F., Zheng, Y., Bruzzo, P., Singh, V., Ouaaz, F., Medzhitov, R. M., and Beg, A. A. (2001). An essential role of the NF-kappa B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. J.Immunol. *166*, 7128-7134.

Li, M. O., Sarkisian, M. R., Mehal, W. Z., Rakic, P., and Flavell, R. A. (2003). Phosphatidylserine receptor is required for clearance of apoptotic cells. Science. *302*, 1560-1563.

Liu, Q. A. and Hengartner, M. O. (1998). Candidate adaptor protein CED-6 promotes the engulfment of apoptotic cells in C. elegans. Cell. *93*, 961-972.

Liu, Y., Cousin, J. M., Hughes, J., Van Damme, J., Seckl, J. R., Haslett, C., Dransfield, I., Savill, J., and Rossi, A. G. (1999). Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes. J.Immunol. *162*, 3639-3646.

Lugini, L., Lozupone, F., Matarrese, P., Funaro, C., Luciani, F., Malorni, W., Rivoltini, L., Castelli, C., Tinari, A., Piris, A., Parmiani, G., and Fais, S. (2003).

Potent phagocytic activity discriminates metastatic and primary human malignant melanomas: a key role of ezrin. Lab Invest. *83*, 1555-1567.

Mackay, C. R., Terpe, H. J., Stauder, R., Marston, W. L., Stark, H., and Gunthert, U. (1994). Expression and modulation of CD44 variant isoforms in humans. J.Cell Biol. *124*, 71-82.

Mariani, T. J., Sandefur, S., Roby, J. D., and Pierce, R. A. (1998). Collagenase-3 induction in rat lung fibroblasts requires the combined effects of tumor necrosis factor-alpha and 12-lipoxygenase metabolites: a model of macrophage-induced, fibroblast-driven extracellular matrix remodeling during inflammatory lung injury. Mol.Biol.Cell. *9*, 1411-1424.

Marshall, J. G., Booth, J. W., Stambolic, V., Mak, T., Balla, T., Schreiber, A. D., Meyer, T., and Grinstein, S. (2001). Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fc gamma receptor-mediated phagocytosis. J.Cell Biol. *153*, 1369-1380.

Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S., and Tsukita, S. (1998). Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. J.Cell Biol. *140*, 647-657.

May, R. C., Caron, E., Hall, A., and Machesky, L. M. (2000). Involvement of the Arp2/3 complex in phagocytosis mediated by FcgammaR or CR3. Nat.Cell Biol. *2*, 246-248.

McCutcheon, J. C., Hart, S. P., Canning, M., Ross, K., Humphries, M. J., and Dransfield, I. (1998). Regulation of macrophage phagocytosis of apoptotic neutrophils by adhesion to fibronectin. J.Leukoc.Biol. *64*, 600-607.

McGreal, E. P., Martinez-Pomares, L., and Gordon, S. (2004). Divergent roles for C-type lectins expressed by cells of the innate immune system. Mol.Immunol. *41*, 1109-1121.

McKee, C. M., Penno, M. B., Cowman, M., Burdick, M. D., Strieter, R. M., Bao, C., and Noble, P. W. (1996). Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. J.Clin.Invest. *98*, 2403-2413.

McKnight, A. J. and Gordon, S. (1998). Membrane molecules as differentiation antigens of murine macrophages. Adv.Immunol. *68*, 271-314.

Meszaros, A. J., Reichner, J. S., and Albina, J. E. (2000). Macrophage-induced neutrophil apoptosis. J.Immunol. *165*, 435-441.

Meuret, G. and Hoffmann, G. (1973). Monocyte kinetic studies in normal and disease states. Br.J.Haematol. *24*, 275-284.

Mevorach, D. (2000). Opsonization of apoptotic cells. Implications for uptake and autoimmunity. Ann.N.Y.Acad.Sci. *926*, 226-234.

Mevorach, D., Zhou, J. L., Song, X., and Elkon, K. B. (1998). Systemic exposure to irradiated apoptotic cells induces autoantibody production. J.Exp.Med. *188*, 387-392.

Michiels, F., Habets, G. G., Stam, J. C., van der Kammen, R. A., and Collard, J. G. (1995). A role for Rac in Tiam1-induced membrane ruffling and invasion. Nature. *375*, 338-340.

Mikecz, K., Brennan, F. R., Kim, J. H., and Glant, T. T. (1995). Anti-CD44 treatment abrogates tissue oedema and leukocyte infiltration in murine arthritis. Nat.Med. *1*, 558-563.

Miller, Y. I., Viriyakosol, S., Binder, C. J., Feramisco, J. R., Kirkland, T. N., and Witztum, J. L. (2003). Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. J.Biol.Chem. *278*, 1561-1568.

Milstone, L. M., Hough-Monroe, L., Kugelman, L. C., Bender, J. R., and Haggerty, J. G. (1994). Epican, a heparan/chondroitin sulfate proteoglycan form of CD44, mediates cell-cell adhesion. J.Cell Sci. *107 (Pt 11)*, 3183-3190.

Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M. (1995). Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J.Cell Biol. *131*, 791-804.

Moffatt, O. D., Devitt, A., Bell, E. D., Simmons, D. L., and Gregory, C. D. (1999a). Macrophage recognition of ICAM-3 on apoptotic leukocytes. J.Immunol. *162*, 6800-6810.

Moffatt, O. D., Devitt, A., Bell, E. D., Simmons, D. L., and Gregory, C. D. (1999b). Macrophage recognition of ICAM-3 on apoptotic leukocytes. J.Immunol. *162*, 6800-6810.

Moon, S. Y. and Zheng, Y. (2003). Rho GTPase-activating proteins in cell regulation. Trends Cell Biol. *13*, 13-22.

Mortensen, R. F., Osmand, A. P., Lint, T. F., and Gewurz, H. (1976). Interaction of C-reactive protein with lymphocytes and monocytes: complement-dependent adherence and phagocytosis. J.Immunol. *117*, 774-781.

Mosser, D. M. (2003). The many faces of macrophage activation. J.Leukoc.Biol. *73*, 209-212.

Muller-Eberhard, H. J. (1986). The membrane attack complex of complement. Annu.Rev.Immunol. *4*, 503-528.

Nagata, S. (1997). Apoptosis by death factor. Cell. *88*, 355-364.

Nakamura, F., Amieva, M. R., and Furthmayr, H. (1995). Phosphorylation of threonine 558 in the carboxyl-terminal actin-binding domain of moesin by thrombin activation of human platelets. J.Biol.Chem. *270*, 31377-31384.

Nakamura, T., Yamashita, H., Nagano, Y., Takahashi, T., Avraham, S., Avraham, H., Matsumoto, M., and Nakamura, S. (2002). Activation of Pyk2/RAFTK induces tyrosine phosphorylation of alpha-synuclein via Src-family kinases. FEBS Lett. *521*, 190-194.

Nakano, T., Ishimoto, Y., Kishino, J., Umeda, M., Inoue, K., Nagata, K., Ohashi, K., Mizuno, K., and Arita, H. (1997). Cell adhesion to phosphatidylserine mediated by a product of growth arrest-specific gene 6. J.Biol.Chem. *272*, 29411-29414.

Nathan, C. F. (1987). Secretory products of macrophages. J.Clin.Invest. *79*, 319-326.

Navratil, J. S., Korb, L. C., and Ahearn, J. M. (1999). Systemic lupus erythematosus and complement deficiency: clues to a novel role for the classical complement pathway in the maintenance of immune tolerance. Immunopharmacology. *42*, 47-52.

Ng, T., Parsons, M., Hughes, W. E., Monypenny, J., Zicha, D., Gautreau, A., Arpin, M., Gschmeissner, S., Verveer, P. J., Bastiaens, P. I., and Parker, P. J. (2001). Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility. EMBO J. *20*, 2723-2741.

Nibbering, P. H., Leijh, P. C., and van Furth, R. (1987). Quantitative immunocytochemical characterization of mononuclear phagocytes. II. Monocytes and tissue macrophages. Immunology. *62*, 171-176.

Noble, P. W., Lake, F. R., Henson, P. M., and Riches, D. W. (1993). Hyaluronate activation of CD44 induces insulin-like growth factor-1 expression by a tumor necrosis factor-alpha-dependent mechanism in murine macrophages. J.Clin.Invest. *91*, 2368-2377.

Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., and Nagata, S. (1993). Lethal effect of the anti-Fas antibody in mice. Nature. *364*, 806-809.

Okamoto, I., Kawano, Y., Tsuiki, H., Sasaki, J., Nakao, M., Matsumoto, M., Suga, M., Ando, M., Nakajima, M., and Saya, H. (1999). CD44 cleavage induced by a membrane-associated metalloprotease plays a critical role in tumor cell migration. Oncogene. *18*, 1435-1446.

Oliferenko, S., Kaverina, I., Small, J. V., and Huber, L. A. (2000). Hyaluronic acid (HA) binding to CD44 activates Rac1 and induces lamellipodia outgrowth. J.Cell Biol. *148*, 1159-1164.

Oliver, T., Dembo, M., and Jacobson, K. (1999). Separation of propulsive and adhesive traction stresses in locomoting keratocytes. J.Cell Biol. *145*, 589-604.

Orr, W., Varani, J., and Ward, P. A. (1978). Characteristics of the chemotactic response of neoplastic cells to a factor derived from the fifth component of complement. Am.J.Pathol. *93*, 405-422.

Parkos, C. A. (1997). Molecular events in neutrophil transepithelial migration. Bioessays. *19*, 865-873.

Peach, R. J., Hollenbaugh, D., Stamenkovic, I., and Aruffo, A. (1993). Identification of hyaluronic acid binding sites in the extracellular domain of CD44. J.Cell Biol. *122*, 257-264.

Peiser, L., Mukhopadhyay, S., and Gordon, S. (2002). Scavenger receptors in innate immunity. Curr.Opin.Immunol. *14*, 123-128.

Picker, L. J., Nakache, M., and Butcher, E. C. (1989). Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. J.Cell Biol. *109*, 927-937.

Pierres, A., Lipcey, C., Mawas, C., and Olive, D. (1992). A unique CD44 monoclonal antibody identifies a new T cell activation pathway. Eur.J.Immunol. *22*, 413-417.

Pittoni, V., Ravirajan, C. T., Donohoe, S., MacHin, S. J., Lydyard, P. M., and Isenberg, D. A. (2000). Human monoclonal anti-phospholipid antibodies selectively bind to membrane phospholipid and beta2-glycoprotein I (beta2-GPI) on apoptotic cells. Clin.Exp.Immunol. *119*, 533-543.

Poltorak, A., Ricciardi-Castagnoli, P., Citterio, S., and Beutler, B. (2000). Physical contact between lipopolysaccharide and toll-like receptor 4 revealed by genetic complementation. Proc.Natl.Acad.Sci.U.S.A. *97*, 2163-2167.

Pommier, C. G., Inada, S., Fries, L. F., Takahashi, T., Frank, M. M., and Brown, E. J. (1983). Plasma fibronectin enhances phagocytosis of opsonized particles by human peripheral blood monocytes. J.Exp.Med. *157*, 1844-1854.

Price, E. A., Coombe, D. R., and Murray, J. C. (1996). Endothelial CD44H mediates adhesion of a melanoma cell line to quiescent human endothelial cells in vitro. Int.J.Cancer. *65*, 513-518.

Priddle, H., Hemmings, L., Monkley, S., Woods, A., Patel, B., Sutton, D., Dunn, G. A., Zicha, D., and Critchley, D. R. (1998). Disruption of the talin gene compromises focal adhesion assembly in undifferentiated but not differentiated embryonic stem cells. J.Cell Biol. *142*, 1121-1133.

Pulford, K. A., Sipos, A., Cordell, J. L., Stross, W. P., and Mason, D. Y. (1990). Distribution of the CD68 macrophage/myeloid associated antigen. Int.Immunol. *2*, 973-980.

Rameshwar, P., Chang, V. T., and Gascon, P. (1996). Implication of CD44 in adhesion-mediated overproduction of TGF-beta and IL-1 in monocytes from patients with bone marrow fibrosis. Br.J.Haematol. *93*, 22-29.

Ravetch, J. V. and Bolland, S. (2001). IgG Fc receptors. Annu.Rev.Immunol. *19*, 275-290.

Regoli, D. and Barabe, J. (1980). Pharmacology of bradykinin and related kinins. Pharmacol.Rev. *32*, 1-46.

Ren, Y. and Savill, J. (1995). Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis. J.Immunol. *154*, 2366-2374.

Ridley, A. J. (2001). Rho proteins, PI 3-kinases, and monocyte/macrophage motility. FEBS Lett. *498*, 168-171.

Ristamaki, R., Joensuu, H., Gron-Virta, K., Salmi, M., and Jalkanen, S. (1997). Origin and function of circulating CD44 in non-Hodgkin's lymphoma. J.Immunol. *158*, 3000-3008.

Rossi, A. G., McCutcheon, J. C., Roy, N., Chilvers, E. R., Haslett, C., and Dransfield, I. (1998). Regulation of macrophage phagocytosis of apoptotic cells by cAMP. J.Immunol. *160*, 3562-3568.

Sachs, L. (2005). The control of growth and differentiation in normal and leukemic blood cells. In "Accomplishments in cancer research". J. G. Fortner and J. E. Rhoads, Eds., pp. 145-164. JB Lippencott, Philadelphia.

Sampath, R., Gallagher, P. J., and Pavalko, F. M. (1998). Cytoskeletal interactions with the leukocyte integrin beta2 cytoplasmic tail. Activation-dependent regulation of associations with talin and alpha-actinin. J.Biol.Chem. *273*, 33588-33594.

Savani, R. C., Cao, G., Pooler, P. M., Zaman, A., Zhou, Z., and DeLisser, H. M. (2001). Differential involvement of the hyaluronan (HA) receptors CD44 and receptor for HA-mediated motility in endothelial cell function and angiogenesis. J.Biol.Chem. *276*, 36770-36778.

Savill, J. (1994). Apoptosis in disease. Eur.J.Clin.Invest. *24*, 715-723.

Savill, J. (1997). Apoptosis in resolution of inflammation. J.Leukoc.Biol. *61*, 375-380.

Savill, J., Dransfield, I., Gregory, C., and Haslett, C. (2002). A blast from the past: clearance of apoptotic cells regulates immune responses. Nat.Rev.Immunol. *2*, 965-974.

Savill, J., Hogg, N., Ren, Y., and Haslett, C. (1992). Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. J.Clin.Invest. *90*, 1513-1522.

Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M., and Haslett, C. (1989). Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. J.Clin.Invest. *83*, 865-874.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. EMBO J. *17*, 1675-1687.

Schmidt, S., Diriong, S., Mery, J., Fabbrizio, E., and Debant, A. (2002). Identification of the first Rho-GEF inhibitor, TRIPalpha, which targets the RhoA-specific GEF domain of Trio. FEBS Lett. *523*, 35-42.

Scott, C. F., Silver, L. D., Purdon, A. D., and Colman, R. W. (1985). Cleavage of human high molecular weight kininogen by factor XIa in vitro. Effect on structure and function. J.Biol.Chem. *260*, 10856-10863.

Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., Earp, H. S., and Matsushima, G. K. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature. *411*, 207-211.

Screaton, G. R., Bell, M. V., Jackson, D. G., Cornelis, F. B., Gerth, U., and Bell, J. I. (1992). Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc.Natl.Acad.Sci.U.S.A. *89*, 12160-12164.

Sengelov, H., Kjeldsen, L., Diamond, M. S., Springer, T. A., and Borregaard, N. (1993). Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. J.Clin.Invest. *92*, 1467-1476.

Shi, M., Dennis, K., Peschon, J. J., Chandrasekaran, R., and Mikecz, K. (2001). Antibody-induced shedding of CD44 from adherent cells is linked to the assembly of the cytoskeleton. J.Immunol. *167*, 123-131.

Shtivelman, E. and Bishop, J. M. (1991). Expression of CD44 is repressed in neuroblastoma cells. Mol.Cell Biol. *11*, 5446-5453.

Sims, P. J. and Wiedmer, T. (2001). Unraveling the mysteries of phospholipid scrambling. Thromb.Haemost. *86*, 266-274.

Song, E., Ouyang, N., Horbelt, M., Antus, B., Wang, M., and Exton, M. S. (2000). Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. Cell Immunol. *204*, 19-28.

Spector, W. G., Walters, M. N., and Willoughby, D. A. (1965). The origin of the mononuclear cells in inflammatory exudates induced by fibrinogen. J.Pathol.Bacteriol. *90*, 181-192.

Stamenkovic, I., Amiot, M., Pesando, J. M., and Seed, B. (1989). A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell. *56*, 1057-1062.

Steimle, P. A., Hoffert, J. D., Adey, N. B., and Craig, S. W. (1999). Polyphosphoinositides inhibit the interaction of vinculin with actin filaments. J.Biol.Chem. *274*, 18414-18420.

Steinman, R. M., Mellman, I. S., Muller, W. A., and Cohn, Z. A. (1983). Endocytosis and the recycling of plasma membrane. J.Cell Biol. *96*, 1-27.

Stern, M., Savill, J., and Haslett, C. (1996). Human monocyte-derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis. Mediation by alpha v beta 3/CD36/thrombospondin recognition mechanism and lack of phlogistic response. Am.J.Pathol. *149*, 911-921.

Susin, S. A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., and Kroemer, G. (1996). Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J.Exp.Med. *184*, 1331-1341.

Svee, K., White, J., Vaillant, P., Jessurun, J., Roongta, U., Krumwiede, M., Johnson, D., and Henke, C. (1996). Acute lung injury fibroblast migration and invasion of a fibrin matrix is mediated by CD44. J.Clin.Invest. *98*, 1713-1727.

Teder, P., Vandivier, R. W., Jiang, D., Liang, J., Cohn, L., Pure, E., Henson, P. M., and Noble, P. W. (2002). Resolution of lung inflammation by CD44. Science. *296*, 155-158.

Teriete, P., Banerji, S., Noble, M., Blundell, C. D., Wright, A. J., Pickford, A. R., Lowe, E., Mahoney, D. J., Tammi, M. I., Kahmann, J. D., Campbell, I. D., Day, A. J., and Jackson, D. G. (2004). Structure of the regulatory hyaluronan binding domain in the inflammatory leukocyte homing receptor CD44. Mol.Cell. *13*, 483-496.

Thoma-Uszynski, S., Stenger, S., Takeuchi, O., Ochoa, M. T., Engele, M., Sieling, P. A., Barnes, P. F., Rollinghoff, M., Bolcskei, P. L., Wagner, M., Akira, S., Norgard, M. V., Belisle, J. T., Godowski, P. J., Bloom, B. R., and Modlin, R. L. (2001). Induction of direct antimicrobial activity through mammalian toll-like receptors. Science. *291*, 1544-1547.

Toole, B. P. (2001). Hyaluronan in morphogenesis. Semin.Cell Dev.Biol. *12*, 79-87.

Toyama-Sorimachi, N., Sorimachi, H., Tobita, Y., Kitamura, F., Yagita, H., Suzuki, K., and Miyasaka, M. (1995). A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. J.Biol.Chem. *270*, 7437-7444.

Tsukamoto, Y., Helsel, W. E., and Wahl, S. M. (1981). Macrophage production of fibronectin, a chemoattractant for fibroblasts. J.Immunol. *127*, 673-678.

Tsukita, S., Oishi, K., Sato, N., Sagara, J., Kawai, A., and Tsukita, S. (1994). ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J.Cell Biol. *126*, 391-401.

Tsukita, S. and Yonemura, S. (1999). Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins. J.Biol.Chem. *274*, 34507-34510.

Tyurina, Y. Y., Shvedova, A. A., Kawai, K., Tyurin, V. A., Kommineni, C., Quinn, P. J., Schor, N. F., Fabisiak, J. P., and Kagan, V. E. (2000). Phospholipid signaling in apoptosis: peroxidation and externalization of phosphatidylserine. Toxicology. *148*, 93-101.

Ueyama, H., Kiyohara, T., Sawada, N., Isozaki, K., Kitamura, S., Kondo, S., Miyagawa, J., Kanayama, S., Shinomura, Y., Ishikawa, H., Ohtani, T., Nezu, R., Nagata, S., and Matsuzawa, Y. (1998). High Fas ligand expression on lymphocytes in lesions of ulcerative colitis. Gut. *43*, 48-54.

Ugolini, V., Nunez, G., Smith, R. G., Stastny, P., and Capra, J. D. (1980). Initial characterization of monoclonal antibodies against human monocytes. Proc.Natl.Acad.Sci.U.S.A. *77*, 6764-6768.

van Furth, R. and Diesselhoff-den Dulk, M. M. (1984). Dual origin of mouse spleen macrophages. J.Exp.Med. *160*, 1273-1283.

van Furth, R., Goud, T. J., Van der Meer, J. W., Blusse van Oud, A. A., Diesselhoff-den Dulk, M. M., and Schadewijk-Nieuwstad, M. (1982). Comparison of the in vivo and in vitro proliferation of monoblasts, promonocytes, and the macrophage cell line J774. Adv.Exp.Med.Biol. *155*, 175-187.

van Furth, R., Hirsch, J. G., and Fedorko, M. E. (1970). Morphology and peroxidase cytochemistry of mouse promonocytes, monocytes, and macrophages. J.Exp.Med. *132*, 794-812.

van Furth, R., Langevoort, H. L., and Schaberg, A. (1975). Mononuclear phagocytes in human pathology - proposal for an approach to improve classification. In "Mononuclear phagocytes in immunity, infection and pathology". R. van Furth, Ed., pp. 1-14. Blackwell Scientific Publications, Oxford.

van Furth, R. and van Zwet, T. L. (1992). Production and migration of monocytes and kinetics of macrophages. In R. van Furth, Ed., Kluwer Academic Publishers, Dordrecht.

van Leeuwen, F. N., van der Kammen, R. A., Habets, G. G., and Collard, J. G. (1995). Oncogenic activity of Tiam1 and Rac1 in NIH3T3 cells. Oncogene. *11*, 2215-2221.

Verdrengh, M., Holmdahl, R., and Tarkowski, A. (1995). Administration of antibodies to hyaluronanreceptor (CD44) delays the start and ameliorates the severity of collagen II arthritis. Scand.J.Immunol. *42*, 353-358.

Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell. *102*, 43-53.

Volanakis, J. E. and Wirtz, K. W. (1979). Interaction of C-reactive protein with artificial phosphatidylcholine bilayers. Nature. *281*, 155-157.

Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R., and Girkontaite, I. (1997). Immunosuppressive effects of apoptotic cells. Nature. *390*, 350-351.

Wagner, J. G. and Roth, R. A. (2000). Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. Pharmacol.Rev. *52*, 349-374.

Wahl, S. M., Hunt, D. A., Wakefield, L. M., McCartney-Francis, N., Wahl, L. M., Roberts, A. B., and Sporn, M. B. (1987). Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. Proc.Natl.Acad.Sci.U.S.A. *84*, 5788-5792.

Wahl, S. M., Wong, H., and McCartney-Francis, N. (1989). Role of growth factors in inflammation and repair. J.Cell Biochem. *40*, 193-199.

Walker, T. R., Ruchaud-Sparagano, M. H., McMeekin, S. R., and Dransfield, I. (2004). A critical 'threshold' of beta 2-integrin engagement regulates augmentation of cytokine-mediated superoxide anion release. Br.J.Pharmacol. *141*, 1131-1140.

Wallach, D., Varfolomeev, E. E., Malinin, N. L., Goltsev, Y. V., Kovalenko, A. V., and Boldin, M. P. (1999). Tumor necrosis factor receptor and Fas signaling mechanisms. Annu.Rev.Immunol. *17*, 331-367.

Weber, G. F., Ashkar, S., Glimcher, M. J., and Cantor, H. (1996). Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science. *271*, 509-512.

Wielenga, V. J., Heider, K. H., Offerhaus, G. J., Adolf, G. R., van den Berg, F. M., Ponta, H., Herrlich, P., and Pals, S. T. (1993). Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression. Cancer Res. *53*, 4754-4756.

Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., Lesavre, P., and Halbwachs-Mecarelli, L. (2000). Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest. *80*, 617-653.

Wright, S. D. and Griffin, F. M., Jr. (1985). Activation of phagocytic cells' C3 receptors for phagocytosis. J.Leukoc.Biol. *38*, 327-339.

Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980). Cell death: the significance of apoptosis. Int.Rev.Cytol. *68*, 251-306.

Yin, H. L. and Hartwig, J. H. (1988). The structure of the macrophage actin skeleton. J.Cell Sci.Suppl. *9*, 169-184.

Yu, Q. and Toole, B. P. (1996). A new alternatively spliced exon between v9 and v10 provides a molecular basis for synthesis of soluble CD44. J.Biol.Chem. *271*, 20603-20607.

Yu, Q., Toole, B. P., and Stamenkovic, I. (1997). Induction of apoptosis of metastatic mammary carcinoma cells in vivo by disruption of tumor cell surface CD44 function. J.Exp.Med. *186*, 1985-1996.

Yu, W. H., Woessner, J. F., Jr., McNeish, J. D., and Stamenkovic, I. (2002). CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. Genes Dev. *16*, 307-323.

Yukawa, M., Iizuka, M., Horie, Y., Yoneyama, K., Shirasaka, T., Itou, H., Komatsu, M., Fukushima, T., and Watanabe, S. (2002). Systemic and local evidence of increased Fas-mediated apoptosis in ulcerative colitis. Int.J.Colorectal Dis. *17*, 70-76.

Zamir, E. and Geiger, B. (2001). Molecular complexity and dynamics of cell-matrix adhesions. J.Cell Sci. *114*, 3583-3590.

Zamir, E., Katz, B. Z., Aota, S., Yamada, K. M., Geiger, B., and Kam, Z. (1999). Molecular diversity of cell-matrix adhesions. J.Cell Sci. *112 (Pt 11)*, 1655-1669.

Zembala, M., Siedlar, M., Ruggiero, I., Wieckiewicz, J., Mytar, B., Mattei, M., and Colizzi, V. (1994). The MHC class-II and CD44 molecules are involved in the induction of tumour necrosis factor (TNF) gene expression by human monocytes stimulated with tumour cells. Int.J.Cancer. *56*, 269-274.

Zhang, G., Gurtu, V., Kain, S. R., and Yan, G. (1997). Early detection of apoptosis using a fluorescent conjugate of annexin V. Biotechniques. *23*, 525-531.

Zhang, Y., Broser, M., Cohen, H., Bodkin, M., Law, K., Reibman, J., and Rom, W. N. (1995). Enhanced interleukin-8 release and gene expression in macrophages after exposure to Mycobacterium tuberculosis and its components. J.Clin.Invest. *95*, 586-592.

n Exp Immunol 2004; **138**:447–452

doi:10.1111/j.1365-2249.2004.02638.x

# Divalent cation-dependent and -independent augmentation of macrophage phagocytosis of apoptotic neutrophils by CD44 antibody

VIVERS, S. J. HEASMAN, S. P. HART & I. DRANSFIELD *Rayne Laboratory, MRC Centre for Inflammation Research, University of Edinburgh Medical School, Edinburgh, UK*

*(Accepted for publication 6 September 2004)*

## SUMMARY

Phagocytosis of apoptotic neutrophils by macrophages is required for resolution of an inflammatory response. Removal of intact apoptotic neutrophils prevents the release of cytotoxic granules that would otherwise cause tissue damage and may lead to development of fibrosis. Importantly, macrophage phagocytosis of apoptotic neutrophils fails to induce release of proinflammatory mediators, consistent with a 'safe' pathway for disposal of potentially harmful inflammatory cells. One pathway for increasing phagocytosis of apoptotic cells to allow matching of tissue phagocyte capacity to apoptotic cell load *in vitro* is via antibody-mediated cross-linking of CD44, providing a mechanism for limiting tissue damage during resolution of inflammation. In this study, we have defined divalent cation-dependent and -independent actions of the CD44 antibody. For the divalent cation-independent CD44 antibody effect, we provide evidence that 'enabled' CD32 on the apoptotic neutrophil binds to intact CD44 antibody on the macrophage surface. One implication is that macrophages can phagocytose apoptotic neutrophils that are 'tethered' to the macrophage surface in a manner that is independent of defined apoptotic mechanisms. These data also provide an explanation for the greater efficacy of intact CD44 antibody when compared with $F(ab')_2$ fragments.

**Keywords** apoptosis inflammation granulocyte macrophage phagocytosis

## INTRODUCTION

acrophage phagocytosis of apoptotic neutrophils is an essential ocess in the normal resolution of an inflammatory response . Although the physiological fate of neutrophils recruited to lammatory sites is to undergo apoptosis, a process which intains membrane integrity and prevents the release of cyto- xic granules, uncontrolled granule content release may contrib- e to host tissue damage that is associated with development of ease. Apoptotic neutrophils are therefore swiftly recognized d then phagocytosed by local macrophages [1]. Importantly, gestion of apoptotic neutrophils by macrophages does not mulate the release of proinflammatory mediators, thereby pro- ling a mechanism for the safe disposal of potentially harmful lammatory cells [2]. However, if the rate of cell death by apo- osis exceeds the rate of clearance by macrophages, apoptotic ls can undergo secondary necrosis, resulting in the release of totoxic substances and damage to surrounding tissues. Indeed, paired clearance of apoptotic neutrophils has been linked to development of inflammatory conditions such as acute respiratory distress syndrome (ARDS), fibrosing alveolitis, rheumatoid arthritis and ulcerative colitis [3]. We have previously identified CD44 as a potentially novel mechanism for rapidly matching tissue phagocyte capacity to apoptotic cell load that is distinct from soluble mediators or cell–cell interactions [4,5]. Ligation of CD44 may therefore determine the rate of apoptotic neutrophil clearance by macrophages, a finding supported by recent *in vivo* studies [6].

CD44 is a cell-surface glycoprotein expressed on many cell types such as leucocytes, erythrocytes, fibroblasts and a number of tumour cells [7]. It is heavily glycosylated and can be expressed as a number of variant isoforms that may influence its function [8,9]. CD44 acts as a receptor for a variety of extracellular matrix molecules including hyaluronan [10,11], fibronectin [12], fibrin [13] and collagen [14,15]. Many diverse functions have been attributed to CD44 including involvement in cellular adhesion and migration, activation and proliferation of lymphocytes, cytocidal activity of natural killer (NK) cells and tumour metastasis [7]. Binding of bivalent CD44 monoclonal antibody (MoAb) has been shown to augment macrophage phagocytosis of apoptotic neutrophils [16]. This effect appears to be specific for apoptotic neutrophils, as phagocytosis of apoptotic lymphocytes or erythrocytes opsonized with immunoglobulin was not augmented after MoAb-induced cross-linking of macrophage CD44. Although the mechanism of

Correspondence: S. P. Hart, Rayne Laboratory, MRC Centre for lammation Research, University of Edinburgh Medical School, Teviot ce, Edinburgh EH8 9AG, UK.

E-mail: s.hart@ed.ac.uk

2004 Blackwell Publishing Ltd

this effect remains to be fully characterized, CD44 MoAb may promote redistribution of cell surface CD44 facilitating phagocyte–apoptotic neutrophil interactions, or alternatively may transduce a signal within the macrophage that alters phagocytic capacity.

To characterize further the molecular basis for the augmentation of macrophage phagocytosis of apoptotic neutrophils following CD44 MoAb binding, we have undertaken studies of the effects of depletion of divalent cations. In previous studies using microscopy-based assay systems, vigorous washing of adherent macrophages was required to ensure removal of bound target cells leading to detachment of macrophages (approximately 20% of total cells [17]), an effect that was greater when divalent cation-free conditions were used. We have therefore used a carefully validated technique for assessment of phagocytosis by flow cytometry which obviates the need for washing, minimizing the impact of cell losses that limit microscopy-based studies. In this report, we have identified both divalent cation-independent and -dependent components to the augmentation of phagocytosis following CD44 MoAb binding to macrophages. For the divalent cation-independent effect, blockade of CD32 on the apoptotic neutrophil was found to be inhibitory. In support of this finding, $F(ab')_2$ fragments of CD44 MoAb fail to augment phagocytosis in a cation-deplete environment. While these findings are somewhat counter-intuitive, as both apoptotic and non-apoptotic neutrophils express CD32, our recent studies have demonstrated that CD32 is enabled on apoptotic neutrophils [18]. In summary, augmentation of macrophage phagocytosis of apoptotic neutrophils by CD44 MoAb involves two distinct effects: first, a direct effect of cross-linking that is observed with $F(ab')_2$ fragments of CD44 MoAb that requires the presence of divalent cations; and secondly, a divalent cation-independent tethering of apoptotic neutrophils via CD32 to the Fc portion of MoAb bound to the macrophage surface.

## MATERIALS AND METHODS

### *Reagents and antibodies*

All cell culture materials were from Invitrogen (Paisley, UK) and other reagents were from Sigma (Poole, UK) unless stated otherwise. The following MoAb were used: 5A4 (anti-CD44, a kind gift of Dr Graeme Dougherty, San Francisco, CA, USA) IV3 (anti-CD32 Fab′ fragments for function blocking studies; Medarex, Annandale, NJ, USA), IVC7 (anti-CD36; CLB, Amsterdam), 61D3 (anti-CD14 provided by Dr A. Devitt, Edinburgh, UK), DFT-1 (anti-CD43, Serotec, Oxford, UK) and T29/33 (anti-CD45, DakoCytomation, Ely, UK). IgG1 isotype control MoAb was purchased from Beckman-Coulter, High Wycombe (UK). All MoAb were used at saturating concentrations as determined by indirect immunofluorescence techniques and flow cytometric analysis (FACSCaliber, Becton-Dickinson, Oxford, UK).

### *Purification of CD44 $F(ab)_2$ antibodies*

CD44 MoAb (5A4) was purified from hybridoma supernatant using protein A affinity chromatography and pepsin digestion (1% wt/wt) to generate $F(ab')_2$ fragments. Residual intact antibody was removed by three rounds of depletion using protein A agarose affinity resin and the resulting $F(ab')_2$ fragments were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions to confirm purity. Indirect immunofluorescence analysis together with flow cytometry was used to confirm that preparation of antibody fragments did not compromise binding and that antibody fragments were used at saturating concentrations in other assays.

### *Cell isolation*

Monocytes and neutrophils were isolated from human peripheral blood by dextran sedimentation and discontinuous Percoll™ (Amersham Pharmacia, St Albans, UK) gradient centrifugation, as described previously [19]. Mononuclear cells were suspended at $4 \times 10^6$/ml in IMDEM and allowed to adhere to cell culture plates during incubation at 37°C for 1 h. Non-adherent cells (mostly lymphocytes) were removed and adherent cells were washed three times in Hanks' balanced salt solution without $Ca^{2+}$/$Mg^{2+}$. Adherent monocytes (approximately 90% $CD14^+$) were then cultured in Iscove's modification of Dulbecco's modified Eagles' medium (IDMEM) containing 10% autologous serum for 5 days (monocyte-derived macrophages). Neutrophils were incubated at $2 \times 10^7$/ml with 22 $\mu$M 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes, Oregon, USA) at 37°C for 15 min. Labelled neutrophils were then cultured at $4 \times 10^6$/ml in IDMEM with 10% autologous serum for 24 h, during which time they underwent spontaneous apoptosis [1]. Apoptosis was assessed either by microscopic inspection of cytocentrifuge preparations that had been stained with Diff-Quik™ (Baxter Healthcare, Glasgow, UK) as per the manufacturer's protocol, or alternatively assessed by dual immunofluorescence analysis using annexinV-FITC in combination with propidium iodide to determine the proportions of apoptotic and necrotic cells.

### *Phagocytosis of apoptotic neutrophils*

Macrophage phagocytosis of apoptotic neutrophils was assessed using a flow cytometric assay that has been validated carefully to quantify internalization of apoptotic targets and not surface binding [17]. Monocyte-derived macrophages were washed with IDMEM and incubated with CD44 MoAb diluted in IDMEM for 15 min at 37°C. Excess MoAb was removed and monocyte-derived macrophages washed with 25 mM Hepes (pH 7·4) buffered saline (HBS) prior to adding $2 \times 10^6$ CMFDA-labelled apoptotic neutrophils in 0·5 ml of either IDMEM, chelex-treated IDMEM (divalent cation-depleted) or HBS (divalent cation-free) to each well for 60 min at 37°C. At the end of the assay, a solution of 0·25% trypsin/1 mM EDTA was added to each well and the cells incubated at 37°C for 15 min to detach all monocyte-derived macrophages and remove non-internalized neutrophils. After a further 15 min incubation on ice, the proportion of macrophages that exhibited increased fluorescence (corresponding to phagocytosis of fluorescently labelled apoptotic neutrophils) was determined by flow cytometry using a FACSCalibur analyser with post-acquisition data analysis performed using CELLQUEST software (Becton-Dickinson).

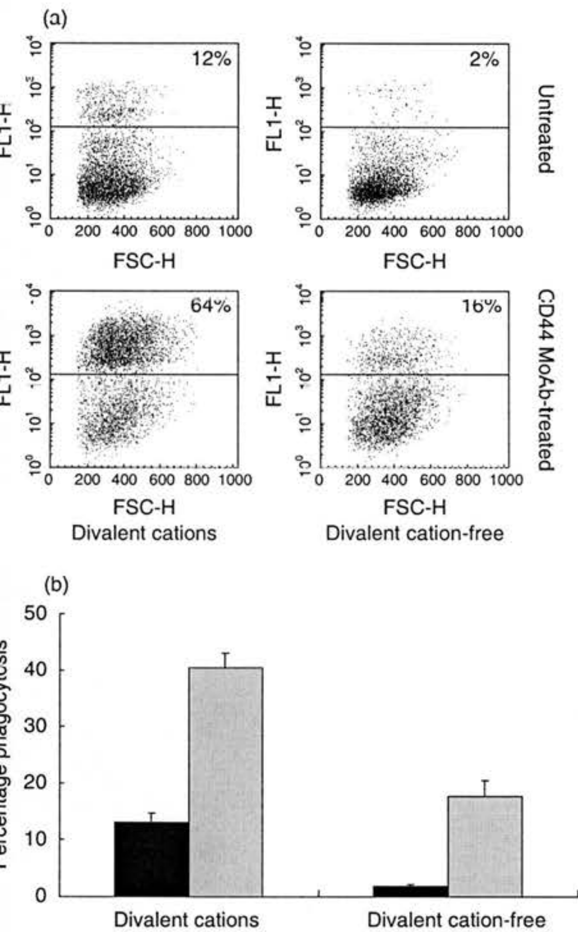
### *The effects of antibody/ligand inhibitors upon phagocytosis*

Monocyte-derived macrophages were washed with HBS without $Ca^{2+}$/$Mg^{2+}$ and incubated with CD44 MoAb diluted in HBS for 15 min at 37°C. Unbound antibody was washed off and monocyte-derived macrophages were pretreated with saturating concentrations of putative inhibitory MoAb, or 1 mg/ml fucoidan (scavenger receptor inhibitor) or 50 U/ml heparin (heparan sulphate proteoglycan inhibitor) prior to assessment of phagocytosis of apoptotic neutrophils using the flow cytometric assay described above.

## RESULTS

*acrophage phagocytosis of apoptotic neutrophils in divalent-tion depleted conditions is augmented by CD44 MoAb*

e requirement for the presence of either $Ca^{2+}$ or $Mg^{2+}$ for hesion molecules to bind to their cognate ligands provides a nple method for discrimination of potential receptor pathways volved in CD44 MoAb-augmented phagocytosis of apoptotic utrophils. Control (untreated) or CD44 MoAb-treated mono-te-derived macrophages were challenged with apoptotic neu-phils in the presence or absence of divalent cations and the rcentage of phagocytosis was determined (Fig. 1). For control nocyte-derived macrophages, the absence of divalent cations BS) virtually eliminated phagocytosis. To confirm that this ect was not due to a change in media conditions used, we deter-ned the levels of phagocytosis in IDMEM that had been depleted specifically of divalent cations using chelex resin. Phagocytosis in IDMEM was reduced from 34·2 ± 6 to 6·6 ± 2 in chelex-treated IDMEM. In contrast, CD44 MoAb-treated macrophage phagocytosis of apoptotic neutrophils was still observed in the absence of divalent cations, suggesting that CD44 MoAb binding to monocyte derived macrophages may allow engagement of distinct divalent cation-independent apoptotic cell recognition mechanisms.




. 1. CD44 MoAb-augmented macrophage phagocytosis of apoptotic trophils in the presence and absence of divalent cations. Adherent nan monocyte-derived macrophages were preincubated with either 44 MoAb or medium alone for 15 min, washed and then incubated with -CFDA-labelled apoptotic neutrophils in the presence or absence of alent cations as indicated for 60 min. (a) Typical flow cytometric profiles nonstrating macrophage phagocytosis of fluorescent apoptotic neutro-ls are shown. (b) Phagocytosis of apoptotic neutrophils by macrophages owing incubation with CD44 MoAb (grey bars) or IDMEM (black s) was determined in duplicate samples by flow cytometry. Results are ressed as the mean ± s.e.m. of 22 independent experiments.

*CD32 is required for CD44 MoAb augmented phagocytosis of apoptotic neutrophils in the absence of divalent cations*

To investigate the molecular basis for the CD44 MoAb augmented phagocytosis, we tested the effects of inhibitors of putative divalent cation-independent recognition pathways. Pretreatment with fucoidan (scavenger receptor inhibitor), heparin (heparan sulphate proteoglycan inhibitor), anti-CD36 or anti-CD14 prior to incubation with apoptotic neutrophils under divalent cation free conditions failed to inhibit the CD44 MoAb augmented phagocytosis (Fig. 2). In contrast, CD32 MoAb completely blocked CD44 MoAb augmented macrophage phagocytosis of apoptotic neutrophils in the absence of divalent cations (Fig. 2). We did not observe inhibition of phagocytosis by CD32 MoAb in the presence of divalent cations (data not shown).

*Role of macrophage or apoptotic neutrophil CD32 in CD44 MoAb augmented phagocytosis*

We next used MoAb preincubation strategies to determine whether CD32 was required on the macrophage or the neutrophil or both cell types. When macrophages were pretreated with CD32 MoAb there was no inhibitory effect upon CD44 MoAb-augmented phagocytosis in divalent cation free conditions (Fig. 3). These data suggested that macrophage CD32 was not mediating the divalent cation-independent CD44 MoAb-augmented phagocytosis. In contrast, when neutrophils were preincubated with CD32 MoAb, divalent cation-independent phagocytosis was inhibited. Although neutrophils also express

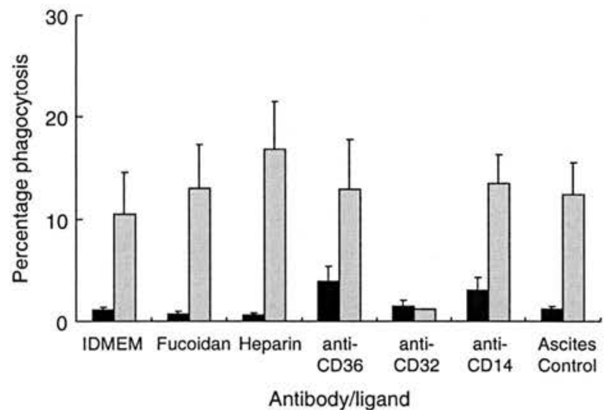



**Fig. 2.** The effect of inhibitory antibodies and ligands upon CD44 MoAb-augmented phagocytosis of apoptotic neutrophils by macrophages. Macrophages were incubated with CD44 MoAb (grey bars) or IDMEM (black bars) for 15 min, washed and then incubated with the antibody/ligand indicated for a further 15 min. Macrophages were incubated with apoptotic neutrophils for 60 min in the absence of divalent cations. Macrophage phagocytosis of apoptotic neutrophils in duplicate samples was then determined by flow cytometry. Results are expressed as mean ± s.e.m. of at least three independent experiments.

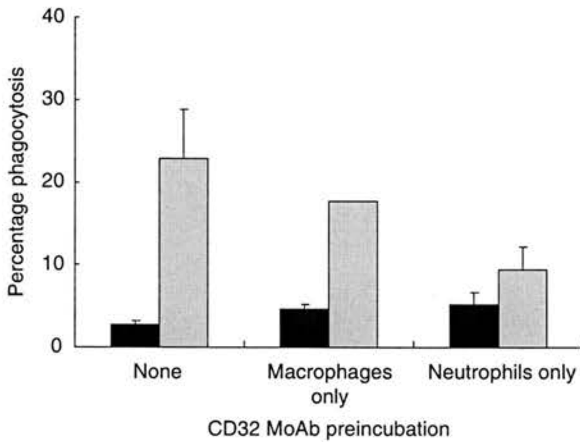



**Fig. 3.** Effect of pretreatment of macrophages or neutrophils with anti-CD32 MoAb. Macrophages or apoptotic neutrophils were preincubated with CD32 MoAb for 15 min and then washed with medium. Macrophages were the incubated with either CD44 MoAb (grey bars) or IDMEM (black bars) for 15 min, washed and incubated with apoptotic neutrophils in the absence of divalent cations for 60 min. Macrophage phagocytosis of apoptotic neutrophils in duplicate samples was then determined for each experiment by flow cytometry. Results shown are mean ± s.e.m. of at least three independent experiments.

another receptor for IgG (CD16), apoptosis is associated with >90% reduction in surface expression of this receptor [19] and CD16 blockade does not prevent aggregated murine IgG binding to apoptotic neutrophils [18]. These data imply that CD32 on the apoptotic neutrophil is able to mediate binding to intact CD44 MoAb bound to macrophages. Based upon these results, one prediction would be that binding of antibody to any highly expressed macrophage surface receptor would also augment phagocytosis under divalent cation-free conditions.

*Effects of MoAb binding to other highly expressed macrophage receptors*

We next tested whether binding of MoAb to other highly expressed surface receptors would also augment macrophage phagocytosis of apoptotic neutrophils. Macrophages were incubated with MoAb of the same isotype (IgG1) against either CD43, CD44 or CD45, molecules that are expressed at similar levels on the macrophage surface (data not shown). We found that preincubation of macrophages with CD43 MoAb resulted in a slight augmentation of phagocytosis in the presence of divalent cations (from 18·8 ± 7% to 33·5 ± 7%, $n = 3$) when compared to medium alone. In a separate series of experiments, CD45 MoAb pretreatment also increased phagocytosis of apoptotic neutrophils in the presence of divalent cations from 24·4 ± 3% to 37·7 ± 6% ($n = 3$). In contrast, CD44 treatment resulted in a robust increase of phagocytosis in the presence of divalent cations (66·6 ± 8% and 64·4 ± 5% for the CD43 MoAb and CD45 MoAb experiments, respectively). These observations would be consistent with macrophage-bound antibody providing a ligand for neutrophil receptors for IgG and thereby contributing to apoptotic cell uptake. Based upon these observations, we would have predicted that CD43 antibodies would also augment phagocytosis under divalent cation-free conditions. However, examination of macrophage phagocytosis following treatment with CD43 MoAb did not support this assertion (Fig. 4).

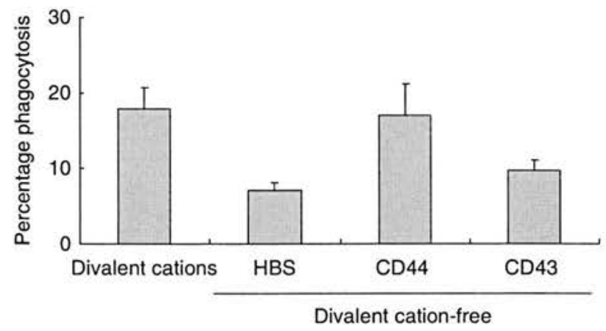



**Fig. 4.** Distinct effects of MoAb binding to highly expressed receptors and CD44 MoAb. Macrophages were preincubated with either CD44 MoAb or CD43 MoAb or untreated control for 15 min, washed and then incubated with apoptotic neutrophils in the absence of divalent cations for 60 min. Macrophage phagocytosis of apoptotic neutrophils in duplicate samples was then determined by flow cytometry. Results are shown as mean ± s.e.m. of six independent experiments.

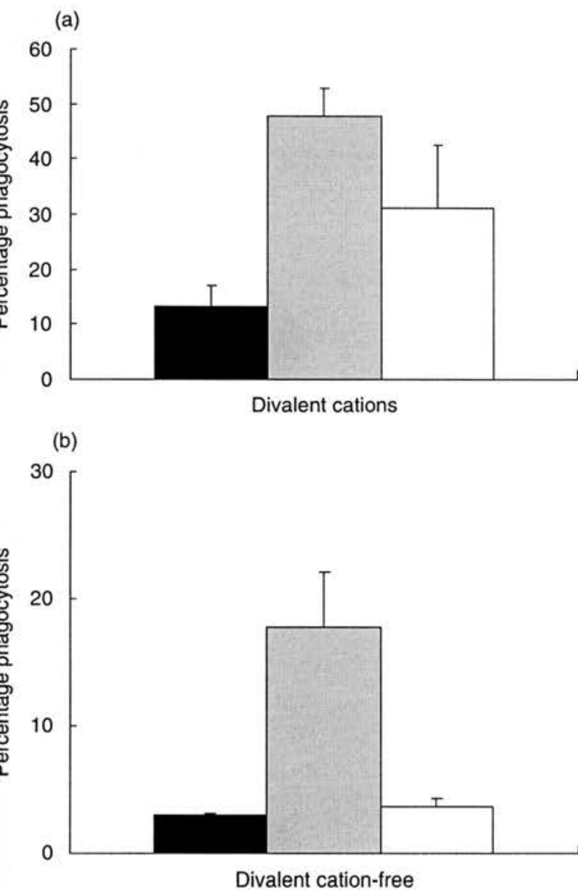
*Effects of CD44 F(ab′)$_2$ fragments upon phagocytosis of apoptotic neutrophils*

We next investigated the effects of F(ab′)$_2$ fragments of CD44 MoAb. In the presence of divalent cations, treatment of macrophages with F(ab′)$_2$ resulted in augmentation of phagocytosis of apoptotic neutrophils. In contrast, in the absence of divalent cations there was no effect (Fig. 5). These data demonstrate a specific effect of ligation of CD44 by MoAb upon macrophage phagocytic function that is independent of involvement of CD32.

## DISCUSSION

In this study, we have used removal of divalent cations to eliminate the potential contribution of integrins [20] or C-type lectins [21] to CD44 MoAb-mediated augmentation of macrophage phagocytosis of apoptotic neutrophils. We have confirmed that phagocytosis of apoptotic neutrophils by untreated macrophages requires exclusively divalent cation-dependent recognition pathways. In contrast, we found that CD44 MoAb augmented macrophage phagocytosis had both divalent cation-dependent and independent components. Our data suggest that augmentation of apoptotic cell phagocytosis by macrophages following CD44 MoAb treatment in the absence of divalent cations requires tethering of the apoptotic neutrophils via CD32 to CD44 antibodies bound to the macrophage surface. This interaction may facilitate engagement of apoptotic cell recognition pathways that lead subsequently to internalization. Despite similar levels of binding of CD44 and CD43 MoAb (both IgG1) to the macrophage surface, we failed to observe increased phagocytosis by CD43 MoAb in the absence of divalent cations. At present the reason for this difference is not clear. One possibility is that this may reflect the differences in the distribution of CD44 and CD43 in the macrophage membrane.

Data from *in vitro* studies of macrophage recognition of apoptotic cells has implicated involvement of a number of distinct, unrelated molecular pathways (reviewed in [22]). It has been proposed recently that many of these molecules or even well-established opsonins [23] may be involved in tethering of apoptotic targets. It is possible that internalization of tethered




;. 5. Effects of CD44 F(ab′)₂ fragments upon macrophage phagocytosis apoptotic neutrophils. (a) Macrophages were preincubated with intact )44 MoAb (grey bars), CD44 F(ab′)₂ fragments (white bars) or medium ne (black bars) for 15 min, washed and then incubated with apoptotic ıtrophils for 60 min in the presence of divalent cations. Macrophage ıgocytosis of apoptotic neutrophils in duplicate samples was then deter- ıed by flow cytometry. Results are shown as the mean ± s.e.m. of five ependent experiments. Macrophages were incubated with intact )44 MoAb (grey bars), CD44 F(ab′)₂ fragments (white bars) or medium ne (black bars) for 15 min, washed and then incubated with apoptotic ıtrophils for 60 min in the absence of divalent cations. Macrophage ıgocytosis of apoptotic neutrophils in duplicate samples was then deter- ıed by flow cytometry. Results are shown as the mean ± s.e.m. of five ependent experiments.

ɔptotic targets is then driven via a common phosphatidylserine ;eptor-dependent mechanism [24]. Recently we have demon- ated that CD32 expressed on apoptotic neutrophils becomes ıabled', mediating binding to multimeric IgG [18]. Thus, despite overall reduction in levels of expression of CD32 on apoptotic ıtrophils when compared with non-apoptotic neutrophils, we ve demonstrated that only apoptotic neutrophils become teth- ;d to CD44 MoAb bound to the macrophage surface and that ıding is mediated by CD32 on the neutrophil. Although it is ssible that both apoptotic and non-apoptotic neutrophils are e to bind to CD44 MoAb on the macrophage surface and that r assay conditions only allow detection of phagocytosis, we sug- st that our data indicate that apoptotic neutrophils tether spe- cally to antibody bound to the macrophage surface.

In this study we have demonstrated that anti-CD44 $F(ab')_2$ fragments were capable of augmenting phagocytosis of apoptotic neutrophils, eliminating possible Fc receptor-dependent effects in the augmentation of phagocytosis of apoptotic neutrophils following CD44 ligation. As found for untreated macrophages, phagocytosis requires the presence of divalent cations, raising the possibility that CD44 MoAb mediated cross-linking increases the efficiency of existing recognition pathways. One cautionary note is that promotion of apoptotic neutrophil tethering by MoAb bound to the macrophage surface (see Fig. 4) may act to counter inhibitory effects of antibodies in other studies of apoptotic targets bearing Fc receptors and potentially 'mask' true inhibition. Such a possibility may account for lesser inhibition of macrophage uptake of CD32 bearing cell types compared with CD32 negative apoptotic targets [25]. Finally, our data have important implications for use of antibodies to investigate apoptotic cell clearance *in vivo*. While the shorter *in vivo* half-life of proteolytic fragments of antibodies may restrict their use therapeutically and in functional studies [26], intact antibodies may have additional regulatory effects upon macrophage clearance of CD32 expressing apoptotic cells.

## ACKNOWLEDGEMENTS

This work was supported by an MRC Clinician Scientist fellowship (SPH) and MRC studentship (SV). We are grateful to colleagues in the Centre for Inflammation Research for help with leucocyte preparation and helpful comments relating to this work.

## REFERENCES

1 Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest 1989; **83**:865–75.
2 Meagher LC, Savill JS, Baker A, Fuller RW, Haslett C. Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane $B_2$. J Leukoc Biol 1992; **52**:269–73.
3 Weiss SJ. Mechanisms of disease: tissue destruction by neutrophils. N Engl J Medical 1989; **320**:365–76.
4 Ren Y, Savill JS. Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis. J Immunol 1995; **154**:2366–74.
5 Rossi AG, McCutcheon JC, Roy N, Chilvers ER, Haslett C, Dransfield I. Regulation of macrophage phagocytosis of apoptotic cells by cAMP. J Immunol 1998; **160**:3562–8.
6 Teder P, Vandivier RW, Jiang D *et al.* Resolution of lung inflammation by CD44. Science 2002; **296**:155–8.
7 Lesley J, Kincade PW, Hyman R. Antibody-induced activation of the hyaluronan receptor function of CD44 requires multivalent binding by antibody. Eur J Immunol 1993; **23**:1902–9.
8 Isacke CM, Yarwood H. The hyaluronan receptor, CD44. Int J Biochem Cell Biol 2002; **34**:718–21.
9 Katoh S, Zheng Z, Oritani K, Shimozato T, Kincade PW. Glycosylation of CD44 negatively regulates its recognition of hyaluronan. J Exp Med 1995; **182**:419–29.
10 Aruffo A, Stamenkovic I, Melnick M, Underhill C, Seed B. CD44 is the principal cell surface receptor for hyaluronate. Cell 1990; **61**:1303–13.
11 Culty M, Miyake K, Kincade PW, Silorski E, Butcher EC, Underhill C. The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins. J Cell Biol 1990; **111**:2765–74.
12 Jalkanen S, Jalkanen M. Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. J Cell Biol 1992; **116**:817–25.
13 Faassen AE, Schrager JA, Klein DJ, Oegema TR, Couchman JR,

McCarthy JB. A cell surface chondroitin sulphate proteoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. J Cell Biol 1992; **116**:521–31.

14 Henke CA, Roongta U, Mickelson DJ, Knutson JR, McCarthy JB. CD44-related chondroitin sulfate proteoglycan, a cell surface receptor implicated with tumor cell invasion, mediates endothelial cell migration on fibrinogen and invasion into a fibrin matrix. J Clin Invest 1996; **97**:2541–52.

15 Svee K, White J, Vaillant P *et al.* Acute lung injury fibroblast migration and invasion of a fibrin matrix is mediated by CD44. J Clin Invest 1996; **98**:1713–27.

16 Hart SP, Dougherty GJ, Haslett C, Dransfield I. CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages. J Immunol 1997; **159**:919–25.

17 Jersmann HP, Ross KA, Vivers S, Brown SB, Haslett C, Dransfield I. Phagocytosis of apoptotic cells by human macrophages: analysis by multiparameter flow cytometry. Cytometry 2003; **51A**:7–15.

18 Hart SP, Jackson C, Kremmel LM *et al.* Specific binding of an antigen–antibody complex to apoptotic human neutrophils. Am J Pathol 2003; **162**:1011–8.

19 Dransfield I, Buckle A-M, Savill JS, McDowall A, Haslett C, Hogg N. Neutrophil apoptosis is associated with a reduction in CD16 (FcγRIII) expression. J Immunol 1994; **153**:1254–63.

20 Dransfield I, Cabanas C, Barrett J, Hogg N. Interaction of leukocyte integrins with ligand is necessary but not sufficient for function. J Cell Biol 1992; **116**:1527–35.

21 Feizi T. Carbohydrate-mediated recognition systems in innate immunity. Immunol Rev 2000; **173**:79–88.

22 Giles KM, Hart SP, Haslett C, Rossi AG, Dransfield I. An appetite for apoptotic cells: controversies and challenges. Br J Haematol 2000; **109**:1–12.

23 Hart SP, Smith JR, Dransfield I. Phagocytosis of opsonized apoptotic cells: roles for 'old-fashioned' receptors for antibody and complement. Clin Exp Immunol 2004; **135**:181–5.

24 Henson PM, Bratton DL, Fadok VA. Apoptotic cell removal. Curr Biol 2001; **11**:R795–805.

25 Flora PK, Gregory GD. Recognition of apoptotic cells by human macrophages: inhibition by a monocyte/macrophage-specific monoclonal antibody. Eur J Immunol 1994; **24**:2625–32.

26 Covell DG, Barbet J, Holton OD, Black CD, Parker RJ, Weinstein JN. Pharmacokinetics of monoclonal immunoglobulin G1, F(ab')2, and Fab' in mice. Cancer Res 1986; **46**:3969–78.

© 2002 Wiley-Liss, Inc.

Cytometry Part A 51A:7–15 (2003)

# Phagocytosis of Apoptotic Cells by Human Macrophages: Analysis by Multiparameter Flow Cytometry

**Hubertus P. A. Jersmann, Katherine A. Ross, Sharon Vivers, Simon B. Brown, Christopher Haslett, and Ian Dransfield***

MRC Centre for Inflammation Research, University of Edinburgh Medical School, Edinburgh, United Kingdom

Received 1 April 2002; Revision Received 12 August 2002; Accepted 4 October 2002

**Background:** Phagocytic removal of apoptotic cells is an important regulatory event in development, tissue homoeostasis, and inflammation. There are several methodologic problems with most in vitro studies of the molecular mechanisms of apoptotic cell phagocytosis. First, cell loss occurs during rigorous washing of adherent macrophages required to ensure removal of noningested particles. Second, discrimination of adherent or internalised apoptotic cells is difficult. Third, microscopic quantification is time consuming and has the potential for significant interobserver error. Fourth, subsequent analysis of phagocyte populations is difficult.

**Methods:** We used a flow cytometric method that allows quantification of phagocytosis of fluorescently labelled apoptotic cells with the use of multiparameter flow cytometric analysis.

**Results:** Phagocytosis of apoptotic cells was validated by use of inhibitors (cytochalasins) or low temperature and counterstaining with cell surface markers for the phagocytic targets to exclude binding to the phagocytic surface. Populations of phagocytic macrophages were sorted, and the presence of internalized apoptotic material was validated by microscopy.

**Conclusions:** The technique we used in this study allows observer-independent analysis of phagocytosis of apoptotic cells by macrophages. Importantly, phagocytic or nonphagocytic populations could be subjected to further characterization with the use of flow cytometry with additional fluorochrome reagents and can be re-cultured to study underlying regulatory mechanisms. Cytometry Part A 51A:7–15, 2003. © 2002 Wiley-Liss, Inc.

**Key terms:** phagocytosis; macrophage; apoptotic cells

Since the first description of apoptosis as a mechanism of cell death required for the physiologic removal of cells in many processes (1), there has been an increasing interest in the mechanisms that underlie the rapid recognition and engulfment of apoptotic cells (2,3). Current assays for the quantification of the uptake of apoptotic cells by phagocytes are broadly based on assays that measure cell-to-cell adhesion and are potentially flawed by several problems. Adherent phagocyte populations are overlaid with apoptotic targets in suspension and then cocultured for different periods according to the cell population under study. Rigorous washing used to dislodge adherent cells before assessment of phagocytosis may lead to loss of phagocytes, thereby skewing the apparent magnitude of the overall phagocytic response. Further, use of mixed populations of apoptotic and nonapoptotic "targets" makes discrimination of internalisation from adherence of cells to the macrophage surface difficult. In addition, separation of phagocytic and nonphagocytic cells is not readily achieved with conventional plate- or slide-based assays, thus precluding further culture or examination of other functional capacities of phagocytic cells. Moreover, counting plates is time consuming and carries a risk of significant intra- and interobserver errors.

We therefore developed a flow cytometry-based quantitation method to specifically address these problems. We used the distinct scatter characteristics of macrophages and apoptotic targets and specific fluorochromes to allow discrimination of nonphagocytosed apoptotic targets, nonphagocytic macrophages, and macrophages that have internalised apoptotic cells, thereby eliminating the requirement for washing. Acquisition of large amounts of

Contract grant sponsor: MRC; Contract grant number: 9016491; Contract grant sponsor: Sanofi-Winthrop Scholarship, Royal Australian College of Physicians.

*Correspondence to. Ian Dransfield, MRC Centre for Inflammation Research, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH8 9AG, UK.

E-mail: i.dransfield@ed.ac.uk

Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.a.10005

data is rapid and observer-independent. This cytometry-based assay is robust, with similar data acquired using FACSCalibur (Becton-Dickinson, Oxford, UK) and Epics XL (Beckman Coulter, High Wycombe, UK) cell analysers. The methodology described herein may be readily adapted to analysis of other phagocytes or phagocytic targets, including cell lines. We found that cell populations of interest can be collected by cell sorting, allowing further analysis of such cells by direct inspection (microscopy), counterstaining of any antigens required, or reculturing the cells for further functional analysis. This assay will provide unique opportunities for the characterisation of the process of apoptotic cell phagocytosis.

## MATERIALS AND METHODS

### Reagents

CellTracker Green (5-chloromethylfluorescein diacetate; CMFDA) was purchased from Molecular Probes (Leiden, The Netherlands). Cytochalasin D and propidium iodide were obtained from Sigma (St. Louis, MO). Annexin V Fluo was obtained from Boehringer Mannheim (Mannheim, Germany). The following monoclonal antibodies (mAbs) were used: CD44 mAb (5A4, immunoglobulin [Ig] $G_1$, provided by Dr. G. Dougherty, University of California, San Francisco), CD14 mAb (UCHM1, IgG2a), and CD16 mAb (3G8, $IgG_1$, provided by Dr. J. Unkeless, Mount Sinai School of Medicine, New York, NY); all hybridoma culture supernatants were used at saturating concentrations. Phycoerythrin indodicarbocyanine (PECy5)-conjugated CD15 mAb was purchased from Beckman Coulter. Polyclonal goat anti-mouse IgG $F(ab')_2$ RPE antibody was obtained from DAKO (Copenhagen, Denmark). CD62L mAb (Leu-8, $IgG_{2b}$) was supplied by Becton-Dickinson. Trypsin/ethylene-diaminetetra-acetic acid (EDTA) solution was obtained from GIBCO Life Technologies (Paisley, UK). Tissue culture plastics were manufactured by Falcon (Becton-Dickinson).

### Cellular Isolation and Culture

Human peripheral blood mononuclear cells and neutrophils were isolated by using dextran sedimentation and centrifugation with discontinuous Percoll gradients, as described elsewhere (4,14). Monocytes were enriched by selective adherence and monocyte-derived macrophages, typically greater than 85% $CD14^+$, were differentiated by culture for 5-7 days in Iscove's modification of Dulbecco's modified Eagle's medium (IDMEM; GIBCO Life Technologies) containing 10% autologous serum. Polymorphonuclear cells, with more than 95% neutrophils when Giemsa-stained cytocentrifuge preparations were examined by microscopy, were resuspended at $20 \times 10^6$/ml in IDMEM and incubated with CMFDA at a final concentration of 22 μM (20 min, 37°C). The neutrophil suspension was then diluted with IDMEM to a final cell density of $4 \times 10^6$/ml and cultured in IDMEM containing 10% autologous serum (20 h, 37°C). A group I Burkitt lymphoma cell line, Mutu I, induced into apoptosis by 16 h of incubation with 1 μg/ml of the calcium ionophore ionomycin (CN Biosciences, Nottingham, UK), was used as the source of apoptotic lymphocytes (5) and labelled with CMFDA as described for neutrophils. Before use in the phagocytosis assay, the percentage of apoptotic cells was estimated by microscopic examination of cytocentrifuge preparations and Annexin V/propidium iodide staining and flow cytometry.

### Microscopy-Based Phagocytosis Assay

Adherent macrophages were incubated with apoptotic neutrophils or lymphocytes suspended at $5 \times 10^6$/ml for 30 min at 37°C, thus providing a phagocyte-to-target ratio of approximately 1:10. Adherent macrophages were vigorously washed with ice-cold Hank's balanced saline solution, fixed in 2.5% glutaraldehyde, and stained for myeloperoxidase with 0.1 mg/ml of dimethoxy benzidine and 0.03% (v/v) hydrogen peroxide in phosphate buffered saline (PBS), as previously described (6). The percentage of macrophages that had interacted with one or more peroxidase-positive apoptotic neutrophils was quantified by examination with an inverted microscope with at least 500 cells per well counted per replicate.

### Flow Cytometry–Based Phagocytosis Assay

The assay was performed essentially as described above. However, after incubation of macrophages with apoptotic targets, the medium was removed by careful pipetting to minimise disturbance of the sedimented cells. Wells were then incubated with Trypsin/EDTA for 20 min at 37°C followed by another 20 min at 4°C. Cells were then harvested with a 1,000-μl micropipette; optimal removal of macrophages required vigorous pipetting. Plates were inspected with a phase-contrast microscope to verify that all macrophages had been successfully removed. The unfixed samples were then analysed by flow cytometry with a FACSCalibur with CellQuest software (Becton-Dickinson) or an Epics XL (Beckman Coulter) with EXPO32 software. Neutrophil and macrophage populations were identified by their distinct laser scatter properties. A minimum of 6,000 events within the macrophage gate was acquired. The number of the $FL1^+$ events in the macrophage gate was divided by the total number of macrophages to obtain the percentage of macrophages that had internalised neutrophils. Cells harvested from wells were incubated with 10 μl of rabbit serum (10 min, 4°C) to minimise nonspecific antibody binding to Fc receptors and then incubated with anti-CD15-PECy5 alone, CD14, or isotype control antibody followed by anti-mouse IgG-PECy5 (45 min, 4°C), washed twice, and then analysed by flow cytometry.

### Cell Sorting

Subpopulations of cells identified as described above were sorted with a FACSVantage (Becton-Dickinson). All cells were kept at 4°C in medium containing 1% human serum albumin to minimise further phagocytosis and/or degradation of internalised particles. Cytocentrifuge preparations of the sorted cell populations were prepared, immediately fixed in methanol, and stained with DiffQuik.

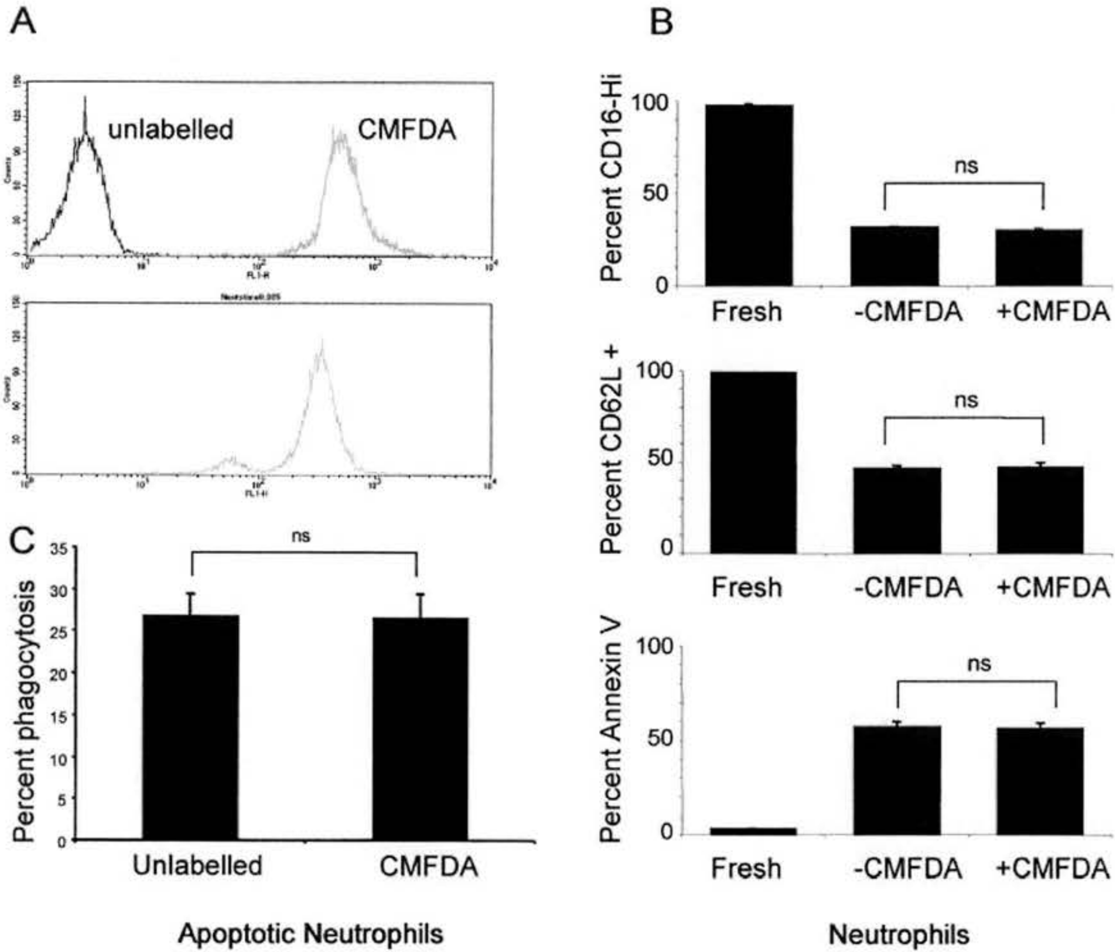



FIG. 1. Labelling of neutrophils with 5-chloromethylfluorescein diacetate (CMFDA) does not affect constitutive neutrophil apoptosis or macrophage phagocytosis. **A:** After incubation of neutrophils with CMFDA in serum-free medium for 20 min, more than 99% of cells became $FL1^+$ (top). Histogram overlay: left, unlabelled cells; right, labelled cells. After undergoing constitutive apoptosis for 20 h, the fluorescence intensity was slightly reduced, with the appearance of an FL1-low subpopulation (bottom). **B:** Neutrophils aged with or without CMFDA lost their surface markers CD16 and CD62L to similar extents. In addition, similar proportions of neutrophils labelled with or without CMFDA become Annexin V positive. The mean ± standard error of the mean of three experiments are shown for CD16 and CD62L, and those of six experiments are shown for Annexin V. **C:** Neutrophils were aged for 20 h in the presence or absence of CMFDA, and phagocytosis was assessed with a well-validated plate assay performed as described in Materials and Methods. The data (a minimum of 500 cells per replicate) represent the mean ± standard error of the mean of five independent experiments, each carried out in quadruplicate.

Images of sorted cells were obtained with a CoolSNAP Camera and OpenLAB software on a G4 Power Macintosh desktop computer using a Zeiss Axiovert S100 inverted microscope.

### Fixing Protocol

Cell samples as harvested from the wells were washed in PBS and resuspended in paraformaldehyde (1%, methanol free, 20°C). After 20 min the samples were washed in PBS and "quenched" with ammonium sulphate (50 mM, 10 min) and then stored at 4°C in the dark in PBS containing 0.1% azide and 0.1% human serum albumin.

### Statistics

All results are expressed as mean ± standard error of the mean, where n represents the number of independent experiments using macrophages from different donors, conducted in triplicate unless stated otherwise. Results were analysed by analysis of variance and the Tukey-Kramer multiple comparison test.

## RESULTS

### CMFDA Reliably Labels Neutrophils and Does Not Affect the Apoptotic Process

Flow cytometric analysis of samples from unlabelled and CMFDA-labelled neutrophil preparations confirmed that high levels of uniformly $FL1^+$ cells were present when compared with unlabelled cells (Fig. 1A). Neutrophils that had undergone constitutive apoptosis during in vitro culture for 18–24 h still exhibited high levels of fluorescence, although we consistently noted a small de-

crease in overall levels of fluorescence. To be certain that CMFDA labelling did not adversely alter the apoptotic program in a way that might accelerate constitutive cell death or affect functional studies of phagocytosis, we examined changes in surface molecule expression. Data shown in Figure 1B demonstrate that CMFDA labelling does not affect the changes in surface expression of CD16 and CD62L, which we and others previously demonstrated to be markedly downregulated when neutrophils undergo constitutive apoptotic cell death (7). In addition, we assessed the binding of Annexin V to measure exposure of phosphatidylserine on the outer leaflet of the cell membrane (8). Importantly, we found no significant difference in the percentage of labelled or unlabelled neutrophils that had undergone apoptosis (Fig 1B), suggesting that CMFDA labelling does not reduce neutrophil viability. Further, these data illustrate that labelling of neutrophil populations does not affect membrane alterations that represent well-defined attributes associated with the intrinsic apoptotic program. In addition, the percentage of macrophages that had internalised apoptotic cells as quantified by microscopy was similar for labelled and unlabelled apoptotic targets (Fig. 1C). In a separate series of experiments, we tested whether viability of macrophages was altered after internalisation of apoptotic cells. In these experiments, we found no difference in the numbers of adherent macrophages when comparing those that had internalised CMFDA targets with macrophages that had not been exposed to apoptotic targets (91.8 ± 7.2 and 90 ± 7 macrophages per high power field, respectively). Further, we confirmed that macrophages that had been exposed to apoptotic neutrophils and then detached with trypsin/EDTA retained viability (not shown) and adhered equally well to tissue culture plastic when compared with macrophages that had not been exposed to apoptotic cells (106.3 ± 9 vs. 98.6 ± 3.2 cells per high powered field, respectively). We believe these data indicated that fluorescent labelling of apoptotic targets with CMFDA is minimally disruptive for the process of phagocyte recognition and subsequent internalisation.

## Characterisation of Macrophage Phagocytosis of Apoptotic Cells Using Flow Cytometry

Examination of apoptotic cell populations by flow cytometry revealed two distinct neutrophil populations in terms of fluorescence, despite a uniform initial labelling profile. These distinct populations (Figs. 1A and 3A) were sorted and the FL1-bright population was found to comprise intact neutrophils with apoptotic and nonapoptotic morphologies. In contrast, the FL1-dim population represented cellular debris and cells that lacked nuclei, possibly representing late apoptotic cells (6) that had extruded nuclear material (data not shown).

A gate for acquisition of macrophage data was determined from examination of CMFDA-labelled neutrophils (Fig. 2, top) and macrophages labelled with CD14 mAb (Fig. 2, bottom). The appearance of a population of $FL1^+$ macrophages after 30 min of incubation with apoptotic neutrophils is shown in Figure 3A. Although the these cells (R1) were easily distinguished from the $FL1^-$ cells (R2), we also observed cells with intermediate fluorescence (R3). To confirm that R1 and R2 represented phagocytic and nonphagocytic macrophages, respectively, and to investigate the nature of the intermediate R3 macrophages, all cell populations were sorted with a FACSVantage. Cytocentrifuge preparations of the sorted populations (Fig. 3B) were analysed by light microscopy (Fig. 3C) and showed that cells in R1 consisted predominantly of macrophages that had ingested apoptotic cells (96 ± 2%). Consistent with the suggestion that R2 represented nonphagocytic macrophages, we observed very few internalised neutrophils in the sorted R2 population (1.1 ± 0.8%). Interestingly, macrophages in R3, which displayed intermediate fluorescence on flow cytometry, also showed only a very low percentage of uptake of neutrophils (3 ± 1.7%). However, when examined with a fluorescent microscope, these cells displayed a weak, diffuse fluorescence of their cytoplasm, possibly as a result of having internalised cellular debris (data not shown).

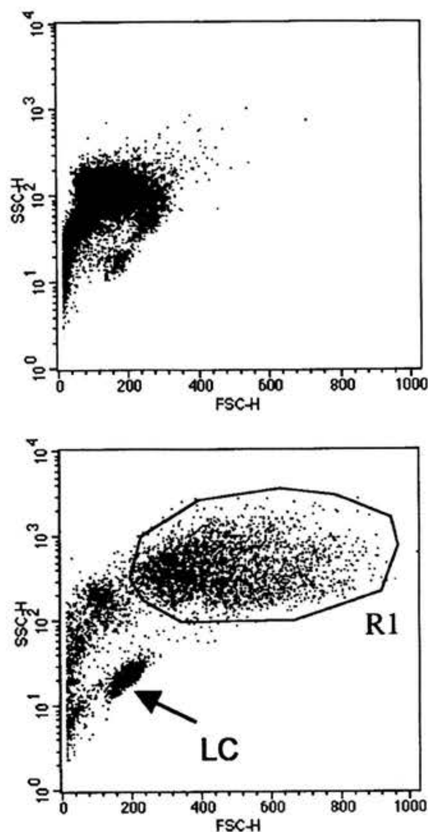



FIG. 2. Typical laser scatter properties of cells used to determine acquisition gates. Dot-plots show forward (FSC) and side scatter (SSC) for aged neutrophils labelled with 5-chloromethylfluorescein diacetate (top). Macrophages harvested from adherent culture as described in Material and Methods were counterstained with CD14 to confirm the laser scatter properties of macrophages (R1; bottom). A few contaminating lymphocytes (LC) are present in these samples. R1 was used as the acquisition gate for phagocytosis assessment. Data are representative of at least six independent experiments.

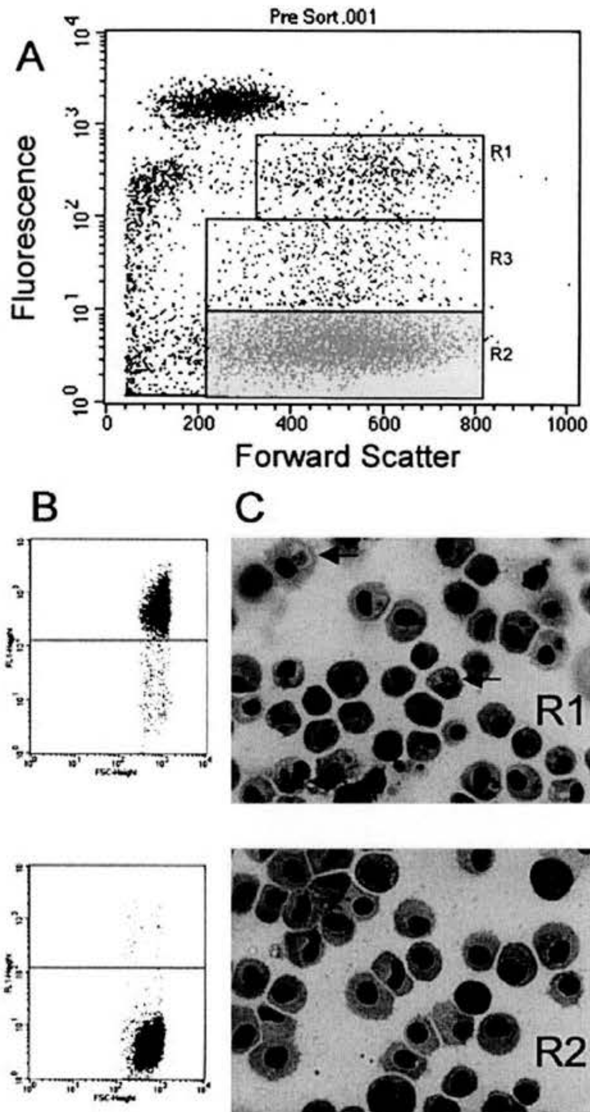



FIG. 3. Flow cytometric analysis of cells detached after phagocytosis assay. **A:** A typical two-parameter dot-plot of untreated macrophage populations after 30 min of incubation. The sort gates R1, R2, and R3 are highlighted. **B:** Sorted populations were reanalysed with a FACSCalibur to confirm that they represent FL1$^+$ and FL-1$^-$ populations. **C:** Cytocentrifuge preparations of sorted populations were examined by microscopy to confirm that most cells in gate R1 had ingested apoptotic neutrophils (96 ± 2%) and that cells in gates R2 and R3 had not (1.1 ± 0.8% and 3 ± 1.7%, respectively). The percentages of macrophages with internalised neutrophils were assessed microscopically by counting at least 200 cells per replicate. Two independent cell sorts were performed, with similar results for subsequent analyses.

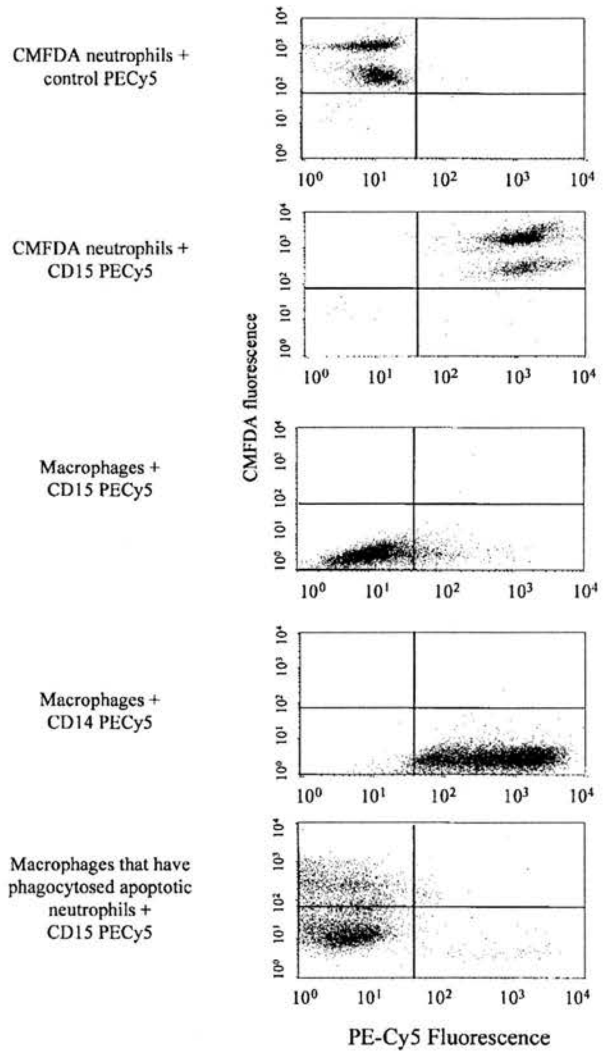



FIG. 4. Discrimination of internalised from bound particles by flow cytometry. Aged neutrophils labelled with 5-chloromethylfluorescein diacetate (CMFDA) were stained with a phycoerythrin indodicarbocyanine (PECy5) control antibody (first panel) or CD15-PECy5 (second panel), demonstrating that CD15 reliably labels apoptotic neutrophils. FL1-bright and FL1-dim populations shift equally (second panel). Macrophages that had not been coincubated with apoptotic neutrophils do not show significant levels of labelling CD15-PECy5 conjugate (third panel) but could be specifically labelled with CD14-PECy5 (fourth panel). Using these controls to set the threshold for FL3 positivity, macrophages that had been coincubated with apoptotic cells were stained with direct CD15-PECy5 antibody (fifth panel). Note the lack of significant FL3 (CD15) positivity in these cells, suggesting that the increase in FL1 fluorescence was due to internalised and not to externally bound neutrophils. Representative sets of dot-plots from one of four independent experiments are shown.

## Differentiating Ingested From Bound Apoptotic Targets

Although we did not observe externally bound neutrophils on the surface of sorted R1 macrophages, we wished to confirm the extent of apoptotic cell binding independently of cell sorting, to eliminate the possibility that shear stress during sorting might have dislodged externally bound cells. Macrophages were therefore counterstained with a PECy5-conjugated CD15 to identify surface bound neutrophils (Fig. 4, upper panels). This antigen was chosen because the carbohydrate determinant was shown to be resistant to trypsin treatment. To confirm resistance of the CD15 epitope to trypsin/EDTA treatment used to detach macrophages, we tested the levels of surface ex-

pression of CD15 on neutrophils treated with and without trypsin/EDTA. In these experiments CD15 levels (MFI, arbitrary values) were found to be increased from 562 ± 10 to 637 ± 30 after trypsin/EDTA treatment. In contrast, CD62L expression was drastically reduced from 200 ± 28 to 4 ± 1.3 after trypsin/EDTA treatment, thus confirming that trypsin treatment was effective. Importantly, no significant FL3 fluorescence was detected in FL1-bright or FL1-dim macrophage populations (Fig. 4, lower panels), demonstrating that bound neutrophils do not contribute significantly to the estimate of percentage of phagocytosis. As reported previously (9,18), although the extent of phagocytosis showed interassay variability (mean, 23.64%; range, 7.6 - 48%), intra-assay variability was low; for example, in one experiment for eight replicate wells, the mean (standard deviation) estimate of phagocytosis was 34.6 (2.1).

### Validation of Flow Cytometric Quantitation

To validate the flow cytometric assessment of phagocytosis, we examined enhancers or inhibitors of apoptotic cell uptake that have been well characterised. Thus, cytochalasin D, EDTA, and low temperature (4°C) inhibit apoptotic cell uptake, whereas cross-linking macrophage CD44 (10) markedly increases phagocytosis (Fig. 5A). Analysis of some of these conditions had proven difficult because cell detachment after treatment with EDTA or low temperatures (4°C) can hinder assessment of phagocytic potential. In contrast, because assessment of phagocytosis by flow cytometry does not involve a wash step, this concern is removed. We also examined the kinetics of macrophage phagocytosis of apoptotic neutrophils in this system. Examination of the percentage of phagocytic macrophages present after 30, 60, 90, and 120 min of coincubation with apoptotic targets showed that maximal phagocytic response occurs after 90 - 120 min, suggesting that conventional 30-min plate-based assays may underestimate macrophage phagocytic capacity. Because the flow cytometric-based quantification eliminates the requirement for washing of macrophage monolayers, we next investigated whether estimates of levels of phagocytosis determined microscopically were affected by cell loss. Apoptotic neutrophils were removed by pipetting without washing, or wells were washed three times before assessment of phagocytosis by flow cytometry as previously described for microscopy-based assays. When assessed by flow cytometry, we found a decrease in the percentage of phagocytic macrophages after three washes, suggesting that conventional assays further underestimate phagocytic potential. Consistent with this suggestion, we found that a small but significant number of phagocytic macrophages was present in the washing fluid (Fig. 6). Interestingly, the proportion of phagocytic macrophages in the washes was higher than that measured for the total population, implying that phagocytic cells may selectively detach.

For reasons of practicality and resource management, we were also interested to determine whether this flow cytometry-based quantitation would allow assessment of phagocytosis if the harvested cell suspensions were fixed by using high-quality fixation methods and stored before analysis. We acquired the samples as live cells and then fixed them in formaldehyde (2.5% or 1%) or methanol-free paraformaldehyde as described in Material and Methods and performed repeated acquisitions over time. However, we found a progressive increase in background fluorescence intensity of the macrophage populations and a diminished separation of neutrophils and macrophages by forward scatter (data not shown). Thus, this assay does not appear to be suited for fixing and storage of samples beyond 24 h.

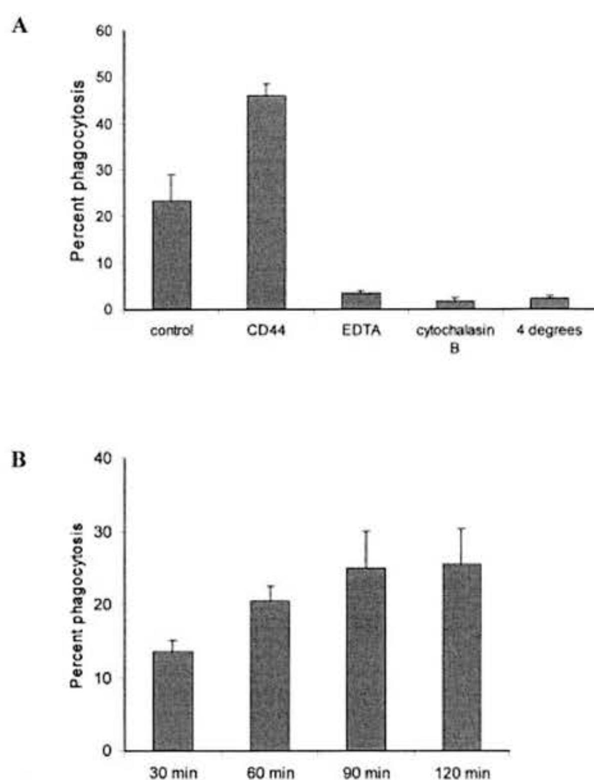



Fig. 5. Characterization of flow cytometric determination of apoptotic cell phagocytosis. **A:** Effect of known enhancers and inhibitors of apoptotic cell phagocytosis. Macrophages were treated with medium (control), CD44 (antibody 5A4, 1:2 dilution of cell culture supernatant, 20 min preincubation), ethylene-diaminetetra-acetic acid (EDTA; 5 mM), or cytochalasin D (5 μg/ml) before coincubation with apoptotic neutrophils at 37°C for 30 min. In parallel, macrophages cultured on a separate plate were incubated with apoptotic neutrophils at 4°C for 30 min. Data represent the mean ± standard error of the mean percentage of fluorescent macrophages from four independent experiments: baseline versus CD44, $P < 0.001$; baseline versus EDTA, cytochalasin D, or 4°C, $P < 0.001$. **B:** Temporal analysis of apoptotic cell phagocytosis. Phagocytosis was determined by flow cytometry after different periods of coincubation of macrophages with apoptotic neutrophils. The data shown are from 5-14 independent experiments and indicated that phagocytosis is not maximal at the 30-min time point used in most studies.

### Flow Cytometric Analysis Is Broadly Applicable to Other Cell Types

To determine whether this convenient flow cytometry-based assay was more generally applicable to examining

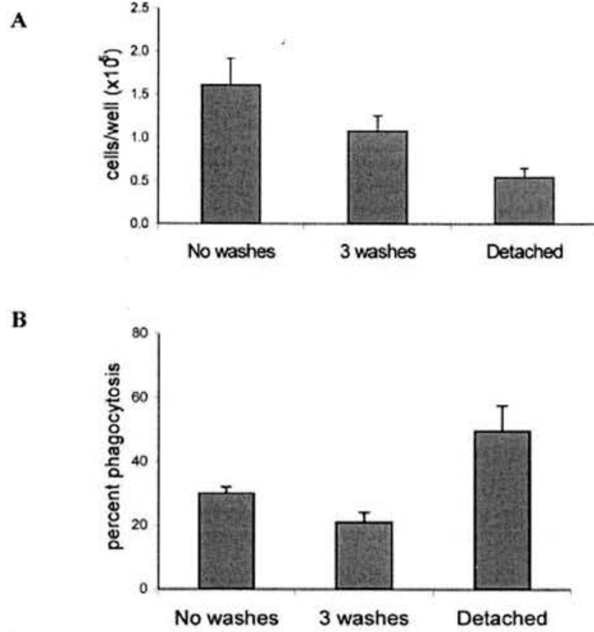
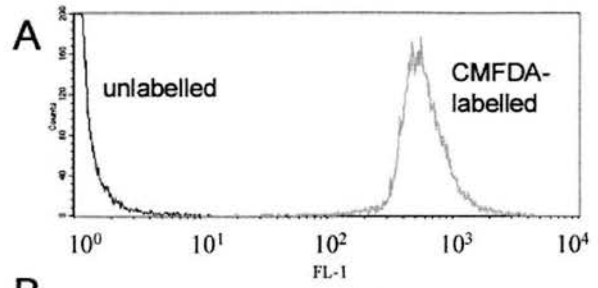
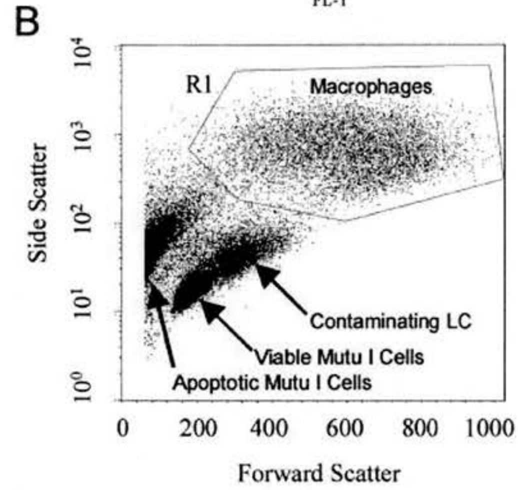



FIG. 6. Effect of washing on the percentage of macrophage phagocytosis recorded. Adherent macrophages in 48-well plates were coincubated with 20-h cultured neutrophils labelled with 5-chloromethylfluorescein diacetate, as described in Materials and Methods. The medium was then carefully removed by aspiration (no wash), and adherent macrophages were then washed three times. **A:** Remaining adherent macrophages were detached by using trypsin/ethylene-diaminetetra-acetic acid, and the numbers of macrophages in samples were determined by microscopic examination with a haemocytometer. **B:** The percentage of phagocytic macrophages in these samples was also assessed. Results are expressed as the mean ± standard error of the mean of five independent experiments conducted in triplicate.



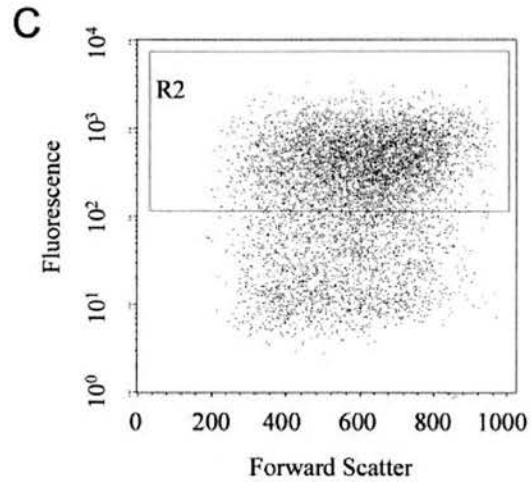




FIG. 7. Typical laser properties of the Burkitt lymphoma cell line Mutu I and human macrophages. **A:** Mutu I populations labelled with 5-chloromethylfluorescein diacetate (CMFDA) are FL1$^+$, with a small proportion of cells with lesser side scatter displaying a lesser fluorescence intensity in FL1 similar to the pattern seen in neutrophils. **B:** A gate for macrophage acquisition was determined based on forward and side scatter properties of the cell populations (R1, verified by CD14 staining). LC, lymphocytes. **C:** Macrophages that had ingested fluorescent Mutu I cells became green (R2). In this representative experiment of three that were performed, 64.7% of macrophages had ingested apoptotic Mutu I cells.

other apoptotic cells, we next used CMFDA-labelled apoptotic lymphocytes as targets. (Fig. 7A). Cell populations were readily distinguished from each other by their forward and side scatter properties and phagocytic macrophages became FL1$^+$ (Fig. 7B). The phagocytic capacity of THP-1 macrophage-like cells and COS-7 fibroblast-like cells, the latter of which is widely used for transfection studies, could also be easily evaluated because the distinct laser scatter properties allowed identification of "free" apoptotic neutrophils or lymphocytes (data not shown). Although the present data were acquired with a FACSCalibur, similar results could be obtained with a different cell analyser (e.g., Epics XL; data not shown).

## DISCUSSION

Apoptosis and the subsequent removal of apoptotic cells by macrophages and other cells have been recognised as important events in development, tissue homoeostasis, initiation of immune responses, and resolution of inflammation (3,11,12). Further understanding of the mechanisms of recognition, engulfment, and especially the fate of the apoptotic target inside the phagocyte requires a robust, reproducible method for quantification and detailed analysis. Plate- or slide-based assays that are widely used to evaluate and quantify the interaction of macrophages with apoptotic cells have multiple shortcomings. Although apoptotic targets have been labelled with fluorescent dyes in the past, the assessment of the

phagocytic response had been made only by fluorescent microscopy, and microscopic quantification has similar problems (5,13). Use of a colorimetric assay for phagocytosis (15), although removing observer bias, still requires vigorous washing of the cells and fails to provide information regarding the proportions of cells that are phagocytic, and phagocytic populations need to be lysed and therefore cannot be recovered.

Surprisingly, many studies of the phagocytosis of apoptotic cells have not used flow cytometry-based assays (16). Galati et al. used flow cytometry to investigate the degree of uptake of labelled apoptotic Jurkat cells by peripheral blood leukocytes in samples of whole blood (17). As expected, there was only low-level uptake in patients pretreated with granulocyte-macrophage colony-stimulating factor and no phagocytosis in healthy individuals, which is consistent with the observation that peripheral blood monocytes show a poor uptake of apoptotic cells (18). Flow cytometry has been used to reliably quantify phagocytosis of bacteria, malarial parasites, liposomes, zymosan particles, latex beads, unopsonised environmental particles, platelets, and erythrocytes (19-26). We have demonstrated that quantification of macrophage phagocytosis by flow cytometry is a robust and reproducible. Large amounts of data could be acquired quickly, particularly in conditions in which conventional assays are known to be unreliable, for example, in the treatment of macrophages with antibodies or cell signalling inhibitors. We also demonstrated that flow cytometry can be used to reliably distinguish bound from internalised apoptotic targets. Flow cytometric quantification of phagocytosis has the technical advantage of removal of observer error, assessment of large numbers of cells, and high sample throughput. We have shown that this technique is readily extended to other apoptotic targets and should be applicable to other phagocytic cells, such as COS-7, a cell line commonly used for transfection of components of the phagocytic machinery (5).

One unique advantage of flow cytometric quantification is that vigorous washing steps can be eliminated. Indeed, the recorded percentage of phagocytosis was decreased after washing of adherent macrophage monolayers. Further, we found that the relative percentage of phagocytic macrophages present in the washing fluid was higher than that for the total population, implying that phagocytic macrophages may be selectively detached during washing. This is likely to have considerable implications if macrophages are treated with agents that disrupt adhesion, such as antibodies, peptides, or cytoskeletal inhibitors. Similarly, highly phagocytic dexamethasone-treated macrophages exhibit altered adhesion status when compared with untreated cells and are prone to detachment (27). Use of flow cytometric quantification also would permit assessment of phagocytosis in suspension culture.

Another important aspect of the flow cytometric assessment of phagocytosis is that the phagocytes studied are available for further analysis. Macrophages that have ingested CMFDA-labelled targets and are subsequently detached for flow cytometric analysis remain viable and can be readily sorted into phagocytic and nonphagocytic subsets. We have shown that re-culturing phagocytic subpopulations for further functional analysis is feasible, allowing investigation of other macrophage functions, including potential for phagocytosis of further apoptotic targets or other particles. It should also be possible to undertake phenotypic and functional analyses, for example, gene profiling of phagocytic subsets of cells and assessment of levels of intracellular cytokines or signalling molecules in permeabilised cells.

## CONCLUDING REMARKS

The advantages of flow cytometry-based quantification of apoptotic cell phagocytosis when using the assay described in this paper will provide new opportunities for the analysis of the mechanisms and consequences of the uptake of apoptotic cells by phagocytes in vitro.

## ACKNOWLEDGMENTS

We thank Ms. Cathy Simpson for her assistance with cell sorting, Dr. Simon Hart, Lynda Stuart, and Dr. James Pryde for helpful comments, and Dr. Graham L. Thomas for his assistance with the imaging software. We are grateful to Dr. Andrew Devitt for providing Mutu-I Burkitt lymphoma cells. Hubertus P. A. Hersmann received a Sanofi-Winthrop Scholarship from the Royal Australian College of Physicians. Sharon Vivers holds an MRC studentship.

## LITERATURE CITED

1. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239-257.
2. Savill J. Recognition and phagocytosis of cells undergoing apoptosis. Br Med Bull 1997;53:491-508.
3. Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. J Clin Invest 2001;108: 957-962.
4. Dooley DC, Simpson JF, Meryman HT. Isolation of large numbers of fully viable human neutrophils: a preparative technique using Percoll density gradient centrifugation. Exp Hematol 1982;10:591-599.
5. Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL, Gregory, CD. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature 1998;392:505-509.
6. Hart SP, Ross JA, Ross K, Haslett C, Dransfield I. Molecular characterization of the surface of apoptotic neutrophils: implications for functional down regulation and recognition by phagocytes. Cell Death Differ 2000;7:493-503.
7. Dransfield I, Buckle A-M, Savill JS, McDowall A, Haslett C, Hogg N, Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. J Immunol 1994;153:1254-1263.
8. Martin SJ, Reutelingsperger CPM, McGahon AJ, Rader JA, van Schie RCAA, LaFace DM, Green DR. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med 1995;182:1545-1556.
9. Licht R, Jacobs CWM, Tax WJM, Berden JHM. An assay for the quantitative measurement of in vitro phagocytosis of early apoptotic thymocytes by murine resident peritoneal macrophages. J Immunol Methods 1999;223:237-248.
10. Hart SP, Dougherty GJ, Haslett C, Dransfield I. CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages. J Immunol 1997;159: 919-925.
11. Haslett C. Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. Clin Sci 1992;83:639-648.
12. Savill J, Fadok V, Henson P, Haslett C. Phagocyte recognition of cells undergoing apoptosis. Immunol Today 1993;14:131-136.
13. Zeira M, Gallily R. Interaction between thymocytes and thymus-

derived macrophages. I. Surface components participating in mutual recognition. Cell Immunol 1988;117:264-276.
14. Haslett C, Guthrie LA, Kopaniak MM, Johnston RB Jr, Henson PM. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. Am J Pathol 1985;119:101-110.
15. Chan H-T, Kedzierska K, O'Mullane J, Crowe SM, Jaworowski A. Quantifying complement-mediated phagocytosis by human monocyte-derived macrophages. Immunol Cell Biol 2001;79:429-435.
16. Lehmann AK, Sornes S, Halstensen A. Phagocytosis: measurement by flow cytometry. J Immunol Methods 2000;243:229-242.
17. Galati G, Rovere P, Citterio G, Bondanza A, Scaglietti U, Bucci E, Heltai S, Fascio U, Rugarlie C, Manfredi AA. In vivo administration of GM-CSF promotes the clearance of apoptotic cells: effects on monocytes and polymorphonuclear leukocytes. J Leukoc Biol 2000;67:174-182.
18. Savill J, Dransfield I, Hogg N, Haslett C. Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature 1990;343:170-173.
19. De Boer EC, Bevers RF, Kurth KH, Schamhart DH. Double fluorescent flow cytometric assessment of bacterial internalization and binding by epithelial cells. Cytometry 1996;25:381-387.
20. Kumaratilake LM, Ferrante A. Opsonization and phagocytosis of Plasmodium falciparum merozoites measured by flow cytometry. Clin Diagn Lab Immunol 2000;7:9-13.
21. Perry DG, Martin WJ 2nd. Fluorescent liposomes as quantitative markers of phagocytosis by alveolar macrophages. J Immunol Methods 1995;181:269-285.
22. Bjerknes R, Bassoe CF. Phagocyte C3-mediated attachment and internalization: flow cytometric studies using a fluorescence quenching technique. Blut 1984;49:315-323.
23. Steinkamp JA, Wilson JS, Saunders GC, Stewart CC. Phagocytosis: flow cytometric quantitation with fluorescent microspheres. Science 1982;215:64-66.
24. Palecanda A, Kobzik L. Alveolar macrophage-environmental particle interaction: analysis by flow cytometry. Methods 2000;3:241-247.
25. Brown SB, Clarke MCH, Magowan L, Sanderson H, Savill J. Constitutive death of platelets leading to scavenger receptor-mediated phagocytosis. A caspase-independent cell clearance program. J Biol Chem 2000;75:987-5996.
26. Bratosin D, Mazurier J, Tissier JP, Slomianny C, Estaquier J, Russo-Marie F, Huart JJ, Freyssinet JM, Aminoff D, Ameisen JC, Montreuil J. Molecular mechanisms of erythrophagocytosis. Characterization of the senescent erythrocytes that are phagocytized by macrophages. C R Acad Sci III 1997;320:811-818.
27. Giles KM, Ross K, Rossi AG, Hotchin NA, Haslett C, Dransfield I. Glucocorticoid augmentation of macrophage capacity for phagocytosis of apoptotic cells is associated with reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation, and high levels of active Rac. J Immunol 2001;167:976-986.

Clinical Science (2002) **103**, 441–449 (Printed in Great Britain) 

GLAXO/MRS PAPER

# Role of macrophage CD44 in the disposal of inflammatory cell corpses*

**Sharon VIVERS, Ian DRANSFIELD and Simon P. HART**
MRC Centre for Inflammation Research, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

ABSTRACT

Understanding the cellular and molecular mechanisms that determine whether inflammation resolves or progresses to scarring and tissue destruction should lead to the development of effective therapeutic strategies for inflammatory diseases. Apoptosis of neutrophil granulocytes is an important determinant of the resolution of inflammation, providing a mechanism for down-regulation of function and triggering clearance by macrophages without inducing a pro-inflammatory response. However, if the rate of cell death by apoptosis is such that the macrophage clearance capacity is exceeded, apoptotic cells may progress to secondary necrosis, resulting in the release of harmful cellular contents and in damage to the surrounding tissue. There are many possible ways in which the rate and capacity of the macrophage-mediated clearance of apoptotic cells may be enhanced or suppressed. Ligation of human macrophage surface CD44 by bivalent monoclonal antibodies rapidly and profoundly augments the capacity of macrophages to phagocytose apoptotic neutrophils *in vitro*. The molecular mechanism behind this effect and its potential significance *in vivo* is a current focus of research.

## NEUTROPHIL APOPTOSIS PROMOTES THE RESOLUTION OF INFLAMMATION

During inflammation, neutrophils are stimulated to release granule enzymes, oxidants and inflammatory mediators by bacterial products, cytokines and adhesion to matrix components and neighbouring cells [1]. Uncontrolled or inappropriate release of these histotoxic substances may result in damage to host tissues and prolong the inflammatory response. Indeed, neutrophils have been implicated in the pathogenesis of a variety of serious inflammatory diseases, such as fibrosing alveolitis, adult respiratory distress syndrome, rheumatoid arthritis and ulcerative colitis [2]. Neutrophil apoptosis results in the loss of expression of adhesion molecules [3] and greatly reduced responsiveness to external stimuli [4], so that these cells become functionally isolated from their environment. In contrast with necrosis, apoptosis is associated with preservation of plasma membrane integrity, so that release of harmful neutrophil contents is limited, and the inert neutrophils are phagocytosed by local macrophages. Furthermore, phagocytosis of apoptotic granulocytes by human macrophages *in vitro* does not stimulate the release of macrophage-derived pro-inflammatory mediators (in contrast with ingestion of particles opsonized with immunoglobulin) [5–7]. In this way, by down-regulating neutrophil functions and triggering 'silent' clearance by phagocytes, apoptosis provides a mechanism for the safe disposal of potentially destructive inflammatory cells. Evidence for a role for leucocyte apoptosis in the resolution of inflammation is supported by the observation that apoptotic granulocytes

* This paper was presented at the Glaxo/MRS Young Investigator session at the MRS Meeting, Royal College of Physicians, London, on 1 June 2001.

**Key words:** apoptosis, CD44, inflammation, macrophage, neutrophil, phagocytosis.

**Abbreviations:** CD44S, 'standard' CD44; CD44v, variant isoform of CD44; FAK, focal adhesion kinase; GM-CSF, granulocyte–macrophage colony-stimulating factor; TNF$\alpha$, tumour necrosis factor $\alpha$.

**Correspondence:** Dr Simon P. Hart (e-mail s.hart@ed.ac.uk).

© 2002 The Biochemical Society and the Medical Research Society

can be identified within phagocytic vacuoles in macrophages from inflamed joints [8] and the airways of asthmatic patients [9].

## RECOGNITION OF APOPTOTIC CELLS

Clearly, macrophages must be able to recognize changes on the surface of the apoptotic cell that distinguish it from a healthy viable cell. Multiple macrophage surface receptors have been implicated in the recognition and clearance of apoptotic cells [10–12]. Similarly, in the nematode *Caenorhabditis elegans*, at least six different genes have been discovered that seem to be required for effective removal of dying cells by phagocytes [13]. The reasons for this redundancy are unclear, but a number of the implicated molecules may be revealed to be indirect regulators of apoptotic cell phagocytosis, rather than true apoptotic cell receptors [14].

During the development of *C. elegans*, the generation of 959 somatic nuclei is accompanied by the death of 131 cells, and a number of mutations that affect the processes of cell death and the engulfment of dead cells have been identified. Mutations in one of any six genes, *ced-1*, *-2*, *-5*, *-6*, *-7* or *-10*, prevents some cell corpses from being engulfed, although in all of these mutants most cells were engulfed normally. In contrast, a major defect in engulfment can be found in double mutants in which one mutation is in *ced-2*, *-5* or *-10*, and the other mutation is in *ced-1*, *-6* or *-7* [13]. This observation suggested that the two sets of genes act in parallel processes that are partially redundant. We now know that the proteins encoded by *ced-2*, *ced-5* and *ced-10* are similar to human CrkII, Crk-binding protein DOCK180 and Rac respectively, and that these molecules comprise a pathway that controls

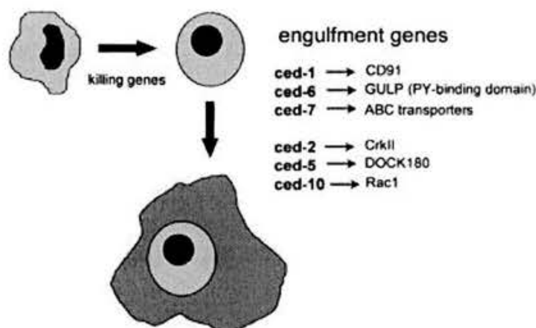



**Figure 1 Engulfment genes in *C. elegans***

The genes that are involved in the engulfment of cell corpses during the development of the nematode *C. elegans* have been divided into two functionally redundant groups (*ced-1*/*ced-6*/*ced-7* and *ced-2*/*ced-5*/*ced-10*). These have been cloned and sequenced, providing evidence for a role for similar molecules in the phagocytosis of apoptotic cells in higher animals.

the organization of the actin cytoskeleton and the extension of cell processes [15]. Mammalian homologues of *ced-1*, *ced-6* and *ced-7* have also been identified (Figure 1). Thus the characterization of *C. elegans* genes that are involved in the phagocytic clearance of cellular corpses in the nematode has elucidated potential roles for a number of key elements of cytoskeletal regulation and signal transduction.

## REGULATION OF THE PHAGOCYTOSIS OF APOPTOTIC CELLS

If the rate of cell death by apoptosis is such that the clearance capacity of macrophages is exceeded, apoptotic cells may become necrotic, resulting in the release of harmful cellular contents and damage to the surrounding tissue (Figure 2). In support of this suggestion, treatment of mice with anti-Fas antibody triggered a massive wave of apoptosis in the liver, and the animals developed extensive hepatic necrosis and died [16]. Similarly, induction of apoptosis in the rat lung led to pulmonary fibrosis [17]. We propose that in these situations the hepatic and pulmonary macrophages respectively were unable to clear the load of apoptotic cells with which they were faced. Similar situations could arise if neutrophil apoptosis were to be induced deliberately as part of a therapeutic strategy for inflammatory diseases. Such potential future approaches may therefore have to include a way of concurrently augmenting the clearance efficiency of local macrophages.

Macrophage phagocytic capacity *in vitro* may be influenced by soluble mediators such as cytokines [18] and prostaglandins [19], or by glucocorticoid hormones [14,20]. In addition, the interaction of surface adhesion molecules with neighbouring cells and components of the extracellular matrix may profoundly influence many aspects of cellular behaviour, including phagocytosis [21]. CD44 is a surface receptor that has been implicated in cell adhesion to a variety of matrix components, including fibronectin, and may be associated with cytoskeletal proteins and intracellular signalling pathways [22]. CD44 was therefore an attractive target for our further investigation of how the phagocytosis of apoptotic cells by macrophages may be regulated.

## STRUCTURE AND FUNCTION OF CD44

The CD44 gene in humans consists of 50–60 kb located on the short arm of chromosome 11. Some of the 20 or more exons are constitutively transcribed, but a sequence of at least 10 exons (exons 6a–14) undergoes variable splicing, giving rise to an additional peptide sequence between amino acids 202 and 203 in the membrane-proximal domain of the CD44 protein [23,24] (Figure 3).

© 2002 The Biochemical Society and the Medical Research Society

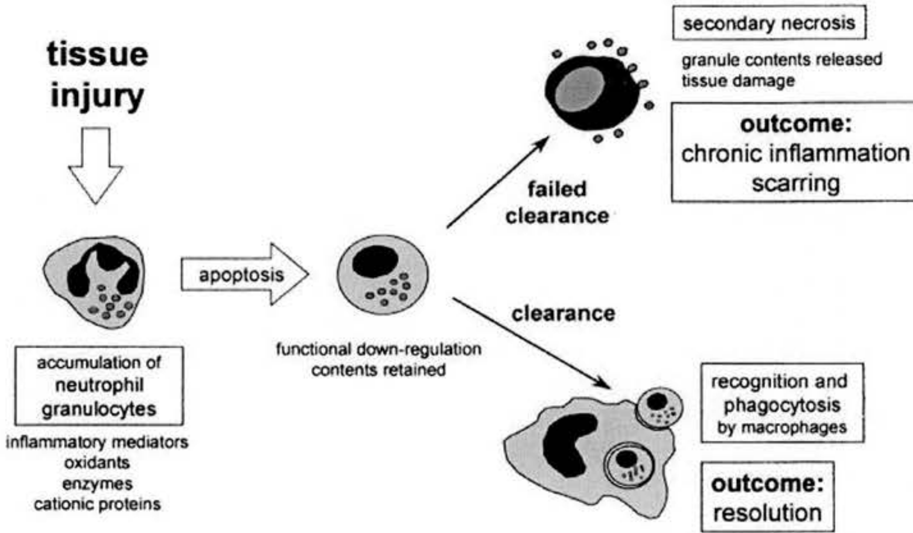



**Figure 2 Neutrophil apoptosis and phagocytic clearance by macrophages in the resolution of inflammation**
See the text for details.

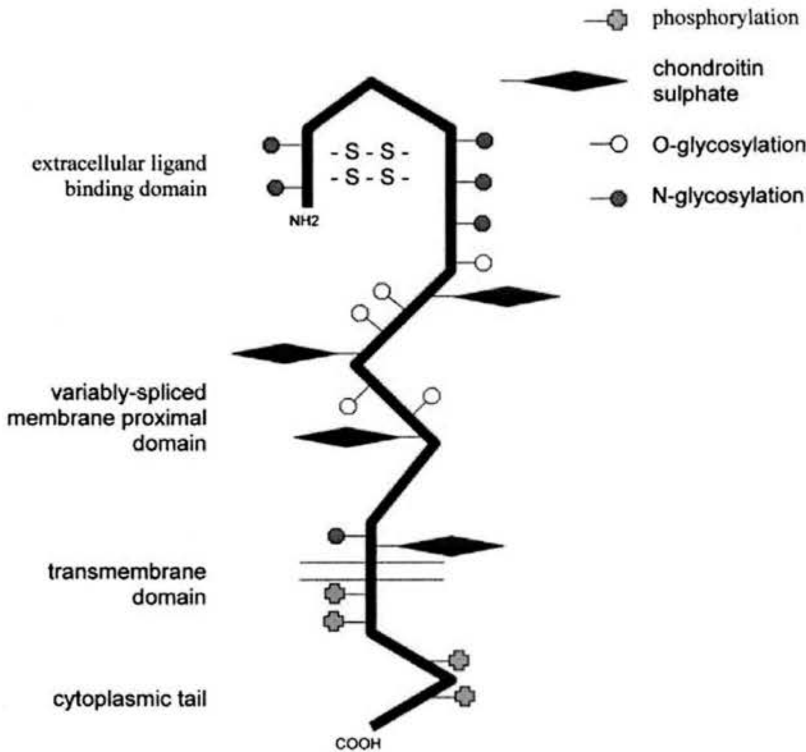



**Figure 3 Diagrammatic representation of CD44**
CD44 comprises a large extracellular ligand-binding domain, an alternatively spliced membrane-proximal domain containing sites for linkage of large sugars, a highly conserved transmembrane sequence, and a short cytoplasmic tail containing phosphorylation sites.

© 2002 The Biochemical Society and the Medical Research Society

In practice, only relatively few variant isoforms of CD44 (CD44v isoforms) containing certain combinations of variably spliced CD44 exons have been found to be expressed in human cells. The most prevalent form of CD44, termed CD44S ('standard') or CD44H ('haematopoietic'), contains no additionally spliced exon products. CD44S is a single-chain type I transmembrane protein containing 341 amino acids, with a predicted molecular mass of 40 kDa. However, extensive post-translational glycosylation results in CD44S having an apparent molecular mass on SDS/PAGE of 80–100 kDa. The expression of variant isoforms along with differential glycosylation alters the range of potential ligands the cell may respond to [25,26].

CD44 is expressed by many cell types, including leucocytes, erythrocytes, fibroblasts, endothelial and epithelial cells, and a variety of tumour cells [22]. Unusually for a cell surface receptor, CD44 seems to have broad ligand specificity, and has been proposed to bind the extracellular matrix molecules hyaluronan [27, 28], fibronectin [29], collagen [30] and fibrin [31,32]. In addition, CD44 may bind to the proteoglycan serglycin found in lymphocyte and mast cell granules [33], to the extracellular phosphoprotein osteopontin [34], and to CD44 molecules on neighbouring cells [35]. The interaction of cell surface CD44 with hyaluronan is thought to be important in a variety of cell adhesion-related phenomena, but the functional relevance of the binding of CD44 to other putative ligands is poorly understood at present. Many diverse functions have been attributed to CD44, including involvement in cellular adhesion and migration, lymphocyte activation and proliferation, cytocidal activity of natural killer cells and tumour metastasis [36]. In other experimental systems, anti-CD44 antibodies can block the migration of murine pro-T cells into the thymus [36,37] and prevent leucocyte extravasation at sites of inflammation [38]. Moreover, they can also stimulate leucocyte adhesion [39] and augment T cell activation [40], suggesting that CD44 does indeed signal within the cell. This idea is supported further by the discovery that CD44 plays a role in cell spreading [41], and cross-linking of CD44 on T cells is known to activate the tyrosine kinase $p56^{lck}$ [42]. Src-family kinases have been shown to participate in CD44-mediated signalling resulting in cell spreading in CD45-deficient T cells [43].

## CD44 IS IMPORTANT IN INFLAMMATION

In recent years, several different observations have implicated CD44 in the regulation of the inflammatory response. Freshly isolated peripheral blood monocytes express CD44S strongly [44], and the differentiation of monocytes into macrophages during *in vitro* culture is associated with increased expression of variant isoforms, while CD44S expression persists at high levels [45,46]. Elevated local concentrations of CD44 ligands (such as hyaluronan and fibronectin) that follow tissue injury are likely to be important mediators of macrophage function as the inflammatory response progresses. Expression of the variant isoforms (CD44v6 and CD44v9) by monocyte-like THP-1 cells can be up-regulated following exposure to the inflammatory cytokines tumour necrosis factor $\alpha$ (TNF$\alpha$) and interferon-$\gamma$ [44], and macrophage expression of some CD44 isoforms is increased at sites of chronic inflammation [46]. *In vivo*, systemic administration of anti-CD44 monoclonal antibodies reduced the severity of joint inflammation in mice with experimental arthritis [38,47].

While these studies provide circumstantial evidence for the involvement of CD44 in inflammation, more direct proof has come very recently from studies of lung inflammation in CD44-deficient knockout mice. Teder and colleagues [48] demonstrated that CD44-deficient mice exhibited unremitting and often fatal inflammation following bleomycin-induced lung injury compared with wild-type animals. The increased propensity to develop severe pneumonitis could be largely reversed by repopulating the bone marrow of the CD44-deficient mice with CD44+ wild-type cells, demonstrating a critical role for CD44 expressed by macrophages or other haematopoietic cells in the resolution of lung inflammation.

## CD44 LIGATION AUGMENTS THE PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS

To assess the role of macrophage CD44 in the phagocytosis of apoptotic neutrophils *in vitro*, anti-CD44 antibodies were used as convenient experimental surrogates for the natural ligand(s) of CD44. Adherent human monocyte-derived macrophages were incubated with anti-CD44 monoclonal antibodies for 20 min immediately prior to assessment of phagocytosis of apoptotic neutrophils using a well characterized assay [8,49]. A control antibody (W6/32, against a non-polymorphic determinant on MHC class I) was chosen which, like the CD44 antibodies, bound strongly to human monocyte-derived macrophages. In this series of experiments, binding of anti-CD44 antibodies resulted in a dramatic increase in the macrophage-mediated phagocytosis of apoptotic neutrophils (Figure 4). Control antibody W6/32 had no significant effect, and neither did antibodies specific for integrin $\alpha5$, $\beta1$ or $\beta2$ subunits [50].

Conventionally, an in-well plate assay has been used to determine the proportion of phagocytic macrophages in experiments like these. However, there are several disadvantages to this approach: counting cells is somewhat subjective and prone to inter-observer variation; there is

© 2002 The Biochemical Society and the Medical Research Society

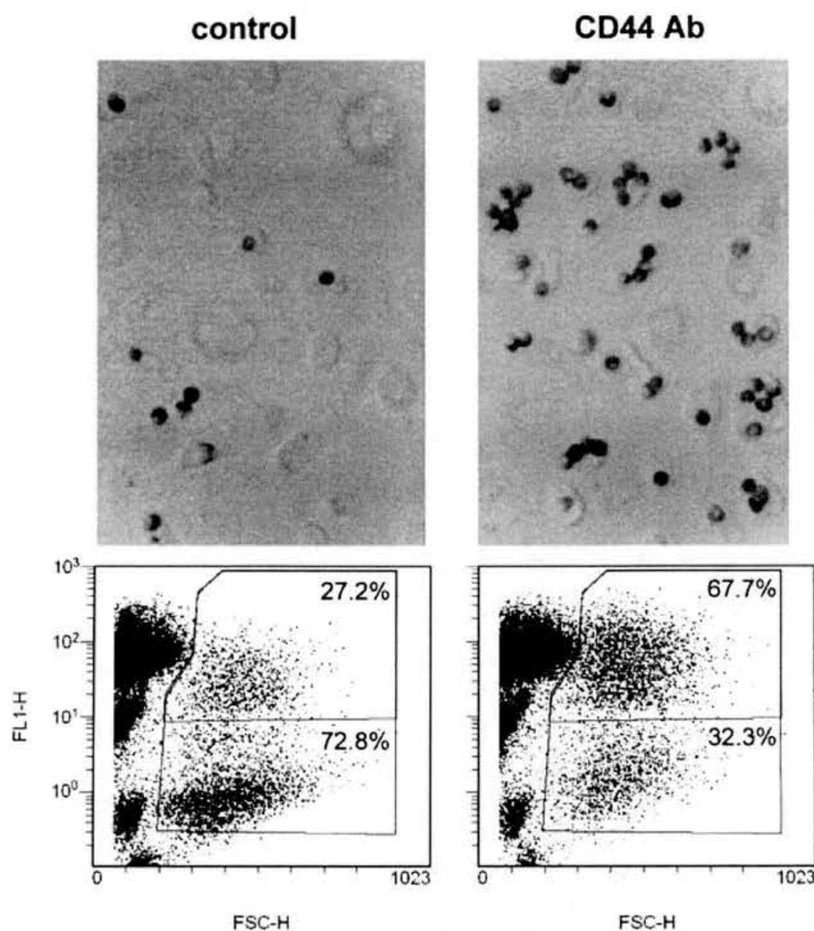



**Figure 4 The anti-CD44 antibody 5A4 augments the macrophage-mediated phagocytosis of apoptotic neutrophils**
Shown are representative micrographs from one experiment demonstrating the macrophage-mediated phagocytosis of apoptotic neutrophils following incubation with medium alone (left panel) or the anti-CD44 antibody 5A4 (right panel). After thorough washing and fixation, cells were stained for myeloperoxidase. Apoptotic neutrophils were 100% myeloperoxidase-positive (dark reaction product), while macrophages were myeloperoxidase-negative. 5A4-treated wells show increases both in the proportion of macrophages that had phagocytosed apoptotic neutrophils and in the number of neutrophils per phagocytosing macrophage. The lower panels show results from a different experiment using a flow-cytometry-based phagocytosis assay to assess the effects of the anti-CD44 antibody 5A4. The percentages of macrophages that had ingested or not ingested fluorescent green-labelled aged neutrophils are shown.

often variability between samples due to the limited number of cells that can be counted; and counting is time consuming. Also, selective detachment of phagocytic macrophages may occur during the washing process required following the assay, leading to an underestimate of phagocytosis. These problems may be circumvented by using a new flow-cytometry-based assay for measuring phagocytosis (H. Jersmann, S. Vivers, K. Ross, S. Brown and I. Dransfield, unpublished work). In this assay, neutrophils are labelled with a fluorescent green cytoplasmic dye and then aged *in vitro* in the usual way to induce apoptosis. When these apoptotic cells are phagocytosed, the green-labelled macrophages can be identified by flow cytometric analysis. This assay can be used to determine the number of macrophages that have phagocytosed apoptotic neutrophils within a certain period of time, without needing to wash the cells, so avoiding the potential problem of selective detachment. Some typical flow cytometry profiles are shown in Figure 4.

Our analysis was extended to include other antibodies that recognize specific variant isoforms of CD44 expressed by human monocyte-derived macrophages. Interestingly, anti-CD44 antibodies that define the CD44v3 (antibody 3G5) or CD44v10 (2G1) isoforms, or a hyaluronan-binding-associated epitope (7F4), failed to augment phagocytosis, despite binding to macrophages. However, it is difficult to completely discount a role for specific isoforms of CD44 in the regulation of phagocytosis of apoptotic neutrophils based on the lack of an

© 2002 The Biochemical Society and the Medical Research Society

effect of single antibodies. Incubation of macrophages with monovalent Fab′ antibody fragments was shown to have no effect on phagocytosis, whereas bivalent $F(ab')_2$ fragments augmented the phagocytosis of apoptotic neutrophils. This observation clearly rules out a role for macrophage Fc$\gamma$ receptors. The lack of an effect of Fab′ fragments of anti-CD44 antibodies indicates that bivalent antibody binding is required for the augmented phagocytosis of apoptotic neutrophils. The requirement for bivalent antibody is important, because it alludes to the possible underlying mechanism of action of CD44 ligation in these experiments. If simple masking of a CD44 epitope by an intact antibody was responsible for the observed effect, then monovalent Fab′ fragments would be expected to act similarly. The requirement for bivalent binding implies that receptor cross-linking is a prerequisite, which then leads to initiation of intracellular signal transduction [51]. In physiological situations it is likely that CD44 on the macrophage surface would be cross-linked by large multivalent ligands in the extracellular matrix. However, we have excluded a role for the principal CD44 ligand hyaluronan, since the addition of soluble hyaluronan or adhesion of macrophages to hyaluronan-coated plastic did not significantly influence the phagocytic uptake of apoptotic neutrophils.

## MECHANISMS OF CD44-AUGMENTED PHAGOCYTOSIS

We have considered a number of possibilities with regard to how the ligation of CD44 exerts its effect on the phagocytosis of apoptotic neutrophils. First, if CD44 were a constitutive negative regulator of apoptotic cell uptake, anti-CD44 antibodies could lead to the redistribution of CD44 into patches on the macrophage surface, thus allowing room for other receptors to interact with the apoptotic target. However, we could see no evidence of CD44 redistribution, as determined by fluorescence microscopy. Secondly, stimulation of CD44 may signal within the cell to promote phagocytosis. In support of this suggestion, the cytoplasmic tail of CD44 has been reported to interact with the cytoskeleton and with intracellular transduction molecules [52]. We believe that the CD44-directed recruitment of these effectors should provide the key to understanding the mechanism behind the augmented phagocytosis of apoptotic neutrophils. The small Rho-like GTPases such as Rac (analogous to ced-10), Rho and cdc42 act as molecular switches that control the organization of the actin cytoskeleton [15], which is critical for particle engulfment during phagocytosis. In mouse mammary epithelial cells, ligation of CD44 triggers activation of Rac1 that results in rearrangements of the actin cytoskeleton [53]. Membrane recruitment of molecules such as Rac1 may be regulated by the guanine nucleotide exchange factor Tiam1, which has been suggested to associate with CD44 in breast tumour cells [54]. The cytoplasmic domain of CD44 may also associate directly with the Src-family kinases Lck and Fyn in lymphocytes [55], and may rapidly induce tyrosine phosphorylation of $p56^{lck}$ [42]. Src kinase activity may in turn result in the phosphorylation of Pyk2, a focal adhesion kinase (FAK) family member. A downstream target of both FAK and Pyk2 is paxillin, an important component of focal adhesions.

## CELL-LINEAGE-SPECIFIC SURFACE ALTERATIONS DURING APOPTOSIS

Ligation of CD44 on human monocyte-derived macrophages by bivalent monoclonal antibodies rapidly and specifically augmented the phagocytosis of apoptotic neutrophils, but had no detectable effect on the phagocytosis of other particles or apoptotic lymphocytes. These results indicate that apoptosis may be associated with the expression of cell-specific markers that signal recognition and phagocytosis by macrophages [50].

## OTHER REGULATORS OF THE CLEARANCE OF APOPTOTIC CELLS

Some inflammatory mediators, such as prostaglandins, signal through activation of adenylate cyclase and elevation of intracellular cAMP levels. Prostaglandins can influence the immune response in a number of different ways, but prostaglandins $E_2$ and $D_2$ are capable of inhibiting the macrophage-mediated phagocytosis of apoptotic neutrophils *in vitro* [19]. Moreover, treatment of macrophages with a membrane-permeable analogue of cAMP also inhibited the uptake of apoptotic neutrophils [19]. Elevated levels of cAMP had no effect on Fc$\gamma$R-mediated phagocytosis, indicating specificity for the inhibition of apoptotic cell uptake. Elevation of cAMP concomitantly induced phenotypic alterations in the macrophage, including changes in the localization of actin and talin into discrete structures. Similar results have been observed when staining for vinculin, paxillin and tyrosine-phosphorylated proteins, suggesting that cAMP may uncouple adhesion receptors from cytoplasmic cytoskeletal elements, resulting in the observed morphological alterations.

Corticosteroids are used widely in the treatment of inflammatory diseases, and are known to inhibit inflammatory cell recruitment and dampen cellular responsiveness. They also have diverse effects on inflammatory cell apoptosis, promoting the apoptosis of thymocytes and eosinophils while inhibiting neutrophil apoptosis [56]. Treatment of human macrophages with glucocorti-

© 2002 The Biochemical Society and the Medical Research Society

coids markedly increased their capacity to phagocytose apoptotic neutrophils *in vitro* [57]. In contrast with CD44 ligation, the effect of glucocorticoids requires several hours of treatment and is inhibited by the protein synthesis blocker cycloheximide [14]. Also, phagocytic effects are not restricted to apoptotic target cells of the neutrophil lineage. Human monocytes treated with the glucocorticoid dexamethasone *in vitro* mature into a homogeneous population of macrophages with a typically smaller, more rounded appearance than untreated cells. This is associated with reduced expression of p130Cas [14], an adaptor molecule that is predicted to disrupt Crk–DOCK180 (ced-2–ced-5) complexes, which are important for cell adhesion. This, combined with reduced phosphorylation and recruitment of paxillin and Pyk2 to sites of adhesion, may account for the changes in cell morphology and phagocytic capacity that accompany steroid hormone treatment [14]. Despite the rounded appearance of dexamethasone-treated cells, they remain highly membrane-active and can be observed rapidly contracting and expanding cellular processes. This may be attributable to increased levels of active GTP-bound Rac in the dexamethasone-treated macrophages [14].

The adhesive state of a macrophage can modulate the phagocytic potential of the cell. The adhesion of macrophages to fibronectin, vitronectin or collagen VI increased the proportion of macrophages that ingested apoptotic neutrophils [58]. Macrophage adhesion to fibronectin may involve CD44, which can be ligated via heparin-binding sites. Heparin completely inhibited the augmentation by fibronectin of macrophage-mediated phagocytosis [58]. Despite this, treatment of macrophages with a proteolytic fragment of fibronectin which contained the major heparin-binding sites was unable to augment phagocytosis. This seems to suggest that, if fibronectin is a ligand for CD44 in this system, generation of a signal for augmented phagocytosis may require binding to more than one receptor. It is possible that the binding of a specific repertoire of macrophage adhesion receptors can influence the cell's potential for phagocytosis of apoptotic neutrophils. Macrophage production and deposition of matrix molecules *in vitro* may play a role in regulating the observed levels of phagocytosis of apoptotic cells.

Cytokines present at sites of inflammation have the potential to modulate cell behaviour, including the phagocytosis of a wide range of targets. A whole host of cytokines, including granulocyte–macrophage colony-stimulating factor (GM-CSF), transforming growth factor-$\beta$1, interferon-$\gamma$, interleukin-1$\beta$ and TNF$\alpha$, have been shown to increase the uptake of apoptotic neutrophils by macrophages after a 4 h incubation. [18]. GM-CSF, interferon-$\gamma$, interleukin-1$\beta$ and TNF$\alpha$ all play important roles in the initiation and amplification of an immune response. Moreover, they are present at sites of inflammation where monocytes mature into macrophages. Therefore these cytokines may assist in preparing macrophages to clear senescent neutrophils once they have undergone apoptosis.

## CONCLUSION

The phagocytosis of apoptotic neutrophils, but not apoptotic lymphocytes, by human monocyte-derived macrophages is rapidly augmented following ligation of CD44 by bivalent antibodies *in vitro*. These observations, together with the lack of effect of anti-CD44 antibodies on the macrophage-mediated phagocytosis of other particles, imply that CD44 may regulate the differential clearance of apoptotic leucocytes during the evolution of inflammatory responses. We reproducibly observe a dramatic effect of CD44 ligation in a simple 30 min in-well phagocytosis assay, but this could feasibly translate into a massive extra capacity for clearance of apoptotic neutrophils *in vivo*, as the inflammatory process evolves over hours or days. The precise molecular pathway recruited following CD44 ligation remains to be defined. The rapidity of the effect of anti-CD44 antibodies and the requirement for bivalent binding suggests that ligation of macrophage CD44 by antibodies may trigger intracellular signalling pathways that lead to increased phagocytosis of apoptotic neutrophils. Rather than continuing to screen individual candidate receptors using inhibitory ligands or antibodies, we believe that a more promising approach will be to pursue the underlying regulatory mechanism by analysis of the intracellular molecular events and the functional responses that occur following macrophage CD44 ligation.

## ACKNOWLEDGMENTS

This work was funded by the Medical Research Council and by a University of Edinburgh Faculty of Medicine fellowship.

## REFERENCES

1 Hogg, N. and Landis, R. C. (1993) Adhesion molecules in cell interactions. Curr. Opin. Immunol. **5**, 383–390
2 Weiss, S. J. (1989) Mechanisms of disease: tissue destruction by neutrophils. N. Engl. J. Med. **320**, 365–376
3 Dransfield, I., Stocks, S. C. and Haslett, C. (1995) Regulation of cell adhesion molecule expression and function associated with neutrophil apoptosis. Blood **85**, 3264–3273
4 Whyte, M. K. B., Meagher, L. C., MacDermot, J. and Haslett, C. (1993) Impairment of function in aging neutrophils is associated with apoptosis. J. Immunol. **150**, 5124–5134
5 Meagher, L. C., Savill, J. S., Baker, A., Fuller, R. W. and Haslett, C. (1992) Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane $B_2$. J. Leukocyte Biol. **52**, 269–273

© 2002 The Biochemical Society and the Medical Research Society

6 Stern, M., Savill, J. S. and Haslett, C. (1996) Human monocyte-derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis: mediation by $\alpha_v\beta_3$/CD36/thrombospondin recognition mechanism and lack of phlogistic response. Am. J. Pathol. **149**, 911–921

7 Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y. and Henson, P. M. (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J. Clin. Invest. **101**, 890–898

8 Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M. and Haslett, C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. J. Clin. Invest. **83**, 865–875

9 Woolley, K. L., Gibson, P. G., Carty, K., Wilson, A. J., Twaddell, S. H. and Woolley, M. J. (1996) Eosinophil apoptosis and the resolution of airway inflammation in asthma. Am. J. Respir. Crit. Care Med. **154**, 237–243

10 Savill, J. S., Fadok, V. A., Henson, P. M. and Haslett, C. (1993) Phagocyte recognition of cells undergoing apoptosis. Immunol. Today **14**, 131–136

11 Hart, S. P., Haslett, C. and Dransfield, I. (1996) Recognition of apoptotic cells by phagocytes. Experientia **52**, 950–956

12 Devitt, A., Moffatt, O. D., Raykundalia, C., Capra, J. D., Simmons, D. L. and Gregory, C. D. (1998) Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature (London) **392**, 505–509

13 Ellis, R. E., Yuan, J. and Horvitz, H. R. (1991) Mechanisms and functions of cell death. Annu. Rev. Cell Biol. **7**, 663–698

14 Giles, K. M., Hart, S. P., Haslett, C., Rossi, A. G. and Dransfield, I. (2000) An appetite for apoptotic cells: controversies and challenges. Br. J. Haematol. **109**, 1–12

15 Caron, E. (2000) Phagocytosis: Rac and roll over the corpses. Curr. Biol. **10**, R489–R491

16 Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M. et al. (1993) Lethal effect of the anti-Fas antibody in mice. Nature (London) **364**, 806–809

17 Hagimoto, N., Kuwano, K., Miyazaki, H. et al. (1997) Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen. Am. J. Respir. Cell. Mol. Biol. **17**, 272–278

18 Ren, Y. and Savill, J. S. (1995) Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis. J. Immunol. **154**, 2366–2374

19 Rossi, A. G., McCutcheon, J. C., Roy, N., Chilvers, E. R., Haslett, C. and Dransfield, I. (1998) Regulation of macrophage phagocytosis of apoptotic cells by cAMP. J. Immunol. **160**, 3562–3568

20 Liu, Y., Cousin, J. M., Hughes, J. et al. (1999) Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes. J. Immunol. **162**, 3639–3646

21 Brown, E. J. (1986) The role of extracellular matrix proteins in the control of phagocytosis. J. Leukocyte Biol. **39**, 579–591

22 Lesley, J., Hyman, R. and Kincade, P. W. (1993) CD44 and its interaction with the extracellular matrix. Adv. Immunol. **54**, 271–335

23 Screaton, G. R., Bell, M. V., Jackson, D. G., Cornelis, F. B., Gerth, U. and Bell, J. I. (1992) Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc. Natl. Acad. Sci. U.S.A. **89**, 12160–12164

24 Cooper, D. L. and Dougherty, G. J. (1995) To metastasize or not? Selection of CD44 splice sites. Nat. Med. (N.Y.) **1**, 635–637

25 Katoh, S., Zheng, Z., Oritani, K., Shimozato, T. and Kincade, P. W. (1995) Glycosylation of CD44 negatively regulates its recognition of hyaluronan. J. Exp. Med. **182**, 419–429

26 Lesley, J., English, N., Perschl, A., Gregoroff, J. and Hyman, R. (1995) Variant cell lines selected for alterations in the function of the hyaluronan receptor CD44 show differences in glycosylation. J. Exp. Med. **182**, 431–437

27 Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. and Seed, B. (1990) CD44 is the principal cell surface receptor for hyaluronate. Cell **61**, 1303–1313

28 Culty, M., Miyake, K., Kincade, P. W., Silorski, E., Butcher, E. C. and Underhill, C. (1990) The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins. J. Cell Biol. **111**, 2765–2774

29 Jalkanen, S. and Jalkanen, M. (1992) Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. J. Cell Biol. **116**, 817–825

30 Faassen, A. E., Schrager, J. A., Klein, D. J., Oegema, T. R., Couchman, J. R. and McCarthy, J. B. (1992) A cell surface chondroitin sulphate proteoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. J. Cell Biol. **116**, 521–531

31 Henke, C. A., Roongta, U., Mickelson, D. J., Knutson, J. R. and McCarthy, J. B. (1996) CD44-related chondroitin sulfate proteoglycan, a cell surface receptor implicated with tumor cell invasion, mediates endothelial cell migration on fibrinogen and invasion into a fibrin matrix. J. Clin. Invest. **97**, 2541–2552

32 Svee, K., White, J., Vaillant, P. et al. (1996) Acute lung injury fibroblast migration and invasion of a fibrin matrix is mediated by CD44. J. Clin. Invest. **98**, 1713–1727

33 Toyama-Sorimachi, N., Sorimachi, H., Tobita, Y. et al. (1995) A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. J. Biol. Chem. **270**, 7437–7444

34 Weber, G. F., Ashkar, S., Glimcher, M. J. and Cantor, H. (1996) Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science **271**, 509–512

35 Droll, A., Dougherty, S. T., Chiu, R. K. et al. (1995) Adhesive interactions between alternatively spliced CD44 isoforms. J. Biol. Chem. **270**, 11567–11573

36 Lesley, J., Kincade, P. W. and Hyman, R. (1993) Antibody-induced activation of the hyaluronan receptor function of CD44 requires multivalent binding by antibody. Eur. J. Immunol. **23**, 1902–1909

37 Kawakami, N., Nishizawa, F., Sakane, N. et al. (1999) Roles of integrins and CD44 on the adhesion and migration of fetal liver cells to the fetal thymus. J. Immunol. **163**, 3211–3216

38 Mikecz, K., Brennan, F. R., Kim, J. H. and Glant, T. T. (1995) Anti-CD44 treatment abrogates tissue oedema and leukocyte infiltration in murine arthritis. Nat. Med. **1**, 558–563

39 Aho, R., Jalkanen, S. and Kalimo, H. (1994) CD44-hyaluronate interaction mediates in vitro lymphocyte binding to the white matter of the central nervous system. J. Neuropathol. Exp. Neurol. **53**, 295–302

40 Pierres, A., Lipcey, C., Mawas, C. and Olive, D. (1992) A unique CD44 monoclonal antibody identifies a new T cell activation pathway. Eur. J. Immunol. **22**, 413–417

41 Santos-Argumedo, L., Kincade, P. W., Partida-Sanchez, S. and Parkhouse, R. M. E. (1997) CD44-stimulated dendrite formation ('spreading') in activated B cells. Immunology **90**, 147–153

42 Taher, T. E. I., Smit, L., Griffioen, A. W., Schilder-Tol, E. J. M., Borst, J. and Pals, S. T. (1996) Signaling through CD44 is mediated by tyrosine kinases. J. Biol. Chem. **271**, 2863–2867

43 Li, R., Wong, N., Jabali, M. D. and Johnson, P. (2001) CD44-initiated cell spreading induces Pyk2 phosphorylation, is mediated by Src family kinases, and is negatively regulated by CD45. J. Biol. Chem. **276**, 28767–28773

44 MacKay, C. R., Terpe, H.-J., Stauder, R., Marston, W. L., Stark, H. and Gunthert, U. (1994) Expression and modulation of CD44 variant isoforms in humans. J. Cell Biol. **124**, 71–82

45 Culty, M., O'Mara, T. E., Underhill, C., Yeager, H. and Swartz, R. P. (1994) Hyaluronan receptor (CD44) expression and function in human peripheral blood monocytes and alveolar macrophages. J. Leukocyte Biol. **56**, 605–611


© 2002 The Biochemical Society and the Medical Research Society

46 Levesque, M. C. and Haynes, B. F. (1996) In vitro culture of human peripheral blood monocytes induces hyaluronan binding and up-regulates monocyte variant CD44 isoform expression. J. Immunol. **156**, 1557–1565
47 Verdrengh, M., Holmdahl, R. and Tarkowski, A. (1995) Administration of antibodies to hyaluronan receptor (CD44) delays the start and ameliorates the severity of collagen II arthritis. Scand. J. Immunol. **42**, 353–358
48 Teder, P., Vandivier, R. W., Jiang, D. et al. (2002) Resolution of lung inflammation by CD44. Science **296**, 155–158
49 Newman, S. L., Henson, J. E. and Henson, P. M. (1982) Phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit inflammatory macrophages. J. Exp. Med. **156**, 430–442
50 Hart, S. P., Dougherty, G. J., Haslett, C. and Dransfield, I. (1997) CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages. J. Immunol. **159**, 919–925
51 Sanchez-Mejorada, G. and Rosales, C. (1998) Signal transduction by immunoglobulin Fc receptors. J. Leukocyte Biol. **63**, 521–533
52 Isacke, C. M. (1994) The role of the cytoplasmic domain in regulating CD44 function. J. Cell Sci. **107**, 2353–2359
53 Oliferenko, S., Kaverina, I., Small, J. V. and Huber, L. A. (2000) Hyaluronic acid (HA) binding to CD44 activates Rac1 and induces lamellipodia outgrowth. J. Cell Biol. **148**, 1159–1164
54 Bourguignon, L. Y. W., Zhu, H., Shao, L. and Chen, Y. W. (2000) CD44 interaction with Tiam1 promotes Rac1 signaling and hyaluronic acid-mediated breast tumor cell migration. J. Biol. Chem. **275**, 1829–1838
55 Ilangumaran, S., Briol, A. and Hoessli, D. C. (1998) CD44 selectively associates with active Src family protein tyrosine kinases Lck and Fyn in glycosphingolipid-rich plasma membrane domains of human peripheral blood lymphocytes. Blood **91**, 3901–3908
56 Kato, T., Takeda, Y., Nakada, T. and Sendo, F. (1995) Inhibition by dexamethasone of human neutrophil apoptosis in vitro. Nat. Immun. **14**, 198–208
57 Liu, Q. A. and Hengartner, M. O. (1998) Candidate adaptor protein CED-6 promotes the engulfment of apoptotic cells in C. elegans. Cell **93**, 961–972
58 McCutcheon, J. C., Hart, S. P., Canning, M., Ross, K., Humphries, M. J. and Dransfield, I. (1998) Regulation of macrophage phagocytosis of apoptotic cells by adhesion to fibronectin. J. Leukocyte Biol. **64**, 1–8

© 2002 The Biochemical Society and the Medical Research Society