Characterisation of the Molecular Mechanism Required for Glucocorticoid Augmentation of Macrophage Phagocytosis of Apoptotic Neutrophils

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Presented for the degree of Doctor of Philosophy

The University of Edinburgh

July 2009

DECLARATION

This thesis was composed entirely by myself on the basis of work carried out under the supervision of Prof. Ian Dransfield and Prof. Christopher Haslett in the MRC Centre for Inflammation Research, University of Edinburgh.

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Edinburgh, July 2009

ABSTRACT

The successful resolution of inflammation requires removal of neutrophils from the inflammatory site to prevent release of histotoxic contents that may potentiate inflammatory processes and promote progression to a chronic state associated with impaired repair mechanisms and/or autoimmune responses. Macrophages are "professional" phagocytes required for rapid and efficient clearance of apoptotic neutrophils. Macrophage phagocytic capacity can be critically regulated by a number of environmental factors, including cytokines, bacterial products, and glucocorticoids. We have hypothesised that modulation of macrophage phagocytic capacity may represent an effective strategy for promoting resolution of inflammation in diseases where clearance of neutrophils may be impaired or inefficient. The aim of this thesis was to investigate the molecular mechanisms underlying glucocorticoid-augmentation of macrophage phagocytosis. We have demonstrated that long-term exposure of human peripheral blood monocytes to the synthetic glucocorticoid dexamethasone dramatically increases phagocytic capacity for "early" membrane-intact apoptotic neutrophils. Increased phagocytic potential was associated with a "switch" from a serum-independent to a serum-dependent apoptotic cell recognition mechanism. We initially employed an "add back" approach to rule out several well-defined opsonins in apoptotic neutrophil clearance, including immune complexes, IgG, complement proteins, pentraxin-3, fibronectin, annexin I, and platelet-derived factors. Using a multi-step purification scheme involving anion exchange and gel filtration chromatography, we purified a high molecular weight fraction that contained the pro-phagocytic activity of serum and analysis by mass spectrometry identified C4-binding protein as a candidate protein. C4-binding protein circulates in human plasma bound predominately in a >570kDa complex with protein S and the presence of protein S in high molecular weight fractions was confirmed by immunoblotting. We found that protein S was equivalent to unfractionated serum in its ability to enhance phagocytosis of apoptotic neutrophils by dexamethasone-treated monocyte-derived macrophages (Dex-MDMϕ) and that immunodepletion of protein S resulted in loss of pro-phagocytic activity. Protein S was found to opsonise apoptotic neutrophils in a calcium-dependent manner and enhanced phagocytic potential by Dex-MDMϕ

through stimulation of Mer tyrosine kinase (Mertk), a receptor that is upregulated on the surface of Dex-MDMφ compared to untreated MDMφ.

The studies presented in this thesis have provided novel insight into the underlying molecular mechanisms required for high capacity clearance of apoptotic neutrophils by macrophages following treatment with glucocorticoids and may form the foundations for further studies investigating glucocorticoid action for development of safer and more selective therapies.

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to my supervisors: Prof. Ian Dransfield, for his enthusiasm, inspiration and guidance throughout the last three years, and Prof. Chris Haslett for providing advice and enjoyable discussions. I also wish to express my appreciation to Prof. Adriano Rossi for his support and endless supply of cakes.

Secondly, I would like to thank all of the blood donors, blood takers and suppliers of cells; none of this work could have been performed without your generosity. Thanks also to Tara and Jillian for their limitless patience when teaching me valuable lab techniques. I would like to send out a big thank you to fellow colleagues in the CIR, most importantly Tara, Sylwia, Paula, Sarah, Jillian, and Lynsey, for their support and for making this a fantastic and sociable environment to work in.

Outside of work, I would like to thank my many flatmates over the years, especially Melissa who has always been there for the laughs, the tears and the many beers. Lastly, and most importantly, I wish to thank my parents, Helen and Harry McColl, for their encouragement and emotional support during the course of this work.

FUNDING

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ABBREVIATIONS

AP-1	Activator protein-1
AS	Autologous serum
BSA	Bovine serum albumin
C1q	Complement fragment C1q
C3b	Complement fragment C3b
C4BP	C4-binding protein
C5a	Complement fragment C5a
$CaCl_2$	Calcium chloride
CAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation (as in CD32)
CMFDA	5-chloromethylfluorescein diacetate
COPD	Chronic obstructive pulmonary disease
CR	Complement receptor
CRP	C-reactive protein
DAF	Decay accelerating factor
Dex	Dexamethasone
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular regulated kinase
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
Fc	Fragment crystallizable
FcR	Fc-receptor
FITC	Fluorescein isothiocyanate
FL-1	Log fluorescence-1
FMLP	*N*-formyl-methionyl-leucyl-phenylalanine
FPRL1	Formyl peptide receptor-like 1
G-CSF	Granulocyte-colony stimulating factor

GM-CSF	Granulocyte-macrophage-colony stimulating factor
Gas6	Growth arrest-specific gene 6
GEF	Guanine nucleotide exchange factor
GHR	Globular head region
GILZ	Glucocorticoid-inducible leucine zipper
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HAT	Histone acetyltransferase
HBSS	Hank's buffered saline solution
HDAC	Histone deacetylase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMW	High molecular weight
HRP	Horseradish peroxidase
HSA	Human serum albumin
ICAM-1	Intercellular adhesion molecule-1
IFNγ	Interferon-gamma
Ig	Immunoglobulin (as in IgG)
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
INOS	Inducible nitric oxide synthase
IOA	Iodoacetamide
Kd	Dissociation constant
LFA-1	Leukocyte function-associated antigen-1
LMW	Low molecular weight
LPS	Lipopolysaccharide
LXA4	Lipoxin A4
M-1	Macrophage T_H1 type
M-2	Macrophage T_H2 type
M-CSF	Macrophage-colony stimulating factor
MAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MBL	Mannose-binding lectin

MCP	Membrane cofactor protein
MCP-1	Monocyte chemoattractant protein-1
MDMϕ	Monocyte-derived macrophage
Mertk	Mer tyrosine kinase
MES	4-Morpholineethanesulfonic acid
MFG-E8	Milk fat globule EGF factor 8
MIP-1α	Macrophage inflammatory protein-1 alpha
MKP-1	MAPK phosphatase 1
MNC	Mononuclear cell
MNGC	Multinucleated giant cell
NaCl	Sodium chloride
NaVO3	Sodium vanadate
Na3VO4	Sodium orthovanadate
NFκB	Nuclear factor-κB
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PECAM-1	Platelet endothelial cell adhesion molecule 1
PGE$_2$	Prostaglandin E$_2$
PI	Isoelectric point
PI3K	Phosphatidylinositol 3 kinase
PMN	Polymorphonuclear cell
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RDGS	Arg-Gly-Asp-Ser peptide
RIPA	Radioimmunoprecipitation assay
RPE	Retinal pigmented epithelial cells
SAP	Serum amyloid protein
SCR1	Soluble CR1
SDS	Sodium dodecyl sulphate

SEM	Standard error of the mean
SHBG	Sex hormone-binding globulin
SiRNA	Small interfering RNA
SIRPα	Signal regulatory protein alpha
SLE	Systemic lupus erythematosus
SLPI	Secretory leukocyte proteinase inhibitor
SP-A	Surfactant protein A
SR-A	Scavenger receptor class A
TACE	TNFα-converting enzyme
TAM	Tyro-3/Axl/Mertk
TBS	Tris buffered saline
TGFβ	Transforming growth factor beta
T_H1	Helper CD4+ T cells
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TSP-1	Thrombospondin-1
Tyr	Tyrosine

CHAPTER 1: INTRODUCTION

1.1   Inflammation: an overview

1.1.1 *Polymorphonuclear phagocytes*

Neutrophil granulocytes are terminally differentiated cells produced in the bone marrow from myeloid stem cells. It is estimated that around 100 billion neutrophils are released into the bloodstream daily, making them the most prominent cellular component of the human innate immune system (Athens et al., 1961, Bainton et al., 1971, Walker and Willemze, 1980). Studies of intravenously infused radiolabeled rabbit neutrophils suggest that neutrophils circulate in peripheral blood with a half-life of approximately 6 hours before localising to the spleen, bone marrow or liver (Haslett et al., 1989). However, during acute inflammation, neutrophils are rapidly recruited to inflamed sites via localised expression of chemoattractant signals and upregulation of adhesion molecule expression on vascular endothelial cells (*fig. 1.1*) (Strieter et al., 1993, Carlos and Harlan, 1994, Tekstra et al., 1996). Neutrophils are highly efficient phagocytes and contain or produce an impressive arsenal of degradative enzymes and toxic metabolites, including cationic proteins, lysozyme and reactive oxygen species, which represent the neutrophil's defence against invading microbes (Klebanoff, 1980, Beaman and Beaman, 1984, Passo and Weiss, 1984, Borregaard and Cowland, 1997). Neutrophils can also exert systemic inflammatory effects through the release of pro-inflammatory mediators, including cytokines (IL-1, IL-6 and TNFα) and chemokines (IL-8 and MIP-1α) (Cassatella, 1995), which induce the recruitment of additional inflammatory cells. The importance of neutrophils in host defence is emphasised in patients with deficiencies either in neutrophil number (neutropaenia) or function (leukocyte adhesion deficiency, Chediak Higashi syndrome, chronic granulomatous disease) who are susceptible to recurrent bacterial infections (Kamani and Infante, 2000, Roos and Law, 2001, Boxer and Dale, 2002, Rezaei et al., 2005).
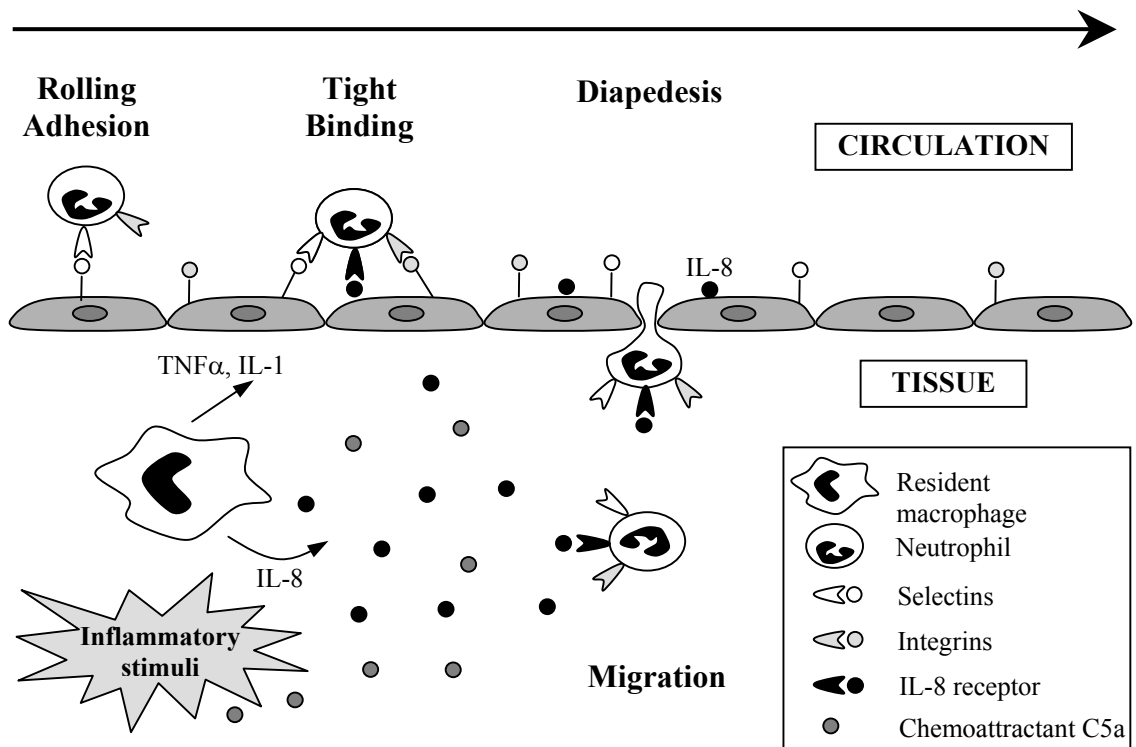
Figure 1.1: Recruitment of neutrophils to inflamed sites.
Neutrophil recruitment is a coordinated process involving attachement to endothelial cells and migration into inflamed tissues. Resident tissue macrophages are often initiators of the inflammatory cascade, responding to microbial infection by secreting TNFα and IL-1 cytokines that locally activate endothelial cells to induce the expression of several adhesion molecules. Sialyl-Lewis^x moieties on neutrophils form weak affinity interactions with P-selectin and E-selectin on the surface of activated endothelial cells, resulting in rolling of neutrophils along the surface of the endothelial wall. Upon neutrophil activation by the chemokine IL-8, LFA-1 integrin is converted to a high affinity state and binds to ICAM-1 induced on the surface of endothelial cells, promoting tight binding of neutrophils to the endothelial wall. Chemokines such as IL-8 then induce extravasation of neutrophils through the endothelial wall *via* homotypic binding of CD31 (PECAM-1) present on both neutrophils and intracellular junctions of endothelial cells. Neutrophils penetrate the basement membrane by the release of proteolytic enzymes in a process known as diapedesis, and migrate toward the chemokine gradient. Neutrophils rapidly accumulate at infected sites where they perform essential anti-microbial functions.

1.1.2   Mononuclear phagocytes

Macrophages are mononuclear cells that differentiate from blood monocytes upon recruitment into tissues in response to chemokines such as monocyte chemoattractant protein-1 (MCP)-1 (Lu et al., 1998). Macrophages reside in different organs and tissues of the body, including the lung (alveolar macrophages), liver (Kupffer cells), kidney (glomerular mesangial cells), spleen, neural tissue (microglia), gut and lymph nodes where they play an essential role in immune surveillance. Macrophages have the capacity to ingest and destroy pathogenic agents using enzymes and toxic mediators such as lysozyme, superoxide radicals and nitric oxide (Vazquez-Torres et al., 2000), and can secrete pro-inflammatory mediators that promote recruitment of neutrophils from the circulation (*fig. 1.1*) (Schroder et al., 1987, Koch et al., 1991).

Macrophages show considerable heterogeneity in terms of morphology and function that may depend on the local microenvironment they are exposed to during differentiation from monocytes (Dougherty and McBride, 1984, Kreutz et al., 1992, Laskin et al., 2001, Gordon and Taylor, 2005). *In vitro* studies by Akagawa and colleagues showed that human monocytes cultured in human serum, or in the presence of macrophage colony-stimulating factor (M-CSF) added to foetal bovine serum (FBS), differentiated into two distinct monocyte-derived macrophage (MDMϕ) populations that differed in morphology and protein expression (Akagawa et al., 1988). Additionally, human monocytes differentiated *in vitro* in human serum are a highly heterogeneous population of MDMϕ in terms of phagocytic potential (Giles et al., 2001). Indeed, serum is a complex source of cytokines and growth factors that may "program" macrophage function during differentiation. IFNγ and TNFα are pro-inflammatory mediators associated with "classical" activation of macrophages (M-1 phenotype), induction of pro-inflammatory responses and propagation of a T_H1-like response (North, 1978, Goerdt et al., 1999, Mills et al., 2000). In contrast, cytokines such as IL-4, IL-10 and IL-13 and glucocorticoids "alternatively" activate macrophages (M-2 phenotype), characterised by downregulation of pro-inflammatory cytokine responses, development of T_H2-like responses, increased debris scavenging activity and wound healing (Becker and

Daniel, 1990, Stein et al., 1992, Bogdan and Nathan, 1993). *In vitro* programming may be dependent on the first stimuli encountered (Erwig et al., 1998), and monocytes infiltrating into the inflamed site at different periods of the inflammatory response may acquire specialised phenotypic characteristics important for performing diverse functions. However, more recent evidence suggests that macrophages may have the capacity to continuously adapt their functional phenotype in response to the changing environment of a progressive inflammatory response (Stout and Suttles, 2004).

Successful restoration of a tissue to its original state after an inflammatory insult requires that the processes involved in the initiation and progression of inflammation must be reversed. Pro-inflammatory cytokine expression is switched off, recruitment of inflammatory cells is ceased, and large numbers of extravasated neutrophils are cleared from the inflamed site. The physiological mechanisms for removal must be efficient and non-inflammatory to ensure that effete neutrophils do not cause host tissue damage through the inappropriate release of intracellular contents (Haslett et al., 1994, Klebanoff, 2005). During this resolution phase of inflammation, recruited neutrophils undergo apoptosis and are subsequently removed by phagocytosis (*fig. 1.2*) (Savill et al., 1989b, Cox et al., 1995). Phagocytic clearance is a rapid and efficient process involving a complex system of phagocyte receptors, soluble bridging molecules and apoptotic cell ligands that importantly does not stimulate pro-inflammatory macrophage responses (Meagher et al., 1992).
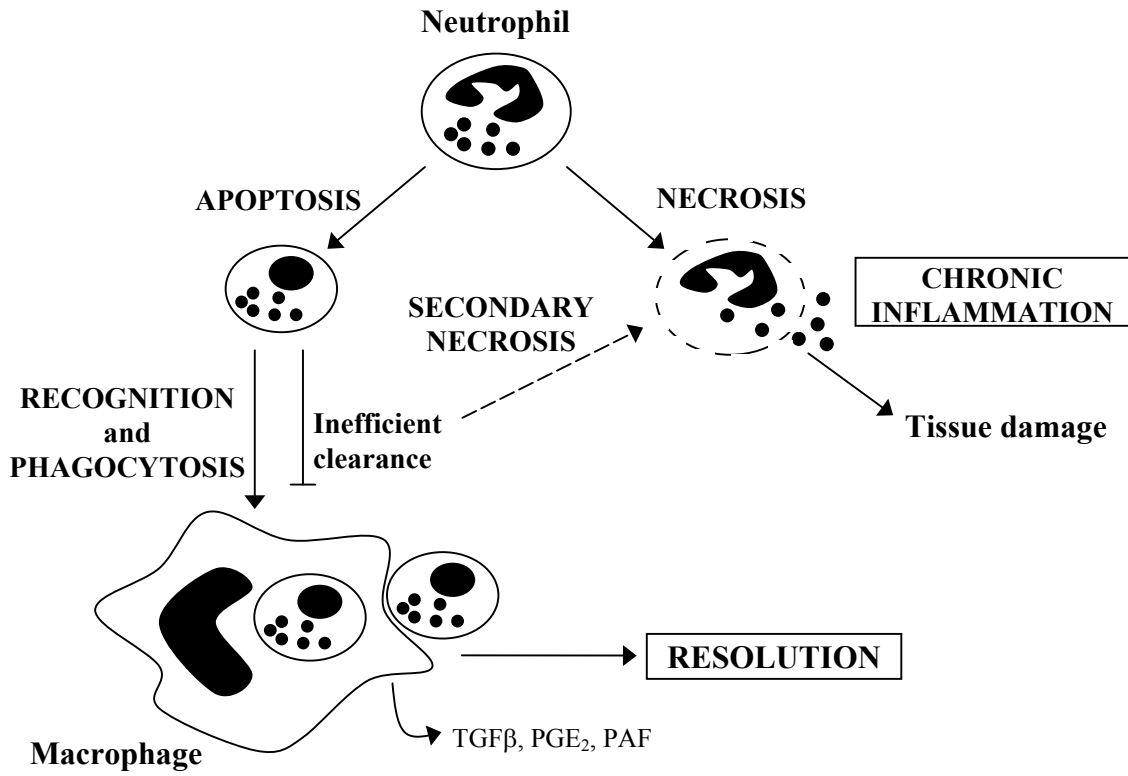
Figure 1.2: Fate of dying neutrophils.
The physiological mechanisms for removal of neutrophils from tissues must be efficient and non-inflammatory to ensure that effete neutrophils do not cause tissue damage through the inappropriate release of intracellular contents. Neutrophils undergo apoptosis followed by recognition and phagocytosis of intact apoptotic neutrophils by macrophages, a process that is associated with the release of anti-inflammatory mediators such as TGFβ, PGE$_2$, PAF by macrophages and the resolution of inflammation. In contrast, necrotic cell death results in loss of membrane integrity and subsequent release of a vast array of histotoxic granular contents that cause tissue injury and induce further recruitment of inflammatory cells. Inefficient or defective clearance of apoptotic cells results in secondary necrosis and induction of a similar chronic inflammatory state.

1.2   Resolution of inflammation

1.2.1   Neutrophil apoptosis

Apoptosis is a programmed form of cell death (Kerr et al., 1972) that regulates the number and fate of neutrophils both *in vitro* and *in vivo* (*fig. 1.2*) (Savill et al., 1989b). There has been extensive research into the molecular mechanisms relating to the regulation of neutrophil apoptosis *in vitro*. During apoptosis, neutrophils activate an intrinsic suicide program that results in a well-choreographed sequence of events such as chromatin condensation (often with margination to the nuclear envelope), increased cytoplasmic density, cell shrinkage, DNA fragmentation and cytoplasmic vacuolation, with little evidence of apoptotic body formation or surface blebbing that has been reported for other cells (Savill et al., 1989b, Payne et al., 1994). Most importantly, the plasma membrane and cytoplasmic granules remained structurally intact in apoptotic neutrophils (Savill et al., 1989b). The morphological changes observed in neutrophils undergoing constitutive apoptosis are concomitant with activation of caspases (Fadeel et al., 1998), cysteine proteases which play a central role in the execution of the apoptotic process by cleaving their target proteins at specific aspartic acid residues (Earnshaw et al., 1999), and are accompanied by downregulation of cellular functions. Neutrophils that have entered the apoptotic process lose the ability of chemokinesis, chemotaxis, phagocytosis, and the potentially injurious responses of oxidative burst and degranulation in response to receptor-mediated stimuli (Whyte et al., 1993). Functional attenuation is also achieved by surface changes during the apoptotic process, including down-regulation of FcγRIII (CD16) and L-selectin expression, and uncoupling of β2 integrins, key receptors that mediate neutrophil phagocytosis and adhesion events (Dransfield et al., 1994, Dransfield et al., 1995). Thus, apoptosis is an important physiological clearance mechanism that acts to limit tissue damage by isolating effete neutrophils from exogenous stimuli and may be a prerequisite for resolution of acute inflammatory processes.

During inflammatory states, for example microbial infection, neutrophils are exposed to multiple cytokines, chemokines and microbe-derived molecules which have

dramatic effects on neutrophil function and survival. Neutrophils have a relatively short half-life in the circulation *in vivo*, but can survive in inflamed tissues for 1 to 2 days (Homburg and Roos, 1996) due to the presence of mediators such as IL-1β, TNFα, IFNγ, G-CSF, GM-CSF, C5a or bacterial-derived LPS that delay the constitutive apoptotic pathway (Colotta et al., 1992, Lee et al., 1993, Cox, 1995, Murray et al., 1997). Delay of apoptosis in neutrophils coincides with preservation of function, indicating that retardation of apoptosis at inflamed sites maintains neutrophil functional longevity to deal with microbial infection (Lee et al., 1993, Cox and Austin, 1997). Treatment with translational inhibitors (e.g. cycloheximide) or transcriptional inhibitors (e.g. actinomycin D) accelerated the constitutive rate of apoptosis, suggesting that delayed apoptosis requires the continual production of a survival protein (Whyte et al., 1997). Indeed, NFκB-dependent gene expression has a central role in the regulation of granulocyte survival (Ward et al., 1999a). Neutrophils also express pro- (Bax, Bid, Bak and Bad) and anti-apoptotic (Mcl-1, A1 and Bcl-xL) Bcl-2 family members that critically regulate the rate at which neutrophils undergo apoptosis in response to exogenous signals (Moulding et al., 1998, Chuang et al., 1998, Dibbert et al., 1999, Akgul et al., 2001).

1.2.2 *Phagocytosis*

Physiological clearance of apoptotic cells is believed to play a critical role during many biological processes including development, tissue remodelling, and normal tissue turnover (Han, 1993, Hopkinson-Woolley et al., 1994). Resolution of inflammation also requires that cells dying by apoptosis be disposed of (*fig. 1.2*). Senescent cells are removed by phagocytosis, a process first described in the late 19th century by Elie Metchnikoff who used a light microscope to observe that neutrophils were "englobed" by macrophages in injured tadpole fins. Studies of the interaction between human MDMφ and neutrophils *in vitro* have shown that while freshly isolated neutrophils are not phagocytosed by MDMφ, time-dependent apoptosis in the aging neutrophil population leads to recognition and ingestion of neutrophils by MDMφ *in vitro* and *in vivo* at a stage when their cell membrane appears structurally and functionally intact (Newman et al., 1982, Savill et al.,

1989b). Although "semi-professional" phagocytes, including fibroblasts, hepatocytes and endothelial cells have the capacity for recognition and removal of apoptotic cells and may play a contributory role to the regulation of apoptotic cell load within tissues (Hall et al., 1994, Dini et al., 1995), macrophages are the "professional" phagocyte that are required for rapid and efficient removal of potentially histotoxic apoptotic neutrophils during the resolution of inflammation (Savill, 1997). The remarkable efficiency by which macrophages ingest apoptotic neutrophils *in vivo* is observed in pneumococcal pneumonia where, despite an immense infiltration of inflammatory neutrophils into the lung, the inflammatory response resolves with neutrophil apoptosis and subsequent clearance by macrophages, and the normal lung architecture is preserved (Haslett et al., 1994).

Phagocytosis is not only an important mechanism for disposing of potentially injurious neutrophils, but also has profound effects on phagocyte function. Phagocytosis of human apoptotic neutrophils fails to induce the release of pro-inflammatory mediators such as eicosanoids or GM-CSF, IL-8, and MCP-1 chemoattractants from the phagocytic cell (Meagher et al., 1992, Hughes et al., 1997, Fadok et al., 1998). Additionally, ingestion of apoptotic cells induces immunosuppressive and anti-inflammatory effects in monocytes/macrophages by decreasing secretion of TNFα, IL-1 and IL-12 and increasing the release of IL-10, TGFβ, PGE$_2$ and platelet-activating factor, mediators that dampen inflammatory responses (Voll et al., 1997, Fadok et al., 1998, McDonald et al., 1999). Thus, rapid recognition, ingestion and degradation of apoptotic neutrophils by macrophages in a non-phlogistic manner is consistent with a 'safe' pathway for disposal of potentially harmful inflammatory cells that is obligatory for the resolution of inflammation.

1.3   Macrophage phagocytosis of apoptotic cells

1.3.1   *Apoptotic cell ligands*

In addition to the morphological changes observed in neutrophils undergoing apoptosis, there are also molecular consequences to the apoptotic process. Human

apoptotic neutrophils display specific surface alterations that serve to identify them as targets for phagocytic removal (*table 1*) (Savill et al., 1989b, Hart et al., 2000, Dransfield et al., 1994). These surface changes occur early on during apoptosis to ensure removal of neutrophils at a stage when they are still intact, and may result from either decreased expression of certain receptors or else the appearance of new surface molecules. Non-apoptotic neutrophils display surface ligands like platelet-endothelial cell adhesion molecule-1 (PECAM-1) or CD31 that protect them from ingestion by MDMϕ (Brown et al., 2002). This function is disabled during apoptosis allowing MDMϕ to bind and ingest apoptotic neutrophils. Neutrophils also express CD47, the ligand for macrophage signal regulatory protein (SIRPα) (Vernon-Wilson et al., 2000). Antibody blockade of the CD47-SIRPα interaction was found to promote engulfment of viable neutrophils (Gardai et al., 2005), indicating that CD47 that may function as a "don't eat me" signal on viable neutrophils. Additional modifications include changes in neutrophil surface charge and the pattern of glycosylation of cell surface proteins (Savill et al., 1989a).

The most characterised surface change associated with apoptosis is exposure of phosphatidylserine (PS) (Martin et al., 1995). In viable cells, PS is normally retained in the inner leaflet of the plasma membrane through the action of aminophospholipid translocase, and inactivation of phospholipid asymmetry during neutrophil apoptosis, possibly as a consequence of loss of phospholipid translocase activity or activation of phospholipid scramblases or flippases, leads to the appearance of PS on the outer leaflet (Homburg et al., 1995, Vermes et al., 1995, Bevers et al., 1999, Daleke and Lyles, 2000, Sims and Wiedmer, 2001). PS surface exposure is an early feature of apoptosis, occurring at a stage when the cell membrane is still intact (Chan et al., 1998). The ability of PS-containing vesicles to partially block apoptotic cell phagocytosis suggests that PS may be an important signal for apoptotic cell ingestion (Fadok et al., 1992b, Savill, 1997). Another molecule exposed on the cell surface during apoptosis is annexin-1. Interestingly, annexin-1 is thought to co-localise with PS on the surface of apoptotic cells and facilitate apoptotic cell recognition, with siRNA-mediated silencing of annexin-1 gene expression associated with defective phagocytosis of apoptotic cells (Arur et al., 2003).

In contrast to *in vitro* assay conditions, apoptotic cells and phagocytes may not be located in close proximity *in vivo*, and additional mechanisms may be required to signal the presence of apoptotic cells to phagocytes before they can undergo lysis. Lauber *et al* identified lysophosphatidylcholine, generated by the actions of a calcium-independent phospholipase A2 (iPLA2) during apoptosis, as a factor released by apoptotic cells that had chemotactic effects on phagocytes (Lauber et al., 2003), raising the possibility that apoptotic cells can produce soluble signals to actively recruit phagocytes *in vivo*. A recent study by Peter *et al* has identified the G-protein-coupled receptor G2A as a putative receptor that may mediate lysophosphatidylcholine-dependent macrophage recruitment (Peter et al., 2008).

1.3.2 *Opsonins*

An important aspect of the immune response is the recognition of pathogen-associated molecular patterns on microbes by soluble host factors such as complement proteins, immunoglobulins, and acute phase proteins which target microbes for removal by phagocytes (Savill et al., 2002). This process of opsonisation also plays a critical role in apoptotic cell clearance, where soluble components bind directly to the altered apoptotic cell surface, for example *via* PS, and facilitate clearance through opsonin-mediated phagocytosis (*table 2*). Interestingly, many of the pattern recognition molecules important for microbial recognition can also modulate apoptotic cell clearance, including complement factors, collectins such as mannan-binding lectin (MBL) and surfactant protein A (SP-A) and SP-D, and pentraxins such as serum amyloid P (SAP), C-reactive protein (CRP) and pentraxin-3 (Mevorach et al., 1998a, Bickerstaff et al., 1999, Gershov et al., 2000, Rovere et al., 2000, Ogden et al., 2001, Gaipl et al., 2001, Schagat et al., 2001, Mold et al., 2002, Nauta et al., 2003b). Additional factors that may be important in apoptotic cell recognition include TSP-1, β2-GPI, MFG-E8, properdin, galectin-3, protein S and Gas6 (Savill et al., 1992, Balasubramanian et al., 1997, Scott et al., 2001, Hanayama et al., 2002, Anderson et al., 2003, Kemper et al., 2008, Karlsson et al., 2009). These components may be constitutively present in serum (e.g. protein S) or generated from inactive precursors (e.g. complement). In some

cases, the source of the opsonins may be the phagocyte itself (Hanayama et al., 2002, Faust et al., 2002, Maderna et al., 2005).

Experimental evidence in support of the role of several of these molecules in apoptotic cell clearance *in vivo* is provided by studies of knockout mice (Vandivier et al., 2002, Sano et al., 2003, Stuart et al., 2005). However, only C1q-deficiency has been reported to have significant *in vivo* consequences both in mice and humans, where impaired clearance of apoptotic cells is thought to contribute to the development of a lupus-like autoimmune disease characterised by high titres of autoantibodies against nuclear antigens and glomerulonephritis (Botto, 1998, Taylor et al., 2000). It is possible that no single opsonin dominates in apoptotic cell clearance *in vivo*, or different opsonins may be important for different locations (Schagat et al., 2001). Moreover, several reports have suggested apoptotic cell opsonisation to be a relatively late event, occurring at a stage when the cell membrane is no longer intact (Nauta et al., 2003c, Hart et al., 2005). The pattern of opsonisation may therefore have significant consequences in terms of the phagocyte response to ingestion of apoptotic cells, both with respect to the phagocyte population engaged by opsonised apoptotic cells and the inflammatory outcome of this interaction.

Apoptosis-associated changes	Macrophage receptor	Reference
Lose "don't eat me" signals:		
CD31	CD31	(Brown et al., 2002)
CD47	SIRPα	(Gardai et al., 2005)
Gain "eat me" signals:		
Phosphatidylserine	TIM4	(Miyanishi et al., 2007)
	Stabilin-2	(Park et al., 2008)
Annexin-1	PS-dependent recognition	(Arur et al., 2003)
Secrete "find me" signals:		
Lysophosphatidylcholine	G2A	(Peter et al., 2008)

Table 1: <u>Changes associates with apoptosis</u>

Abbreviations: PS, phosphatidylserine; SIRPα, signal regulatory protein α.

Apoptotic cell opsonin	Macrophage receptor	Reference
Complement:		
C3b	CR3 and CR4	(Mevorach et al., 1998a)
C1q	Calreticulin/CD91	(Ogden et al., 2001)
Properdin (via C3b opsonisation)	CR3 and CR4	(Kemper et al., 2008)
CRP (via C3b opsonisation)	CR3 and CR4	(Gershov et al., 2000)
Collectins:		
MBL	Calreticulin/CD91	(Ogden et al., 2001)
SP-A and SP-D	Calreticulin/CD91	(Vandivier et al., 2002)
Pentraxins:		
CRP and SAP	Fcγ receptors	(Mold et al., 2002)
TSP-1	CD36/αvβ3	(Savill et al., 1992)
MFG-E8	αvβ3	(Hanayama et al., 2002)
Protein S	Mertk	(Uehara and Shacter, 2008)
Gas-6	Mertk	(Scott et al., 2001)

Table 2: <u>Opsonins and receptors associated with apoptotic cell clearance.</u>

Abbreviations: CRP, C-reactive protein; MBL, mannose-binding lectin; SP-A, surfactant protein A; SAP, serum amyloid protein; TSP-1, thrombospondin-1; MFG-E8, milk fat globule EGF factor 8; Gas6, growth arrest-specific gene 6; CR3, complement receptor 3.

1.3.3 *Receptors for apoptotic cell recognition*

Phagocytes display an impressive array of receptors that bestow the capacity for recognition of a diverse particle range. Many of these receptors have been acknowledged in the direct recognition and engulfment of apoptotic cells by phagocytes (*table 1*), including lectins, scavenger receptors, CD31, CD14 and the putative PS receptors TIM4 and stabilin-2 (Platt et al., 1996, Devitt et al., 1998, Vernon-Wilson et al., 2006, Michlewska et al., 2007, Miyanishi et al., 2007, Park et al., 2008). Other phagocyte receptors can indirectly recognise apoptotic cells that have been opsonised with soluble factors, such as complement receptors, integrins and the tyrosine kinase receptor, Mertk (*table 2*) (Savill et al., 1992, Mevorach et al., 1998a, Finnemann and Rodriguez-Boulan, 1999, Ogden et al., 2001, Scott et al., 2001).

With the exception of Mertk (Scott et al., 2001), blocking individual receptors on phagocytes only partially inhibits phagocytosis of apoptotic cells, suggesting that recognition pathways may operate in parallel to ensure efficient apoptotic cell clearance. CD36, a class B scavenger receptor with a very short C-terminal cytoplasmic domain, has been proposed to cooperate with the vitronectin receptor ($\alpha v \beta 3$) for internalisation of TSP-1-opsonised apoptotic neutrophils by MDMϕ (Savill et al., 1992), and C1q-dependent ingestion of apoptotic cells is thought to require stimulation of calreticulin and CD91 (Ogden et al., 2001, Vandivier et al., 2002). This cross talk between two distinct phagocyte receptors illustrates the extraordinary complexity underlying apoptotic cell phagocytosis. Furthermore, $\alpha v \beta 3$ integrin-dependent phagocytosis can also be stimulated by MFG-E8 on the surface of apoptotic cells (Hanayama et al., 2002), adding another layer of complexity through promiscuity of phagocyte receptors for opsonins and may allow phagocytes to adapt to environmental signals. The contributions of receptors to apoptotic cell clearance likely depends upon the phagocytic and apoptotic target cell types (Fadok et al., 1992a), with the cytokine milieu and presence of activatory signals, for example bacterial LPS, also having an influence over the activation state of the phagocyte, surface receptor expression and the availability of opsonins.

1.4   Disease: Failure of Natural Resolution Process?

Although the mechanisms responsible for tipping the balance towards persistent inflammation are likely multifactoral, failure or inefficient resolution processes may represent a common factor contributing to the pathogenesis of many diverse inflammatory diseases (Haslett et al., 1994). Neutrophils have been implicated in the pathogenesis of a variety of inflammatory disorders, such as chronic obstructive pulmonary disease (COPD), systemic lupus erythematosus (SLE) and rheumatoid arthritis (Stockley, 1999, Wipke and Allen, 2001, Ren et al., 2003). The persistent recruitment and/or survival of activated neutrophils may result from dysregulated expression of pro-inflammatory genes, such as cytokines (IL-1, TNFα, GM-CSF, etc.), chemokines (IL-8, IL-5, MIP-1α), and adhesion molecules (ICAM-1 and E-selectin) (Ottonello et al., 2002, Cosio et al., 2005), contributing to neutrophil accumulation in chronically inflamed tissues. In situations where cells are stimulated to undergo apoptosis at a high rate, as demonstrated *in vivo* by anti-Fas treatment of mice (Ogasawara et al., 1993), the tissue load of apoptotic cells may be in danger of exceeding the removal capacity by phagocytes and failure to remove intact apoptotic cells efficiently before lysis may have pathological consequences. In contrast to apoptosis, neutrophil necrosis is characterised by loss of membrane integrity and release of toxic granular contents that have the capacity to damage the surrounding tissue and stimulate pro-inflammatory macrophage responses (Searle et al., 1982, Taylor et al., 2007). Secondary necrosis of neutrophils has been observed *in vivo* during intense neutrophil-rich inflammation (Rydell-Tormanen et al., 2006). Release of cellular contents with immunogenic potential, such as chromatin and DNA, may contribute to the induction of autoimmune reactions (Mevorach et al., 1998b). Indeed, mice with defects in apoptotic cell removal develop many of the characteristics associated with autoimmune disease (Botto, 1998, Scott et al., 2001), and impaired phagocytosis of apoptotic cell material has been implicated in the severity of disease observed in cystic fibrosis airways and human SLE (Herrmann et al., 1998, Vandivier et al., 2002, Gaipl et al., 2006, Tas et al., 2006). Pharmacological manipulation of the processes involved in physiological clearance

of neutrophils from inflamed sites may therefore represent a therapeutic approach to treatment of inflammatory diseases where ineffective clearance has been implicated.

1.5   Regulation of macrophage phagocytosis of apoptotic cells

1.5.1   *Inflammatory mediators (lipoxins, cytokines, LPS)*

The presence of endogenously produced mediators or bacterial products at inflamed sites can affect the ability of phagocytes to ingest apoptotic cells. Lipoxins (LXs), a family of mediators generated from transcellular metabolism of arachidonic acid, are actively synthesised during the resolution phase of inflammation (Serhan and Savill, 2005, Serhan et al., 2007). LXA4 exerts anti-inflammatory effects by inhibiting further extravasation of neutrophils into inflamed sites and stimulating recruitment of mononuclear cells (Maddox and Serhan, 1996, Hachicha et al., 1999). Furthermore, LXA4 exhibits pro-resolving properties by stimulating non-phlogistic macrophage phagocytosis of apoptotic neutrophils both *in vitro* (Godson et al., 2000, Reville et al., 2006) and *in vivo* (Mitchell et al., 2002). Various inflammatory cytokines (GM-CSF, IL-β, IFNγ, TNFα and by TGF-1β) have been suggested to potentiate apoptotic neutrophil clearance *in vitro*. Short-term incubation (4 hours) of human MDMDϕ with any of these mediators increased both the proportion of MDMϕ capable of recognising apoptotic neutrophils, and the phagocytic capacity of individual MDMϕ, yet the potentiating effect was small (Ren and Savill, 1995). More recent studies have reported that LPS-induced TNFα production may exert inhibitory effects, while IL-10 promoted MDMϕ phagocytosis of apoptotic neutrophils (Michlewska et al., 2009). Thus, the *in vivo* efficacy of apoptotic neutrophil clearance by macrophages is complex and may involve a dynamic interplay between pro- and anti-inflammatory mediators.

1.5.2   *Adhesion signalling (CD44, cAMP)*

The adhesive status of the macrophage also regulates the capacity for apoptotic cell clearance. CD44 is a cell-surface glycoprotein that acts as a receptor for a variety of

extracellular matrix molecules including hyaluronate, fibronectin, and collagen (Aruffo et al., 1990, Culty et al., 1990, Jalkanen and Jalkanen, 1992, Lesley et al., 1997). Many diverse functions have been attributed to CD44, including cellular adhesion and migration, and activation and proliferation of lymphocytes (Lesley et al., 1993). Cross-linking of human MDMφ surface CD44 with a bivalent CD44 monoclonal antibody (mAb) rapidly and specifically augmented uptake of apoptotic neutrophils, but not apoptotic lymphocytes (Hart et al., 1997). Additionally, adhesion to fibronectin rapidly increased MDMφ capacity for internalisation of apoptotic neutrophils (McCutcheon et al., 1998). The critical role of CD44 in the resolution phase of inflammation is evident in CD44-deficient mice (Teder et al., 2002). Following non-infectious lung injury induced by treatment with bleomycin, these mice succumb to an unremitting inflammation characterised by impaired clearance of apoptotic neutrophils from the lung.

Elevation of intracellular cyclic adenosine monophosphate (cAMP) in MDMφ rapidly and specifically inhibited phagocytosis of apoptotic neutrophils (Rossi et al., 1998). Elevation of intracellular cAMP within macrophages results in changes in the localisation of actin and talin cytoskeletal proteins, suggesting that altered adhesion can regulate macrophage phagocytic capacity.

1.6   Inflammatory regulation by glucocorticoids

1.6.1 *Endogenous and synthetic glucocorticoids*

Endogenous glucocorticoids like cortisol and corticosterone are produced by activation of the hypothalamic-pituitary-adrenal axis in response to a variety of stressors (starvation, pain, trauma, infection) and are essential for regulating homeostatic mechanisms associated with metabolic control and inflammation (Munck et al., 1984, Stephanou et al., 1992, Harbuz et al., 1992). Since the seminal work of Hench and his co-workers 50 years ago, where small doses of cortisone were found to dramatically improve the symptoms of patients with rheumatoid arthritis

(Hench et al., 1949), powerful synthetic glucocorticoids were developed (Munck et al., 1984), which, despite their unwelcome side effects, remain the mainstay of anti-inflammatory and immunosuppressive therapy. Glucocorticoids are beneficial in the treatment of diverse inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and asthma through their pleiotropic actions on multiple inflammatory cells and signalling pathways, both during the early and late manifestations of acute inflammation and the subsequent repair phase (Barnes, 1998, Goulding et al., 1998). Recent advances in understanding how these drugs exert their effects at the cellular and molecular level (Barnes and Adcock, 2003, Rhen and Cidlowski, 2005) has provided a valuable insight into how glucocorticoids operate as anti-inflammatory agents.

1.6.2 *Glucocorticoid receptor*

Glucocorticoids exert most of their effects by binding to intracellular glucocorticoid receptors (GR), expressed in many immune cells including monocytes/macrophages, granulocytes, and all lymphocyte subpopulations (Goulding, 2004). Alternative splicing of the *gr* gene can generate isoforms of GR with distinct functions. GRα is the prevalent isoform and mediates glucocorticoid binding, whereas GRβ is a C-terminally truncated variant that lacks glucocorticoid-binding ability and instead heterodimerises with GRα to regulate its effect on transcription (Bamberger et al., 1995, Oakley et al., 1999). The GR consists of a C-terminal ligand-binding domain, a central DNA binding region composed of two zinc finger motifs, plus an N-terminal domain that facilitates trascriptional activity and interation with other transcription factors (Buckingham, 2006). Inactive GR resides in the cytoplasm bound in a multi-protein complex with chaperone molecules including immunophilin and heat shock proteins (HSP90) (Pratt and Toft, 1997). Interaction with HSP90 is essential for maintaining the correct configuration of GR and also masks a nuclear localisation signal to prevent nuclear translocation of the unoccupied GR (Barnes, 2006). Upon binding of glucocorticoid to the GR, the chaperone proteins dissociate from the GR to allow translocation of the ligand-activated complex to the nucleus, where it can

regulate the transcription of multiple target genes at transcriptional and post-transcriptional levels (Barnes, 2006).

1.6.3 *Immunosuppressive effects*

Expression of many inflammatory genes, including cytokines, chemokines, inflammatory enzymes and receptors, is achieved *via* activation of transcription factors such as nuclear factor-κB (NFκB) and activator protein-1 (AP-1). These pro-inflammatory transcription factors are activated during inflammatory disease and play a critical role in amplifying the inflammatory process (Rahman and MacNee, 1998, Di Stefano et al., 2002, Muller-Ladner et al., 2002). Inflammatory gene expression requires NFκB- and AP-1-mediated recruitment and activation of coactivator proteins, such as cAMP response element binding (CREB) binding protein (CBP) and p300/CBP-associated factor, which have intrinsic histone acetyltransferase (HAT) activity to initiate histone acetylation and remodelling of chromatin required for gene transcription (*fig. 1.3*) (Urnov and Wolffe, 2001, Adcock et al., 2004). Repression of genes requires reversal of histone acetylation, controlled by corepressor proteins such as histone deacetylases (HDACs) and nuclear receptor corepressor (NcoR) (Barnes, 1998).

A major effect of glucocorticoids is to switch off the multiple inflammatory genes that have been activated during chronic inflammation by inhibiting the transcriptional effects of NFκB and AP-1, a process known as transrepression (*table 3*) (Barnes and Karin, 1997, Barnes, 1998). Transrepression is thought to occur through protein-protein interactions of the GR with the activated transcription factors, either directly (De Bosscher et al., 2000, Karin and Chang, 2001) or through recruitment of corepressors such as HDACs (Ito et al., 2000), resulting in reversal of histone acetylation and gene "silencing" (*fig. 1.3*). For example, glucocorticoids have been shown to mediate transrepression of IL-1-induced gene activation by inhibiting
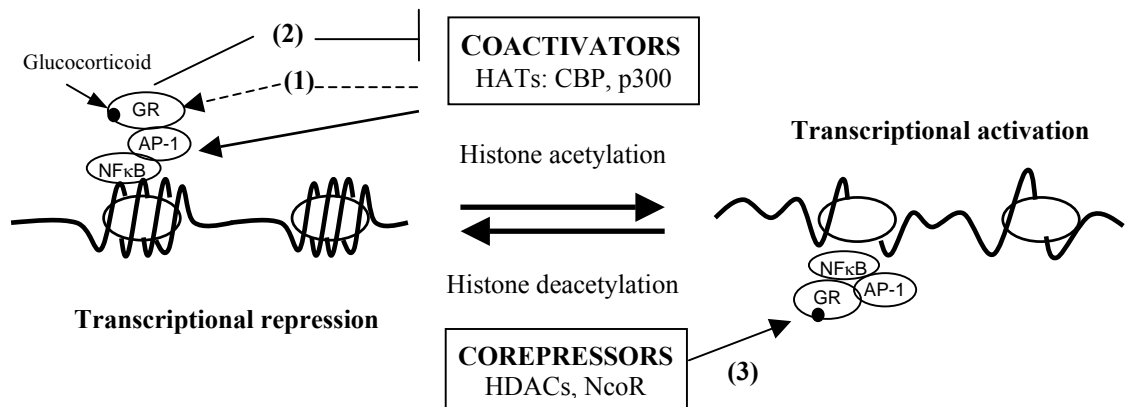
Figure 1.3: Effect of the GR on chromatin remodelling, HATs and HDACs

Gene expression and repression are regulated by modification of core histones. In the resting state, DNA is tightly coiled around histones and is inaccessible to transcriptional cofactors and RNA polymerase II. Upon binding to DNA, NFκB and AP-1 recruit and activate coactivator proteins such as CBP and p300/CBP-associated factor, which have intrinsic HAT activity. Histone acetylation results in slackening of chromatin and unwinding of DNA, allowing the transcriptional machinery to gain access to genes and initiate transcription. Deacetylation is controlled by corepressor proteins such as HDACs and NcoR and is associated with gene repression. Activated GRs may interact with HATs and HDACs to regulate their function. Suggested mechanisms of GR transrepression include; (1) GR competes with NFκB and AP-1 for binding to coactivators, (2) Direct suppression of HAT activity of coactivators by GR, (3) recruitment of corepressors by GR to reverse histone acetylation (McColl et al., 2007). Abbreviations: GR, glucocorticoid receptor; AP-1, activator protein-1; NFκB, nuclear factor κB; HAT, histone acetyltransferase; CBP, cAMP response element binding (CREB) binding protein; HDAC, histone deacetylase; NcoR, nuclear receptor corepressor.

Mechanism of glucocorticoid action	Genes affected
Transactivation: Induction of anti-inflammatory gene expression	SLPI
	IL-1 receptor antagonist
	C1q
	Annexin I
	MKP-1
	GILZ
	IκBα
Transrepression: Suppression of pro-inflammatory gene expression	Cytokines (IL-1, TNFα, GM-CSF)
	Chemokines (IL-8, MIP-1α)
	Adhesion molecules (ICAM-1, E-selectin)

Table 3: Mechanism of glucocorticoid action and genes regulated.

Abbreviations: SLPI, secretory leukocyte proteinase inhibitor; MKP-1, MAPK phosphatase 1; GILZ, glucocorticoid-inducible leucine zipper (McColl et al., 2007).

histone acetylation (Ito et al., 2000). Reichardt and colleagues developed a GR^{dim} mouse, where a point mutation A458T introduced into the D-loop of the central domain produced a GR that was DNA binding-, dimerisation- and transactivation-deficient (Reichardt et al., 1998, Tuckermann et al., 1999, Reichardt et al., 2001). Transrepression of NFκB- and AP-1–mediated gene expression remained intact, suggesting that this process is mediated by monomeric GR. Although it fails to bind palindromic sequences in glucocorticoid-responsive genes, a caveat with the GR^{dim} mutant is the ability to bind an alternative class of glucocorticoid response elements to modulate gene expression (Adams et al., 2003). This must be taken into consideration in studies attempting to dissect out the transactivation action of glucocorticoids based on GR functioning as a homodimer, an approach that may be an oversimplification of the underlying mechanisms of GR action.

1.6.4 *Anti-inflammatory effects*

Glucocorticoids can regulate inflammatory responses by increasing the expression of molecules with anti-inflammatory functions, a process known as transactivation (*fig. 1.3* and *table 3*). Ligand-activated GRs bind to palindromic glucocorticoid-responsive elements (GREs) found in the promoter region of glucocorticoid-responsive genes (Chandler et al., 1983, Drouin et al., 1992) and recruit transcriptional coactivator proteins to initiate transcription (Barnes, 1998). The central domain of the GR contains two zinc fingers essential for GR dimerisation and binding to GRE sequences (Dahlman-Wright et al., 1991, Drouin et al., 1992), with a point mutation in the D-loop of the central domain abolishing transactivation (Reichardt et al., 1998). Thus, transcription of glucocorticoid-responsive genes requires homodimeric GR. This mechanism requires long-term exposure to glucocorticoids, and directly modulates the expression of anti-inflammatory molecules like C1q, annexin-1, secretory leukocyte proteinase inhibitor (SLPI), IL-1 receptor antagonist, and IL-10 (Abbinante-Nissen et al., 1995, Hodge et al., 1999, de Coupade et al., 2001, Pousset et al., 2001, Faust et al., 2002).

Glucocorticoids also enhance the expression of proteins that may be vital for switching off signalling pathways engaged during persistant inflammation, including mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1), IκBα and glucocorticoid-inducible leucine zipper (GILZ). MKP-1 expression is induced by glucocorticoids in a wide variety of cell types including mast cells, monocytes and macrophages (Kassel et al., 2001, Abraham and Clark, 2006, Bhattacharyya et al., 2007). Glucocorticoid-induced expression of MKP-1 in macrophages results in dephosphorylation and inactivation of p38 MAPK to inhibit expression of Cox-2, IFNγ, TNFα and IL-1 (Abraham and Clark, 2006) and destabilise mRNA for Cox-2 (Lasa et al., 2002). This may be an important anti-inflammatory mechanism as it allows glucocorticoids to post-transcriptionally restrict the ongoing production of inflammatory proteins. Glucocorticoids also enhance the expression of IκBα, an inhibitor of NFκB that prevents NFκB translocation to the nucleus (Auphan et al., 1995, Scheinman et al., 1995), and GILZ, which interacts with both NFκB and AP-1 to repress their transactivation function (Riccardi et al., 1999, Mittelstadt and Ashwell, 2001, Berrebi et al., 2003). Thus, glucocorticoids induce the expression of protective proteins that boost innate immune defence, and pro-resolving proteins that suppress pro-inflammatory signalling.

Glucocorticoids can exert anti-inflammatory effects both at transcriptional and post-transcriptional levels via transrepression and transcriptional activation, with inhibition of NFκB representing a common mechanism. However, it is likely that these mechanisms of action are not mutually exclusive and optimal glucocorticoid action probably requires a combination of both, the extent of which may depend on many factors such as cell type, target of suppression or modulation, and requirement for long or short exposure to glucocorticoid.

1.6.5 *Regulation of inflammatory cell survival*

Glucocorticoids can regulate the processes involved in the normal resolution of an inflammatory response. For example, the synthetic glucocorticoids dexamethasone (Dex), methylprednisolone, and hydrocortisone all prolonged neutrophil viability

from 12 to 48 hours by delaying constitutive neutrophil apoptosis *in vitro* (Liles et al., 1995, Meagher et al., 1996, Madsen-Bouterse et al., 2006). The inhibitory effect of glucocorticoids on neutrophil apoptosis was dose-dependent, with maximal effects found with Dex at 10^{-6}M (Cox, 1995, Meagher et al., 1996). The non-glucocorticoid progesterone failed to inhibit the development of apoptosis, indicating that non-specific effects of high dose glucocorticoids are not responsible. Glucocorticoid-induced neutrophil survival was found to be GR-dependent and reversible by inhibitors of protein synthesis and blockade of NFκB signalling (Meagher et al., 1996), suggesting that survival requires continual synthesis of a pro-survival factor such as the anti-apoptotic protein, Mcl-1 (Cox and Austin, 1997, Saffar et al., 2008). Although neutrophil capacity for superoxide generation in response to stimulation with FMLP remains intact (Cox, 1995), glucocorticoids generally exert significant inhibitory effects on neutrophil activation and functions such as chemotaxis, adhesion, phagocytosis, and cytokine release (Cox, 1995, Goulding et al., 1998). Overall, the paradoxical effect of enhancing survival and subsequent accumulation of neutrophils with the potential for superoxide production into an already inflamed tissue may represent an effect that accentuates inflammation and contributes to the resistance of glucocorticoid action in COPD, a neutrophil-mediated disease (Zainudin, 1997).

Direct pharmacological triggering of neutrophil apoptosis pathways may represent a novel therapeutic strategy in the treatment of inflammatory disorders (Serhan et al., 2007, Rossi et al., 2007). To be successful, this approach may require additional tactics to ensure rapid and efficient removal of the apoptotic neutrophils before progression to secondary necrosis and release of cell contents.

1.6.6 *Regulation of macrophage phagocytosis for apoptotic cells*

Glucocorticoids can regulate MDMφ clearance of apoptotic neutrophils *in vitro* (Liu et al., 1999, Giles et al., 2001) and possibly *in vivo* (Gilmour et al., 2006). Freshly isolated human monocytes lack the capacity to ingest apoptotic neutrophils, but acquire phagocytic ability during *in vitro* culture as adherent monocytes differentiate

into MDMφ (Newman et al., 1982). Several drugs of the glucocorticoid family (methylprednisolone, hydrocortisone or Dex) were found to significantly enhance the phagocytic capacity of human MDMφ following *in vitro* culture for 5 days and greater potentiation was observed when monocytes were exposed to glucocorticoids earlier during maturation (Liu et al., 1999, Giles et al., 2001). In contrast to the specificity of CD44-enhanced human MDMφ phagocytosis for apoptotic neutrophils (Hart et al., 1997), glucocorticoids augment human MDMφ phagocytic capacity for alternative apoptotic targets, including Jurkat T cells and eosinophils, and also promote uptake of apoptotic neutrophils by alternative phagocytes, including human glomerular mesangial cells (Liu et al., 1999). Importantly, glucocorticoid-enhanced MDMφ phagocytosis of apoptotic neutrophils did not stimulate the release of pro-inflammatory mediators, including MCP-1 and IL-8 chemokines (Liu et al., 1999). Therefore, glucocorticoids promote "safe" clearance of neutrophils dying by apoptosis and may directly contribute to the resolution of inflammation.

1.7   Glucocorticoid resistance

Although glucocorticoids are the most effective clinical treatment for various inflammatory disorders, a small proportion of patients with severe asthma do not respond well to glucocorticoid treatment, even when given high doses (Barnes and Adcock, 2003). Inhaled glucocorticoids also provide little therapeutic benefit for patients with COPD, with no reduction in inflammatory cells, cytokines or proteases in the inflamed lung (Keatings et al., 1997, Culpitt et al., 1999, Loppow et al., 2001). Several mechanisms have been postulated to contribute to glucocorticoid resistance. Increased expression of the GRβ isotype in response to pro-inflammatory cytokines may interfere with GRα function (Bamberger et al., 1995, Oakley et al., 1999, Webster et al., 2001). Interestingly, the level of GRβ may be increased in peripheral blood mononuclear cells from patients with glucocorticoid-resistant rheumatoid arthritis (Kozaci et al., 2007). The ineffectiveness of glucocorticoids in COPD has been linked to factors such as smoking and oxidative stress that may reduce HDAC2 function, a corepressor protein important for glucocorticoid-mediated gene repression (Ito et al., 2001, Ito et al., 2006).

1.8   Glucocorticoids side effects

There are potential limitations to the applications of glucocorticoids in disease, particularly the undesirable side effects associated with long-term treatment. The nature of glucocorticoid action is pleiotropic and relatively non-specific, and many tissue and organ systems are affected resulting in diabetes, hypertension, osteoporosis, behavioural changes, and sleep disorders (Rosen and Miner, 2005). Many of these effects represent "on target" actions mediated by physiological levels of endogenous glucocorticoid, but result in "undersirable" effects in the presence of high-dose synthetic glucocorticoids. An indication of this is the induction of gluconeogenesis, which under normal physiological conditions represents an important component of the body's response to stress, but represents an underlying cause of glucocorticoid-induced diabetes. The central role of glucocorticoids in the regulation of glucose levels is evident in glucocorticoid-deficient patients with low glucose levels (Addison's disease), and patients with glucocorticoid excess (Cushing syndrome) who exhibit glucose intolerance (Andrews and Walker, 1999).

Despite these adverse effects, glucocorticoids remain one of the most used therapeutic approaches for the treatment of chronic inflammatory diseases. Elucidation of the precise molecular and cellular mechanisms of glucocorticoid action would potentially allow the development of more specific therapies with fewer adverse effects. Side effects have generally been linked to transactivation of gene expression by ligand-activated GR, with a suggested role for glucocorticoid-inducible phosphoenol pyruvate carboxykinase (PEPCK) in regulating gluconeogenesis (Schacke et al., 2002). The finding that transactivation-deficient GR^{dim} mutants retain many of their beneficial anti-inflammatory effects through transrepression of NFκB- and AP-1–mediated gene expression (Reichardt et al., 1998, Tuckermann et al., 1999, Reichardt et al., 2001) raised the possibility that these transcriptional functions of the GR could be separated, and prompted a search for GR ligands that induce transrepression but not transactivation. These 'dissociated' steroids, including RU24858 and RU40066, have anti-inflammatory effects *in vitro* (Vayssiere et al., 1997) and *in vivo* (Schacke et al., 2004), however, RU24858

induced side effects including loss of body weight and bone mass (Belvisi et al., 2001). More recently, Miner *et al* developed a series of GR ligands like LGD5552 that retained strong anti-inflammatory transrepressional activity, but reduced ability to activate transcription at some genes due to diminished interaction with coactivator proteins and an enhanced interaction with corepressor proteins (Miner et al., 2007). Importantly, LGD5552 displayed reduced side effects when compared to glucocorticoids, including weight gain and decreased bone formation. Such compounds may represent an attractive therapeutic strategy for "safer" treatment of inflammatory diseases through this ability to convey the beneficial actions of glucocorticoids with reduced adverse effects (Catley, 2007). However, some side effects such as skin atrophy and suppression of the hypothalamic-pituitary-adrenal axis may be mediated by the transrepressive function of the GR or may even involve a combination of both GR mechanisms that would complicate the use of dissociated steroids in therapy (Schacke et al., 2002). Furthermore, unlike GR-mediated transactivation of other genes, induction of MKP-1 gene expression does not seem to require GR dimerisation and GR^{dim} mutants retain the ability to express MKP-1 (Abraham and Clark, 2006). Upregulation of MKP-1 by glucocorticoids has been suggested to contribute to metabolic dysregulation *via* impairment of insulin-induced glucose uptake by adipocytes (Bazuine et al., 2004). Therefore, dissociation of glucocorticoid-mediated side effects from anti-inflammatory actions based on GR functioning as a monomer or dimer may be an oversimplification of the underlying mechanisms. Additionally, many of the anti-inflammatory effects of glucocorticoids have yet to be fully characterised, and it remains possible that induction of gene expression by glucocorticoids plays a more significant role in the inhibition of inflammatory responses than originally assumed. It is imperative that the specific molecular mechanisms involved in glucocorticoid-mediated immune regulation are delineated to allow the development of more selective therapies.

1.9   Aims

The primary aims of this study were to investigate the mechanisms required for high capacity phagocytosis of apoptotic neutrophils following long-term treatment of human peripheral blood monocyte-derived macrophages with the synthetic glucocorticoid, Dex. Before these studies were initiated, there was limited experimental evidence on the molecular mechanisms involved in glucocorticoid-augmentation of macrophage phagocytosis. Although glucocorticoids have been shown to alter macrophage cytoskeletal organisation and adhesive ability, the phagocytic pathway employed by macrophages following exposure to glucocorticoids has remained uncharacterised. Considering the pivotal role of apoptotic neutrophil clearance in resolution processes, we hypothesised that glucocorticoids specifically induce a distinct mechanism for apoptotic cell clearance. Specifically, we wanted to investigate the following two key issues:

1. The effect of glucocorticoids on the phagocytic potential of human macrophages using confocal and time-lapse microscopy, and, primarily, flow cytometry. In particular, we wished to identify the subpopulation of apoptotic neutrophils that are targeted for clearance.

2. To characterise the molecular basis for apoptotic cell phagocytosis by glucocorticoid-treated macrophages by;

    ▪ Performing inhibitor studies of phagocytosis using receptor antagonists and "blocking" antibodies
    ▪ Evaluating the contribution of serum components
    ▪ Investigating the effect of pro-inflammatory cytokines (IFNγ) and bacterial products (LPS)

CHAPTER 2: MATERIALS AND METHODS

2.1   Sera, serum proteins and other reagents

All chemicals were purchased from Sigma (www.sigmaaldrich.com) unless otherwise stated. Culture media (Iscove's modified Dulbecco's medium (IMDM)), buffers (HBSS and PBS without divalent cations) and Trypsin-EDTA were obtained from PAA Laboratories (www.paa.com). Dextran T500 was from Pharmacosmos (www.pharmacosmos.com). Percoll was obtained from GE Healthcare (www.gehealthcare.com). Dex was from Organon (www.organon.co.uk). Autologous serum was freshly prepared by re-calcification of human platelet-rich plasma (PRP) (220μl of 1M $CaCl_2$/10ml plasma, final concentration 22mM, 37°C for 1 hour). Serum was also obtained from the following species and stored at -70°C for no more than two weeks prior to analysis: murine (C57BL/6 mice; a kind donation from Mauro Perretti), goat (Sigma), and FBS (Biowest; heat-inactivated). Proteins purified from human serum/plasma were obtained form the following sources: protein S (Enzyme Research Laboratories, www.enzymeresearch.co.uk), pentraxin-3 (R&D Systems; www.rndsystems.com), C1q (Merck; www.merckbiosciences.co.uk), IgG (a kind donation from Simon Hart), and fibronectin (R&D Systems). Roscovitine and gliotoxin were from Merck. Human tumour necrosis factor-α (TNFα) was from R&D Systems. Human recombinant interferon-γ (IFNγ) was from Peprotech (www.peprotechec.com). TACE-inhibitor peptide (KD–1X–73.5) was a kind donation from Simon Brown.

2.2   Antibodies

Primary antibodies were from the following sources: polyclonal rabbit anti-human protein S antibody (Dako, www.dako.com), anti-human C4BP mAb (Enzyme Research Laboratories), anti-human Mer mAb (IgG1 isotype, R&D Systems), anti-human αvβ5 (IgG1 isotype, R&D Systems), anti-CD44 mAb (clone 5A4; provided by G. Dougherty, University of California, San Francisco, CA) and anti-FcγRII

(clone IV.3; a kind donation from Stelios Bournazos). Control mouse IgG1 was from Serotec (www.ab-direct.com) and rabbit IgG was from Sigma. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse immunoglobulins, and FITC-conjugated F(ab')$_2$ goat anti-mouse immunoglobulin were from Dako. APC-conjugated annexin V was from Invitrogen (www.invitrogen.com)

2.3   Cell isolation

Mononuclear (MNC) and polymorphonuclear (PMN) leukocytes were isolated as previously described (Dransfield et al., 1994). In brief, freshly drawn peripheral blood from healthy volunteers was collected into sodium citrate (final concentration 0.4%) in 50ml Falcon tubes. Centrifugation at 350 x g for 20min resulted in an upper layer of PRP, which was aspirated for immediate preparation of autologous serum (see 2.1 Sera, serum proteins and other reagents) and a lower cell pellet. Erythrocytes were removed by sedimentation with 0.6% (w/v) dextran T500 (2.5ml dextran/10ml cell pellet, made up to 50ml total volume with saline) for approximately 30min at room temperature. The upper leukocyte-rich layer was then removed and washed with saline (final volume 50ml, 350 x g for 6min) to remove excess dextran. Leukocytes were fractionated over a discontinuous Percoll™ gradient. Percoll was made isotonic with 10x PBS, generating a 90% stock solution from which final concentrations of 55%, 70%, and 81% Percoll were made. Gradients were prepared by overlaying 3ml of each 81% (bottom layer), 70% (middle layer) and 55% (upper layer, used to resuspend leukocyte pellet prior to layering) and centrifuged at 720 x g for 20min at room temperature. MNC were aspirated from the 55/70% interface, and PMN from the 70/81% interface, with residual erythrocytes pelleted at the bottom. Leukocytes were washed twice in PBS (without divalent cations) before assessment of purity (see 2.4 Quality control) and cell culture.

2.4 Quality control

MNC and PMN preparations were assessed for purity by flow cytometry and morphological analysis. Figure 2.1A shows the light scatter profiles of MNC and PMN preparations using a FACScan flow cytometer. MNC and PMN preparations were routinely above 95% purity. Figure 2.1B shows cytospins of MNC and PMN preparations. 4×10^5 cells were cytocentrifuged at 300 x g for 3min, fixed in 100% methanol and visualised with Diff-Quick^{TM} staining and 100x oil immersion light microscopy.

2.5 Generation of monocyte-derived macrophages (MDMϕ)

MNC were resuspended at 4×10^6/ml in IMDM and allowed to adhere to Falcon tissue culture plates for 1 hour at 37°C in 5% CO_2. Non-adherent lymphocytes were removed by washing 3 times with IMDM and adherent monocytes were cultured for 5 days in IMDM (containing penicillin and streptomycin) containing 10% autologous serum ± 1μM Dex. Previous studies showed that >90% of cells are CD14-positive at 5 days (Giles et al., 2001).

2.6 Induction of neutrophil apoptosis

Neutrophils were resuspended at 4×10^6/ml in IMDM ± 10% autologous serum and cultured in Falcon tissue culture flasks at 37°C in 5% CO_2 atmosphere for 20-24 hours, during which time a proportion of the cells underwent apoptosis (Savill et al., 1989b). Alternatively, neutrophils were resuspended in IMDM at 2×10^7/ml and labelled with the fluorescent cell tracker dye 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen), 2μg/ml final concentration for 15min at 37°C. Neutrophils were then washed and cultured for 20-24 hours in IMDM ± 10% autologous serum as before.
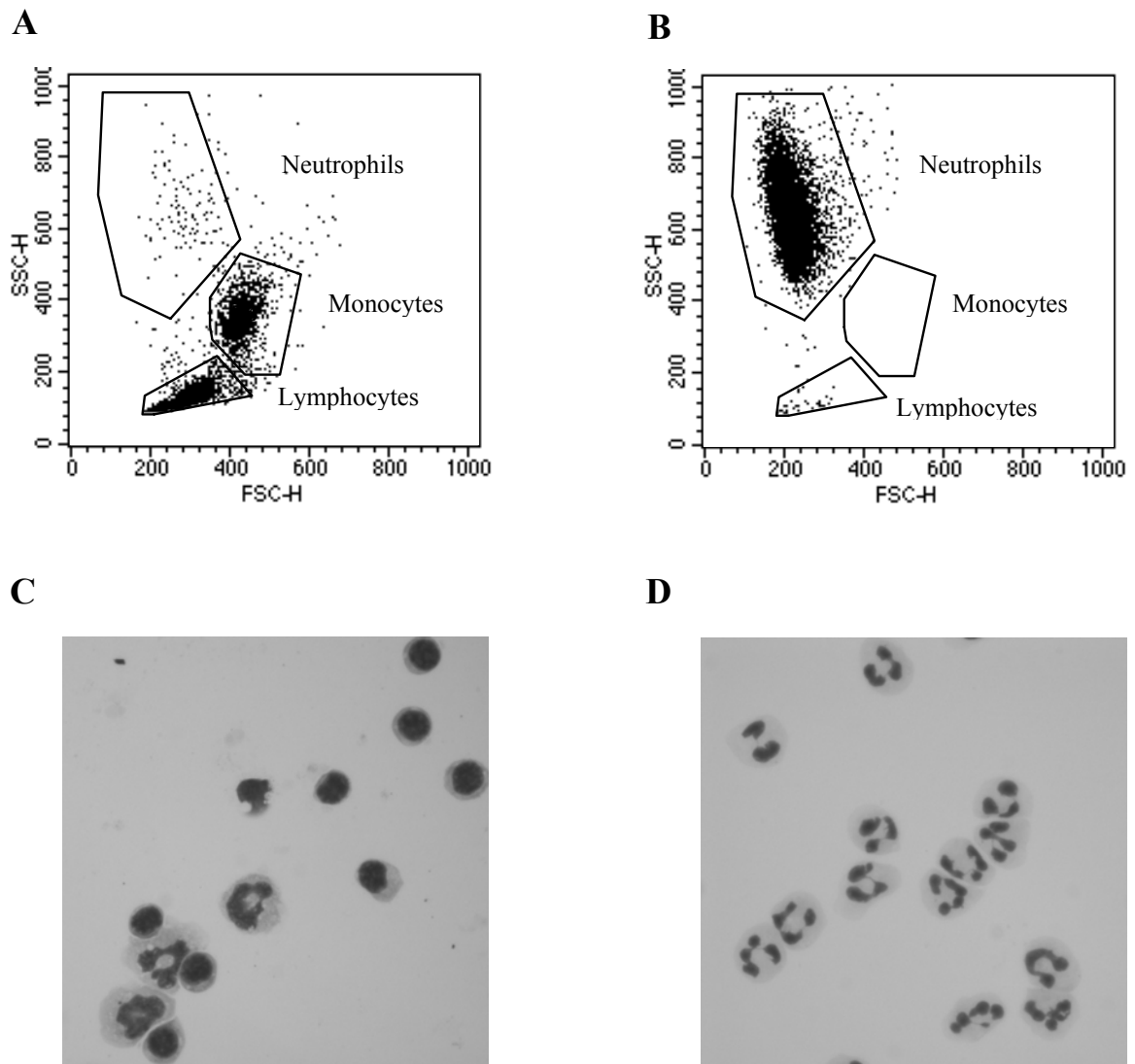
Figure 2.1: Purity of leukocyte preparations.
Mononuclear (MNC) and polymorphonuclear (PMN) leukocytes were isolated from freshly drawn, citrated human blood by dextran sedimentation and centrifugation over a discontinuous Percoll™ gradient. MNC (panels **A** and **C**) and PMN (panels **B** and **D**) preparations were assessed for purity by flow cytometry and morphological analysis. Neutrophils are characterised by the presence of multi-lobed nuclei (panel **D**).
Flow plots were generated with a FACScan flow cytometer. Purity ratios for panel **A**, monocytes: 24.2%, lymphocytes: 72.8%, neutrophils: 1.8%; for panel **B**, monocytes: 0%, lymphocytes: 0.5%, neutrophils: 98.5%.

2.7   Assessment of neutrophil apoptosis

After culture for 20-24 hours, unlabelled neutrophils were pelleted by centrifugation at 200 x *g* for 5min then resuspended in IMDM, and the percentage of apoptotic and secondarily necrotic neutrophils was determined by annexin V-FITC binding (Roche Applied Sciences, www.roche-applied-science.com) and propidium iodide staining (Sigma), respectively.

2.7.1   *Annexin V binding and propidium iodide staining*

PS exposure on apoptotic cells is shown by calcium-dependent binding of fluorescently-labelled annexin V (Vermes et al., 1995). 280μl of annexin V-FITC solution (diluted 1:500 with HBSS containing 5mM $CaCl_2$) was added to 20μl of a neutrophil suspension (2.5 x 10^6/ml), mixed gently, and incubated for 10min on ice. 1μl of 1mg/ml propidium iodide was added to the sample before immediate analysis by two-colour flow cytometry for annexin V binding on FL-1 and propidium iodide staining on FL-2. Neutrophil populations cultured in serum-free conditions for 20-24 hours exhibit a similar percentage (63-70%, n=35, 95% confidence limit) of annexin V+/propidium iodide- (apoptotic) cells when compared with neutrophils cultured in the presence of 10% autologous serum (62-72%, n=10, 95% confidence limit) (*figs. 2.2a and b*). However, there were significantly higher percentages of annexin V+/propidium iodide+ (secondarily necrotic) neutrophils when cultured in serum-free conditions.

2.7.2   *Morphological analysis*

Cytocentrifugation and morphological analysis (see 2.4 Quality control) was used to confirm levels of cell death in neutrophil populations (*figs. 2.2c* and *d*). The multi-lobed nuclear morphology characteristic of viable neutrophils becomes pyknotic in cells undergoing apoptosis (Kerr et al., 1972).

A	
Viable	13 – 24%
Apoptotic	62 – 72%
Necrotic	8 – 17%

B	
Viable	8 – 12%
Apoptotic	63 – 70%
Necrotic	18 – 26%
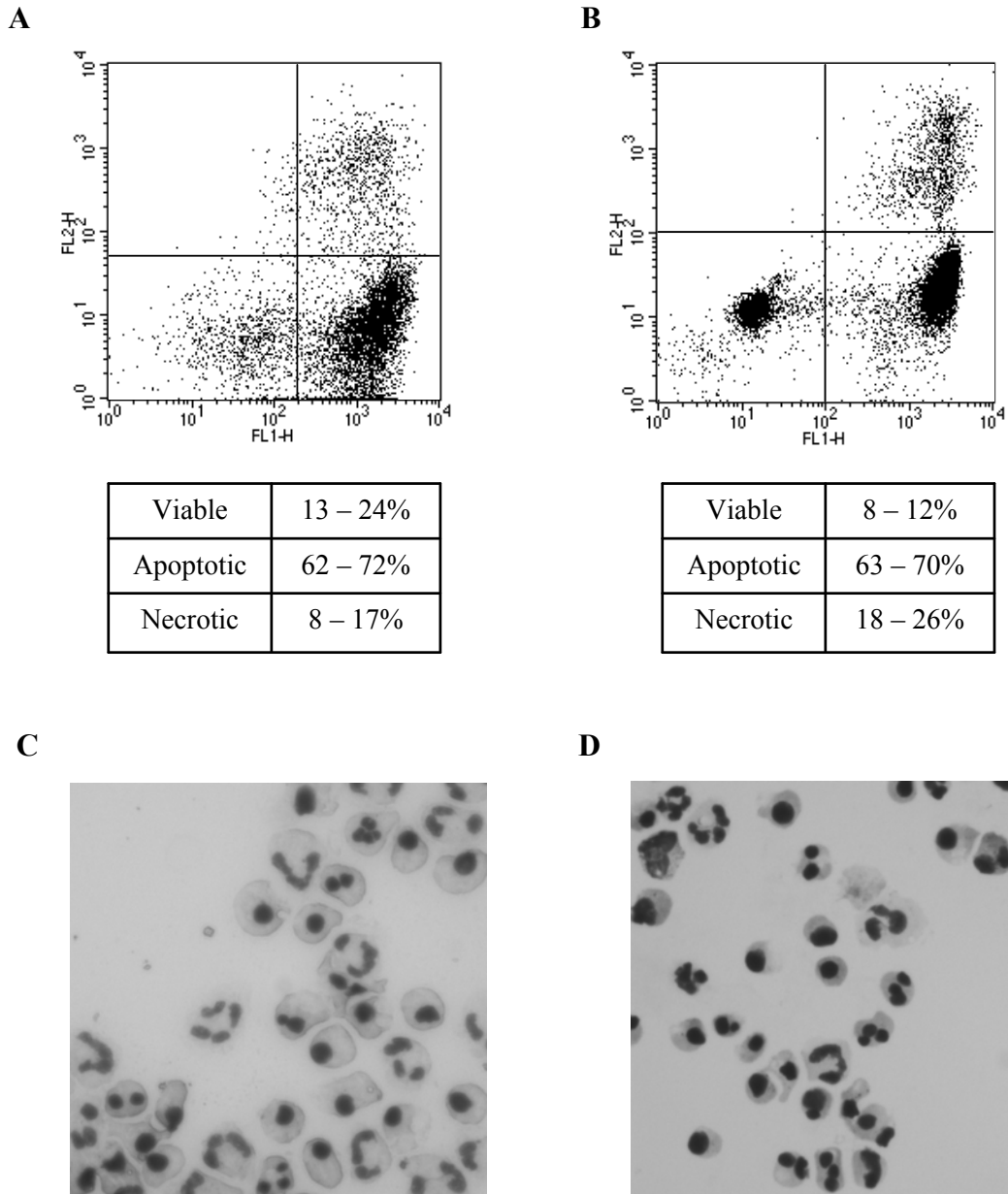
Figure 2.2: <u>Assessment of neutrophil apoptosis.</u>
Peripheral blood neutrophils were cultured for 20-24 hours in IMDM in the <u>presence</u> (panels **A** and **C**) or <u>absence</u> (panels **B** and **D**) of 10% fresh autologous serum. Neutrophil viability was determined by flow cytometric analysis of annexin V-FITC binding on FL-1 and propidium iodide staining on FL-2 (panels **A** and **B**). Data for the proportion of viable (annexin V-/propidium iodide-), apoptotic (annexin V+/propidium iodide-) and secondary necrotic (annexin V+/propidium iodide+) neutrophils are shown as 95% confidence limits. Morphological analysis of cytocentrifuge preparations shows the presence of pyknotic nuclei (condensed and rounded) characteristic for apoptotic neutrophils (panels **C** and **D**).

2.8   Macrophage phagocytosis assay

Phagocytosis was assessed by flow cytometry, confocal microscopy and time-lapse video microscopy. Due to restrictions on blood donations, MDMϕ and neutrophils used for phagocytosis assays were derived from different human donors (allogeneic phagocytosis). Fresh autologous serum used during phagocytosis was obtained from the neutrophil donor, unless otherwise stated.

2.8.1   *Flow cytometry*

MDMϕ were cultured for 5 days ± 1μM Dex in 48-well Falcon tissue culture plates (approximately 200,000/well), washed gently with IMDM then co-incubated with 0.5ml CMFDA-labelled apoptotic neutrophils (centrifuged at 200 x *g* and resuspended at 2.5 x 10^6/ml in IMDM; a ratio of approximately 6 neutrophils per macrophage) for 30min at 37°C in 5% CO_2. Medium was then aspirated from the wells and replaced with 0.5ml Trypsin-EDTA. Cells were incubated for 15min at 37°C, then 15min on ice followed by vigorous pipetting to ensure detachment of all adherent cells. MDMϕ phagocytosis of apoptotic neutrophils was assessed by flow cytometry using a FACScan (Becton-Dickinson, www.bdbiosciences.com). Uningested neutrophils were identified by the combination of their green fluorescence (FL-1 positive) due to CMFDA labelling and their relatively smaller size (FSC) when compared with the MDMϕ (*fig. 2.3a*). MDMϕ populations were gated based on forward and side scatter characteristics, and MDMϕ demonstrating green fluorescence (FL-1 positive) were deemed to have ingested apoptotic neutrophils (*fig. 2.3b*).

This assay has previously been carefully characterised and can be used to discriminate between bound and internalised apoptotic neutrophils, comparing favourably with analysis by light microscopy (Jersmann et al., 2003). For details on receptor inhibitors or purified proteins added to MDMϕ or apoptotic neutrophils, see individual figures for concentrations.
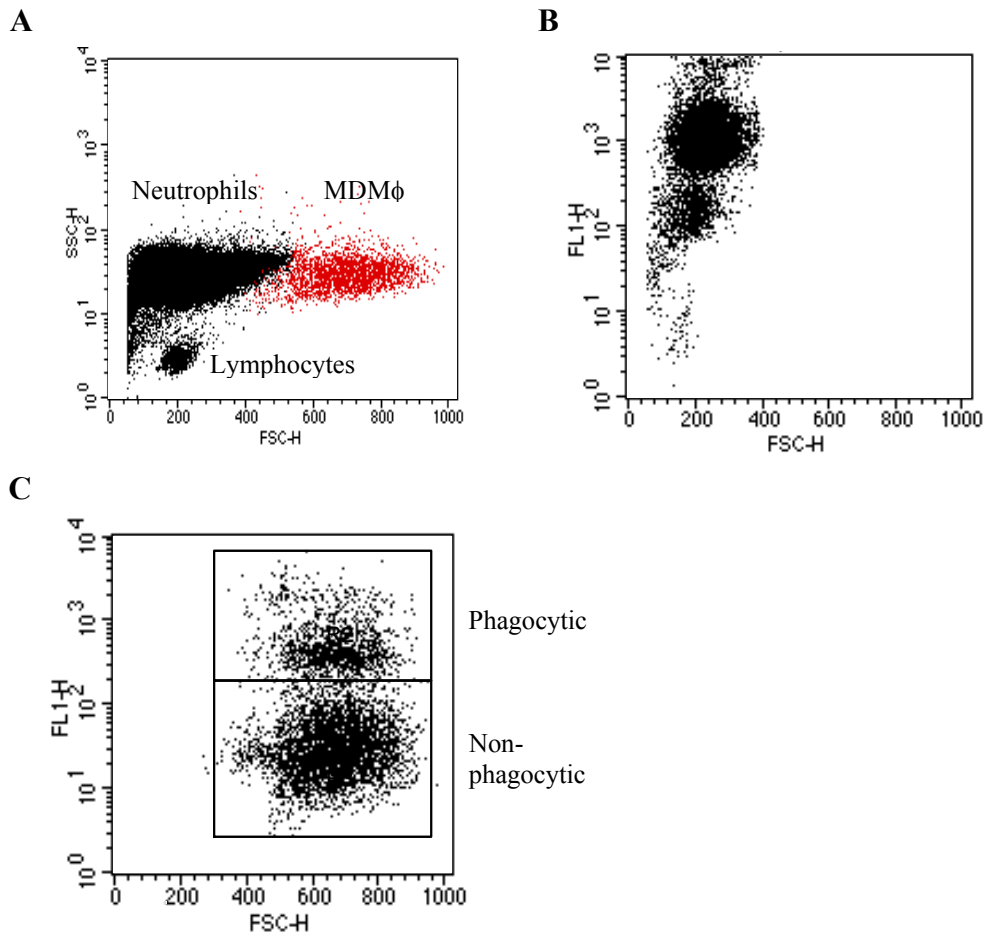
Figure 2.3: Quantitation of MDMφ phagocytosis of apoptotic neutrophils.
The percentage of human MDMφ that could phagocytose CMFDA-labelled apoptotic neutrophils was determined in a 30 minute *in vitro* phagocytosis assay by flow cytometry using a FACScan flow cytometer. **A**, Dot plot showing FSC and SSC of MDMφ and apoptotic neutrophils following phagocytosis assays. **B**, Dot plot of CMFDA-labelled apoptotic neutrophils detected on FL-1. **C**, MDMφ were identified by their distinct forward and side scatter properties. Gate R1 corresponds to FL-1 positive MDMφ that have phagocytosed apoptotic neutrophils, whereas Gate R2 MDMφ are non-phagocytic. A total of 6000 MDMφ events were collected.

2.8.2 *Time-lapse video microscopy*

MDMφ were cultured for 5 days $\pm$ 1µM Dex in plastic chamber slides (12 x 10^6 MNC/chamber slide), washed once in IMDM then overlaid with 3ml of <u>serum-free cultured</u> apoptotic neutrophils (centrifuged at 200 x g for 5min and resuspended at 1 x 10^6/ml in IMDM) $\pm$ 10% autologous serum. Time-lapse video microscopy of phagocytosis was carried out at 37°C using a phase contrast microscope and Leica QWin V3 software with the time lapse sequence set to capture images every 30 seconds for 30 min (see attached CD for videos).

2.8.3 *Confocal microscopy*

MDMφ were cultured for 5 days $\pm$ 1µM Dex in plastic chamber slides (12 x 10^6 MNC/chamber slide), washed once in IMDM then overlaid with 3ml of <u>serum-free cultured</u> apoptotic neutrophils (centrifuged at 200 x g for 5min and resuspended at 2.5 x 10^6/ml in IMDM) $\pm$ 10% autologous serum. After co-incubation for 30min at 37°C in 5% CO_2, medium was aspirated from the wells and MDMφ were washed carefully with IMDM to remove excess neutrophils. Adherent MDMφ were fixed with 0.5ml 3% paraformaldehyde for 15min then washed once with PBS (without divalent cations). Slides were removed from the chambers and mounted with mowial before analysis by confocal laser scanning microscopy using a Carl Zeiss confocal microscope. Analysis of imagery was carried out using Zeiss LSM image browser.

2.9   Serum fractionation

Fractionation experiments were carried out using fresh autologous serum obtained from the neutrophil donor.

2.9.1 *Ultracentrifugation of serum*

Immediately after preparation, human autologous serum (200µl/tube) was ultracentrifuged at 330,000 x g for 1 hour at 4°C using a TL-100 rotor (settings: acceleration 9, brake 9). This centrifugal force generated three distinct serum layers:

a top opalescent lipid layer, a medium straw-coloured protein layer and a pellet containing aggregated protein. The protein layer was carefully removed to prevent perturbation of the pellet, and resuspended to original volume (200µl) with IMDM.

2.9.2 *Ultrafiltration of serum*

Human serum (0.5ml/membrane) was filtered through Centricon YM-50 membranes (Millipore; molecular weight cut off 50-kDa) at 3,000 x *g* for 1 hour in a swinging bucket rotor. At this centrifugal force, solvent and low molecular weight (LMW) components less than 50-kDa in molecular mass were driven through the membrane and collected as the filtrate. Components larger than 50-kDa in molecular mass were retained above the membrane, and this retentate was collected by inverted centrifugation at 300 x *g* for 3min. Both filtrate and retentate were resuspended to original volume (0.5ml) with IMDM. This procedure was repeated using YM-100 membranes (1,000 x *g* for 12min).

2.10 Protein purification techniques

Chromatography techniques were applied for protein purification from human serum. Purifications were performed using fresh serum from a single donor.

2.10.1 *Anion exchange chromatography*

Anion exchange chromatography was carried out using mono Q Sepharose (Sigma), an anion exchanger. A 5cm mono Q Sepharose column was packed under gravity and stored at 4°C. All subsequent steps were performed at 4°C under flow pressure (2ml/min using a pharmacia LKB pump P-1).

2.10.1.1 <u>Optimisation of pH</u>

Buffering solutions were prepared at pH6 (50mM MES and 0.14M NaCl), pH7 and pH8 (50mM HEPES and 0.14M NaCl). Human serum was loaded into dialysis tubing (molecular weight cut off 12,400da, Sigma) and equilibrated overnight at 4°C

to the corresponding pH buffer used to run the column. Mono Q Sepharose was equilibrated with 50ml pH6 buffer before application of 40ml pH6-equilibrated serum. Proteins with an incorrect surface charge at this pH passed through the column and were collected as the flow through. Non-specifically bound proteins were removed by washing the column with 150ml pH6 buffer until A280 of the flow through fell below 0.1. Specifically bound proteins were eluted from the column following application of pH6 buffer containing 0.5M NaCl. 1ml fractions (up to 15) were collected using a fraction collector. Protein concentration in eluted fractions was estimated by measurement of absorbance at 280nm (A280) using a UV spectrophotometer (zero to pH6 buffer containing 0.5M NaCl). To obtain a good protein yield, three fractions containing the highest concentration of protein were combined and dialysed overnight to pH6 buffer (0.14M NaCl) to remove excess salt. The mono Q Sepharose column was regenerated upon washing with 20ml pH6 buffer containing 1M NaCl to remove any remaining bound proteins, and equilibrated with 50ml 0.14M NaCl pH6 buffer to prepare for the next experiment.

This method was repeated using pH7 and pH8 buffering solutions and equilibrated serum. Flow through and elution fractions collected from columns at the various pH values were stored at 4°C for no more than 1 week before assessment for phagocytic activity.

2.10.1.2   Optimisation of ionic strength

Anion exchange chromatography was performed as described above (2.10.1.1 Optimisation of pH). Serum proteins bound to mono Q Sepharose at pH7 were eluted using a salt gradient of 0.2M to 0.5M NaCl prepared in pH7 buffer. A fraction collector was used to collect 1.6ml fractions. Protein concentration in eluted fractions was estimated by measurement of A280 (zero to pH7 buffer of appropriate ionic strength) or by using a Pierce BCA kit. For the BCA kit procedure, 10μl of sample was pipetted on a 96-well microtiter plate and incubated with 200μl of the reagent mixture. After incubation for 30min at 37°C, the plate was allowed to cool to room temperature and the optical density measured at 560nm with an ELISA plate reader. Calibration was performed with BSA in a concentration range of 0.25 to 2mg/ml. Fractions containing the highest concentration of protein were combined and

dialysed overnight to pH7 buffer (0.14M NaCl) to remove excess salt. Protein content in eluted fractions was standardised prior to assessment of phagocytic activity.

2.10.2 *Gel Filtration*

Gel filtration was carried out using Sephacryl S-300 gel (GE Healthcare). 250ml of Sephacryl S-300 was prepared in an equal volume of 1 x TBS buffer (0.5M Tris, 9% NaCl, pH 7.6), loaded into a gel packing reservoir attached to the top of a gel filtration column (a resin bed 90cm in height was used), and filled to the top with 1 x TBS buffer. The column was packed under flow pressure (3ml/min for 2 hours), stored at 4°C and washed with 50ml of 1 x TBS buffer prior to each experiment. All steps were performed at 4°C under flow pressure (2ml/min). Due to the direction of liquid flow during column preparation (top to bottom), the gel was more packed at the bottom and samples were therefore applied to the column in the reverse direction using a three-way tap.

2.10.2.1 <u>Calibration of the gel filtration column</u>

The gel filtration column was calibrated with cytochrome c (12.4-kDa), carbonic anhydrase (29-kDa), human serum albumin (66-kDa), alcohol dehydrogenase (150-kDa), β-amylase (200-kDa) and blue dextran (2,000-kDa) from MW-GF-200 kits (Sigma). Protein standards were reconstituted to 10mg/ml in 1 x TBS running buffer and stored at 4°C. Standards were freshly prepared to the final concentrations recommended in manufacturer guidelines prior to gel filtration. Carbonic anhydrase and alcohol dehydrogenase, and cytochrome c and β-amylase were mixed and run together on the column, and the remaining proteins were run individually. Standards were applied to the column using the same sample volume (250μl sample) and flow rate (2ml/min). 1.6ml fractions were collected from the point of sample application and elution of proteins was detected by absorbance readings at 280nm.

2.10.2.2   Gel filtration of 0.2M anion exchange sample

Pooled 0.2M fractions obtained following anion exchange chromatography of serum at pH7 were concentrated almost 10-fold under pressure using an amicon concentrator, and applied to a Sephacryl S-300 column using the same sample volume (250μl sample) and flow rate (2ml/min) used for blue dextran and protein standards. 1.6ml fractions (up to 80) were collected from the point of sample application and protein concentration determined spectrophotometrically by A280 measurement. Gel filtration fractions containing protein were stored at 4°C for less than 1 week before assessment of phagocytic activity. Fractions were reconstituted with 1mM CaCl$_2$ and normalised for protein content (based upon A280 readings or by Pierce BCA kit microplate procedure; a final concentration of 50μg/ml was used) prior to addition to phagocytosis assays.

2.10.3 *Mass spectrometry*

Gel filtration fractions containing phagocytic activity were resolved by SDS polacrylamide gel electrophoresis (SDS PAGE) (as described in 2.13 SDS polyacrylamide gel electrophoresis) on 12% gels under reducing conditions and the major proteins were identified by tryptic digestion followed by matrix assisted laser desorbtion ionisation (MALDI) and analysis using the protein prophet program (2 separate analyses by Jim Creanor, University of Edinburgh, UK).

2.11   Reduction and alkylation

Fresh autologous serum and high molecular weight (HMW) gel filtration fractions were treated with 5mM dithiothreitol (DTT; prepared at 1M in dH$_2$O) at 37°C for 1 hour at 300 x *g* to break disulfide linkages between cysteine residues of proteins, followed by alkylation with 25mM iodoacetamide (IOA; prepared at 1M in dH$_2$O) at room temperature for 1 hour in the dark to prevent disulphide bonds reforming. Samples were loaded into dialysis tubing and dialysed overnight in 200ml IMDM at 4°C in the dark with continual stirring to remove excess DTT and IOA.

2.12 Protein immunodepletion from human serum

Pooled 0.2M fractions from anion exchange chromatography were incubated with 200µg/ml of rabbit anti-human protein S or mouse anti-human C4BP antibodies for 1 hour on ice. Immunodepletion was achieved by incubation for 1 hour with agarose-coupled goat anti-rabbit IgG (Sigma) or goat anti-mouse IgG (Sigma) at 4°C on a rotator. The serum-agarose mixture was centrifuged at 200 x g for 2min, and the supernatant transferred to a fresh epindorph. Three rounds of depletion were performed to ensure efficient protein depletion from 0.2M fractions. Excess antibody was removed by a final incubation with agarose. Rabbit IgG or mouse IgG added to the same volume of 0.2M fractions was used for mock control depletions. Immunodepleted samples were diluted 1 in 100 with 0.2M NaCl buffer (50mM HEPES, pH7) and proteins resolved by SDS-PAGE using 9% acrylamide gels (see 2.13 SDS polyacrylamide gel electrophoresis) Proteins were transferred electrophoretically onto PVDF membrane and immunoblotted for protein S or C4BP as described in 2.14 Western blotting. See individual figure legends for antibody concentrations used for immunoblotting.

2.13 SDS polyacrylamide gel electrophoresis

Samples were boiled for 5min in 4x sample buffer (125mM Tris pH6.8, 4% v/v SDS, 27.5% v/v glycerol) then resolved by SDS-PAGE typically using 9% acrylamide gels (37.5mM Tris pH 8.8, 0.1% v/v SDS, 0.2% v/v TEMED, 0.04% w/v APS) and 1 x Tris/Glyine running buffer (25mM Tris, 192mM glycine, pH8.3, 0.1% SDS). Gels were run under non-reducing conditions unless otherwise stated, and 2% v/v β-mercaptoethanol was added to sample buffer for reducing conditions. 5µl marker (Invitrogen) was loaded to gels for estimation of protein molecular weight in samples. Protein was detected in resolved gels with Coombassie blue staining (0.5% w/v in 40% v/v ethanol and 10% v/v acetic acid) for 1 hour followed by incubation in destain solution (40% v/v methanol and 10% acetic acid v/v) for up to 4 hours to reduce background staining.

2.14   Western blotting

Protein from unstained gels was transferred electrophoretically (80V, 50 min, 4°C) onto PVDF membrane (Millipore, www.millipore.com) in 25mM Tris, 192mM glycine, pH8.3, 20% v/v methanol transfer buffer. Membranes were blocked overnight at 4°C in 1 x TBS buffer (0.5M Tris, 9% NaCl, pH 7.6) containing 0.1% Tween (TBS/T), then incubated for 1 hour with continual mixing at room temperature with primary and then secondary antibodies prepared in TBS/T. Between incubation steps, membranes were washed three times for 5min each in TBS/T. Detection was performed using enhanced chemilluminescence (GE Healthcare). For antibody concentrations used, see individual figure legends. Total protein content was detected on transferred membranes using colloidal gold stain (Bio-rad; www.bio-rad.com).

2.15   Flow cytometry

MDMφ were cultured for 5 days ± 1μM Dex in 48-well tissue culture plates, washed once in IMDM and detached by incubation in HBSS without divalent cations containing 0.1% BSA and 3mM EDTA (15min at 37°C, then 15min on ice followed by vigorous pipetting to dislodge adherent cells). After washing once with ice-cold HBSS containing 2% FBS, MDMφ (10^5/assay) were pelleted in 96-well U-bottom flexiwell plates by centrifugation at 300 x g for 2min at 4°C. All subsequent incubations were performed on ice to prevent internalisation of antibody. MDMφ were resuspended and incubated for 30min with 10μg/ml anti-Mer or anti-αvβ5 (1:50), 1:50 of an isotype-matched mouse IgG1 as a negative control, or 1:50 anti-CD44 as a positive control, then washed twice in HBSS containing 2% FBS. Binding was detected through incubation for 30min with a FITC-conjugated F(ab')$_2$ goat anti-mouse immunoglobulin (1:50) before analysis using a FACScan flow cytometer. Duplicate wells were used for each experiment, and the experiment was repeated at least three times using different monocyte donors.

2.16   Statistical analysis

Data were analysed using the GraphPad Prism statistical analysis package. Paired tests were chosen as control and treatment groups were obtained from the same donor, and were from a Gaussian distribution. Results were analysed by repeated measures one-way ANOVA with a Bonferroni post test and are presented as mean ± SEM, where n = number of independent experiments using MDMϕ from different donors.

CHAPTER 3: GLUCOCORTICOID AUGMENTATATION OF MACROPHAGE PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS IS SERUM-DEPENDENT

3.1 Introduction

The normal resolution of inflammation requires the coordinated removal of large numbers of extravasated neutrophils from inflamed sites through induction of apoptosis and nonphlogistic removal by macrophages (Savill et al., 1989b, Meagher et al., 1992). However, dysregulation in either apoptosis or phagocytic clearance may result in the accumulation of neutrophils and their potentially injurious contents at inflamed sites, and contribute to the pathogenesis of a number of diverse diseases such as rheumatoid arthritis, COPD and SLE (Haslett et al., 1994, Wipke and Allen, 2001, Ren et al., 2003, Brown et al., 2009). Modulation of the processes involved in physiological clearance of neutrophils from inflamed sites may therefore represent a therapeutic approach to treatment of inflammatory diseases.

NFκB is a pro-inflammatory transcription factor normally held in the cytoplasm in an inactive state by an inhibitory protein from the IκB family, IκBa (Fujihara et al., 2002). Following TNFα treatment, the IκBα subunit is rapidly phosphorylated and ubiquitinated (Fujihara et al., 2002), targeting it for proteolytic degradation by the proteasome, thereby allowing nuclear translocation of NFκB and initiation of gene transcription (Matthews and Hay, 1995, Karin, 1999). In addition to promoting synthesis of pro-inflammatory mediators, NFκB plays a critical role in regulation of neutrophil apoptosis by inducing the synthesis of a pro-survival protein(s) (Ward et al., 1999a, Ward et al., 1999b, Ward et al., 2004) that protects neutrophils against the caspase-dependent pro-apoptotic properties of TNFα (Fujihara et al., 2002). Hence, simultaneous ligation of death receptors (TNFα) along with blocking IκBα degradation and NFκB activation (gliotoxin) induces rapid and synchronous granulocyte apoptosis (Pahl et al., 1996, Ward et al., 1999a, Fujihara et al., 2002). Cyclin-dependent kinases (CDKs), key regulators of cell cycle progression, also

regulate the rate of apoptosis. In a recent study, Rossi *et al* demonstrated that the CDK-inhibitor drug R-roscovitine induced caspase-dependent apoptosis of human neutrophils, and this correlated with a reduction in expression of the anti-apoptotic Bcl-2 family member, Mcl-1 (Rossi et al., 2006).

Despite their undesirable side effects, glucocorticoids are powerful anti-inflammatory drugs often used in the treatment of many inflammatory conditions owing to their capacity to inhibit many processes important in initiation and progression of acute inflammation, including inflammatory cell recruitment and expression of pro-inflammatory molecules, and their ability to manipulate processes important for the resolution of inflammation (Takahira et al., 2001, Schramm and Thorlacius, 2004, Schleimer, 2004, McColl et al., 2007). Synthetic glucocorticoids (methylprednisolone, hydrocortisone or Dex) specifically enhance nonphlogistic macrophage phagocytosis of intact apoptotic neutrophils *in vitro* (Liu et al., 1999), an effect that was associated with altered macrophage morphology, adhesive ability and motility (Giles et al., 2001). Further characterisation revealed that long-term exposure to glucocorticoids (5-days) induced a more homogenous population of smaller, more rounded and less well-spread macrophages with reduced phosphorylation and, hence, recruitment of paxillin and pyk2 to sites of adhesion, and loss of actin- and paxillin-containing podosomes (Giles et al., 2001). Glucocorticoid treatment was also associated with reduced expression of p130cas (Giles et al., 2001), an important adaptor molecule in integrin adhesion signalling through the DOCK180/Crk/p130cas pathway (Hasegawa et al., 1996, O'Neill et al., 2000). Despite their altered adhesion, glucocorticoid-treated macrophages remain highly membrane active through extension and retraction of cellular process, possibly due to high levels of active Rac (Giles et al., 2001). Macrophages exposed to glucocorticoids also display homogeneity in surface receptor expression, including a more uniform expression of HLA-DR, CD14, and CD44 (Giles et al., 2001). Although glucocorticoid-treated macrophages express elevated levels of the haemoglobin scavenger receptor CD163, no single surface receptor was identified in previous studies that could account for the prophagocytic phenotype. A recent study by Ehrchen and coworkers signified the multitide of glucocorticoid action in human

monocytes/macrophages, where glucocorticoids modulated the expression of over 100 genes (Ehrchen et al., 2007). Interestingly, the products of many of these genes are important for monocyte/macrophage functions such as phagocytosis, apoptosis, migration and adhesion. The expression of CD163, FPR1, and Mertk receptors, and MFG-E8 and C1q serum proteins, all known to be associated with apoptotic cell phagocytosis, were upregulated in monocytes exposed to glucocorticoids. However, the relevance of this to the process of glucocorticoid-enhancement of apoptotic neutrophil clearance was unclear.

Despite these advances in understanding the molecular mechanisms associated with acquisition of a pro-phagocytic macrophage phenotype, the receptor mechanism employed by glucocorticoid-treated macrophages for high-capacity clearance of apoptotic neutrophils remains elusive. In this chapter, I describe the characteristics required for augmentation of macrophage phagocytic capacity for apoptotic neutrophils following exposure to glucocorticoids.

3.2 Materials and methods

3.2.1 *Induction of neutrophil apoptosis*

Neutrophils were isolated from peripheral blood as described in Chapter 2, 2.3 Cell isolation. CMFDA-labelled neutrophils were cultured at 4 x 10^6/ml in IMDM ± 10% autologous serum at 37°C in a 5% CO_2 atmosphere for 20-24 hours to render neutrophils apoptotic. Alternatively, apoptosis was actively induced in CMFDA-labelled neutrophil cultures (4 x 10^6/ml) after 4 hour serum-free incubation in IMDM in the absence or presence of either 20μM roscovitine, or 10pg/ml TNFα in combination with 0.1μg/ml gliotoxin. Neutrophil populations were then centrifuged at 200 x g for 5min and resuspended in fresh IMDM to remove treatments prior to assessment of apoptosis and phagocytosis.

3.3   Results

3.3.1   *Glucocorticoids augment macrophage phagocytic capacity for apoptotic*
        neutrophils

Previous studies relating to glucocorticoid-enhanced phagocytosis of apoptotic cells used human MDMϕ and apoptotic neutrophils that had been cultured in the presence of human serum (Liu et al., 1999, Giles et al., 2001). To confirm these observations, human peripheral blood monocytes were cultured *in vitro* for 5 days in the absence or presence of 1μM Dex to generate MDMϕ, human neutrophils were rendered apoptotic following culture in IMDM containing 10% autologous serum for 20-24 hours (see Chapter 2, 2.7 Assessment of neutrophil apoptosis, and Chapter 2, *figs. 2.2a* and *c*) and phagocytosis of <u>serum-cultured</u> apoptotic neutrophils by untreated and Dex-MDMϕ was assessed by flow cytometry (see Chapter 2, 2.8.1 Flow cytometry for characterisation of phagocytosis). Dex treatment was found to significantly increase (over 2-fold) MDMϕ phagocytosis of apoptotic neutrophils, an effect that did not require the addition of autologous serum during the assay (*fig. 3.1a*; *** $p < 0.001$, n=8). Interestingly, when serum-cultured neutrophils were washed once in PBS (without divalent cations) prior to phagocytosis, the ability of Dex-MDMϕ to phagocytose apoptotic neutrophils was diminished (~50% loss in phagocytic activity when compared to untreated control), an effect which could be reversed by addition of 10% autologous serum during the phagocytosis assay (*fig. 3.1b*; * $p < 0.05$, n=5). We were therefore interested in investigating the role of serum in glucocorticoid augmentation of phagocytosis by MDMϕ.
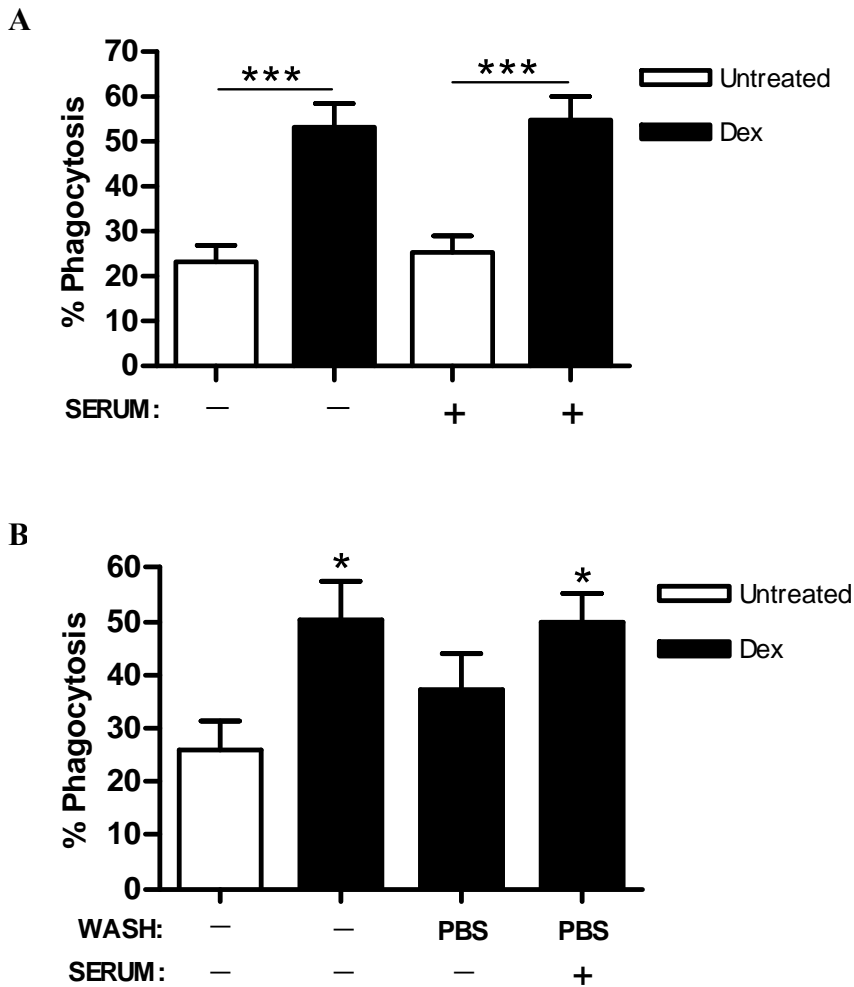
Figure 3.1: Effect of dexamethasone on human MDMφ phagocytosis of serum-cultured apoptotic neutrophils.

Adherent human MDMφ were cultured in the absence or presence of 1μM dexamethasone (Dex) for 5 days, and heterologous neutrophils were cultured in the presence of 10% fresh autologous serum for 20-24 hours. Phagocytosis was measured as the percentage of untreated (white bars) and Dex-treated (black bars) MDMφ that had phagocytosed serum-cultured apoptotic neutrophils in a 30-minute phagocytosis assay. **A**, Dex treatment augments MDMφ phagocytosis of apoptotic neutrophils, and this did not require the presence of autologous serum during the phagocytosis assay. Results presented as mean percent phagocytosis ± SEM for 8 separate experiments. **B**, Dex-enhanced MDMφ phagocytosis of apoptotic neutrophils was suppressed when serum-cultured neutrophils were pre-washed with PBS and could be restored with 10% autologous serum. Mean phagocytosis ± SEM, n=5 where n = the number of different macrophage donors. (*, $p<0.05$; *** $p<0.001$ compared to untreated MDMφ, one-way ANOVA with a Bonferroni post test).

3.3.2 *Glucocorticoid augmentation of macrophage phagocytosis of apoptotic neutrophils is serum-dependent*

To evaluate the potential role of serum opsonisation in promoting apoptotic neutrophil clearance, neutrophils were rendered apoptotic by culture for 20-24 hours in <u>serum-free</u> conditions. Serum-free cultured neutrophils exhibit a similar percentage of annexin V+/propidium iodide- (apoptotic) cells when compared with neutrophils cultured in the presence of 10% autologous serum (see Chapter 2, *figs. 2.2a* and *b*. Serum cultured: 62–72% apoptosis, n=11; serum-free cultured: 63–70% apoptosis, n=35. Data presented as 95% confidence limits). However, there were significantly higher percentages of annexin V+/propidium iodide+ (secondarily necrotic) neutrophils in serum-free cultures (see Chapter 2, *figs. 2.2a* and *b*. Serum cultured: 8–17% necrosis, n=11; serum-free cultured: 18–26% necrosis, n=35). Furthermore, morphological analysis of neutrophil cultures revealed significantly more advanced nuclear pyknosis and cytoplasmic condensation in many of the neutrophils in the serum-free cultures, which mirrored tighter populations of neutrophils observed by flow cytometry (see Chapter 2**,** *figs. 2.2b* and *d*). Thus, neutrophils proceed through the apoptotic process more quickly when cultured in the absence of serum, consistant with the presence of a survival factor in serum.

Phagocytosis of <u>serum-free</u> cultured apoptotic neutrophils by untreated and Dex-MDMϕ was assessed by flow cytometry. Surprisingly, there was no significant augmentation of phagocytosis of serum-free apoptotic neutrophils observed for Dex-MDMϕ, with phagocytosis by Dex-MDMϕ actually slightly lower compared to untreated control (*figs. 3.2a* and *c*; ** $p<0.01$, n = 80) (McColl et al., 2009). The addition of 10% autologous serum to the assay medium revealed increased phagocytic capacity for Dex-MDMϕ (3-fold) (*figs. 3.2b* and *c*; *** $p<0.001$, n=80). The presence of serum also had a small, but statistically significant stimulatory effect upon phagocytosis of apoptotic neutrophils by untreated MDMϕ (*fig. 3.2c*; *** $p<0.001$, n=80). By altering the concentration of serum present during the phagocytosis assay, the effect of serum on Dex-MDMϕ phagocytosis was found to be concentration-dependent and reached significance at 1% (*figs. 3.3a* and *b*). In these experiments, phagocytosis of apoptotic neutrophils by untreated MDMϕ was

not augmented at any concentration of serum (*fig 3.3b*). As the most significant augmentation of Dex-MDMφ phagocytosis was observed using 10% autologousserum, we used this concentration for subsequent experiments.

Although we consistently observed a serum-dependent augmentation in Dex-MDMφ phagocytosis compared to untreated MDMφ for each experiment (*fig. 3.2c*), the inter-donor levels of phagocytosis proved to be highly variable, ranging from 7.4% to 55.9% for untreated MDMφ and from 28.5% to 81.4% for Dex-MDMφ in the presence of 10% autologous serum, and a significant correlation was found between two MDMφ populations (*fig. 3.4,* *** $p < 0.001$, n=80). The "n" value was calculated based upon the number of independent experiments performed using different macrophage donors. However, upon reaching a high n value (n=80, using a pool of ~30 donors) it is highly likely that the cumulative data contained repeat macrophage donors. Therefore, we next sought to compare Dex-MDMφ phagocytic capacity for apoptotic neutrophils obtained from different donors. Significant differences in the phagocytic capacity were observed (donor AR: 49.71%, 61.35% and 72.43% phagocytosis), and did not appear to correlate with the level of necrosis in the phagocytic target population (data not shown). Furthermore, preliminary experiments suggested that phagocytosis was also independent of the proportion of apoptotic cells present in neutrophil cultures, with similar levels of phagocytosis achieved when phagocytic targets were reduced by 50% (2.5×10^6/ml neutrophil targets: 49.8%; 1.25×10^6/ml neutrophil targets: 46.4%. n=2). Although there appears to be a discrepancy in the effect of serum on untreated MDMφ phagocytosis of apoptotic neutrophils (*figs. 2* and *3*), this may be due to the highly variable levels of phagocytosis observed in the presence of serum and random choice of donors within our donor pool for each experiment. Interestingly, in a large series of experiments (n=80), there were a few non-responders to Dex treatment (untreated: 30.9%; Dex 5d: 36.8%, untreated: 32.5%; Dex 5d: 28.5%).
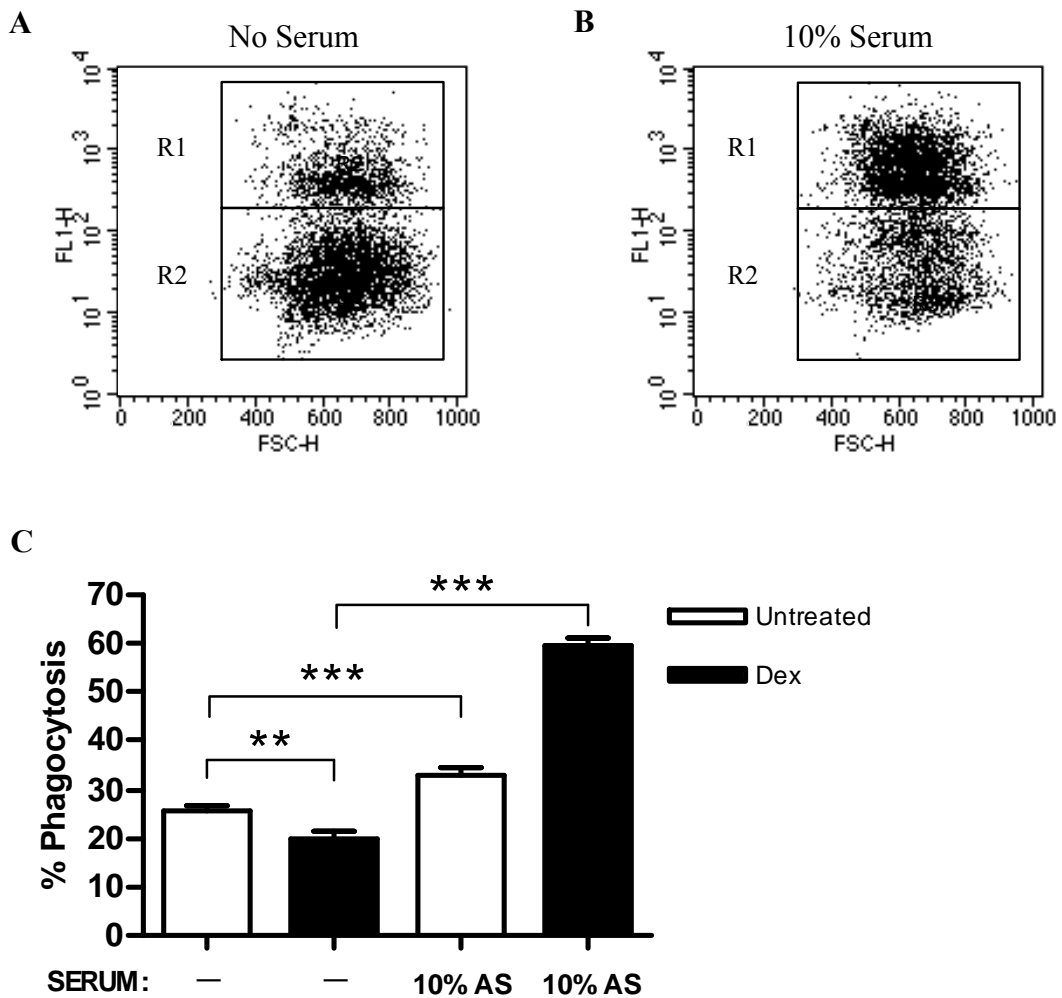
Figure 3.2: <u>Dex-MDMφ phagocytosis of serum-free cultured apoptotic neutrophils is serum-dependent.</u>
MDMφ were cultured in the absence or presence of 1μM Dex for 5 days, and phagocytosis of <u>serum-free cultured</u> heterologous apoptotic neutrophils by untreated (white bars) and Dex-treated (black bars) MDMφ was assessed by flow cytometry. Dot plot showing Dex-MDMφ phagocytosis of <u>serum-free cultured</u> apoptotic neutrophils in the absence (**A**) or presence (**B**) of 10% fresh autologous serum. FL-1 positive MDMφ in gate R1 have phagocytosed CMFDA-labelled apoptotic neutrophils. **C**, Dex-MDMφ phagocytosis of serum-free cultured apoptotic neutrophils required addition of 10% autologous serum (AS) during the phagocytosis assay. Serum also had a small, but statistically significant pro-phagoctyic effect on untreated MDMφ phagocytosis of serum-free cultured apoptotic neutrophils. Mean phagocytosis ± SEM, n=80 where n = the number of phagocytosis assays performing using a pool of ~30 different macrophage donors. (**, $p<0.01$; *** $p<0.001$, one-way ANOVA with a Bonferroni post test).

A

B
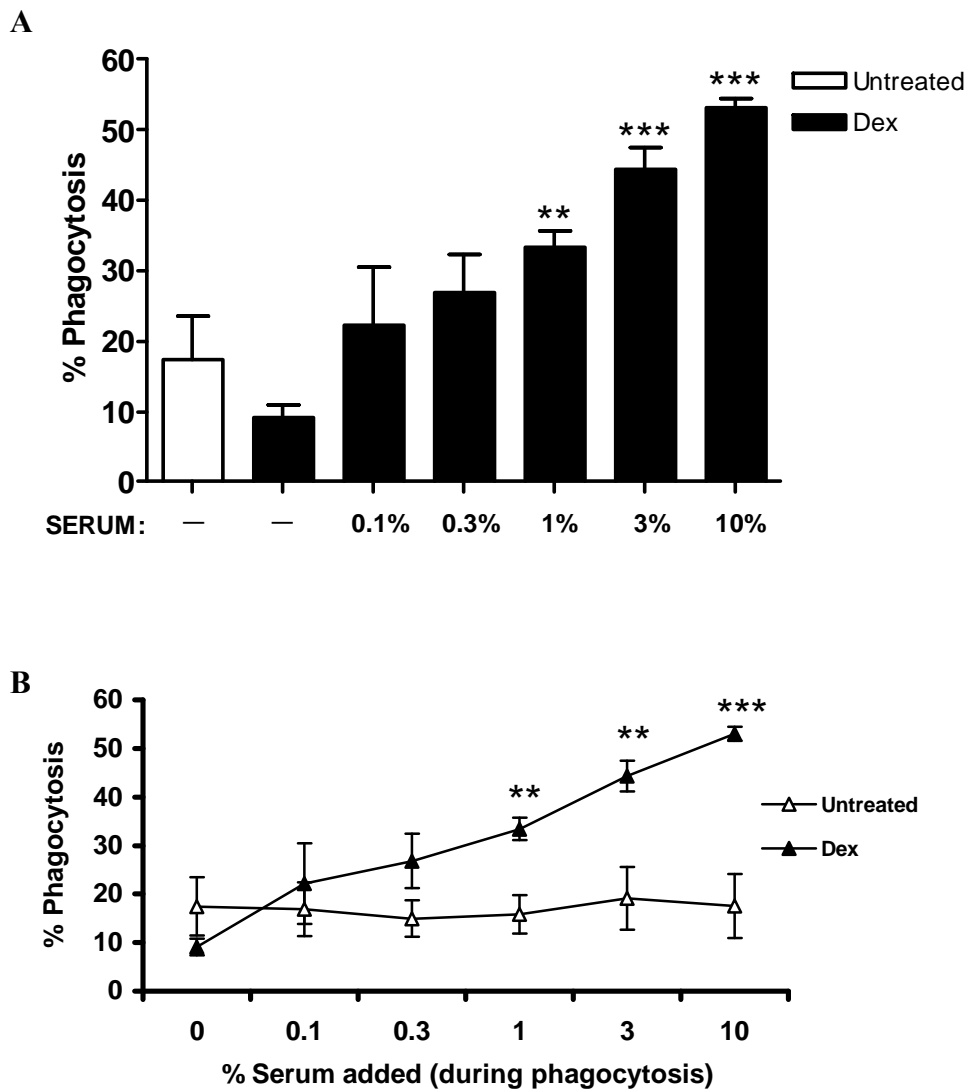
Figure 3.3: Effect of serum serial dilution on Dex-MDMφ phagocytosis of apoptotic neutrophils

Phagocytosis assays were performed as described in Figure 3.2. **A** and **B**, Fresh autologous serum enhanced phagocytosis of serum-free cultured heterologous apoptotic neutrophils by Dex-MDMφ (black bars), but not untreated MDMφ (white bars), in a concentration-dependent manner. Mean phagocytosis ± SEM, n=4 where n = the number of different macrophage donors. (**, $p < 0.01$; *** $p < 0.001$ when compared to uptake by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test).
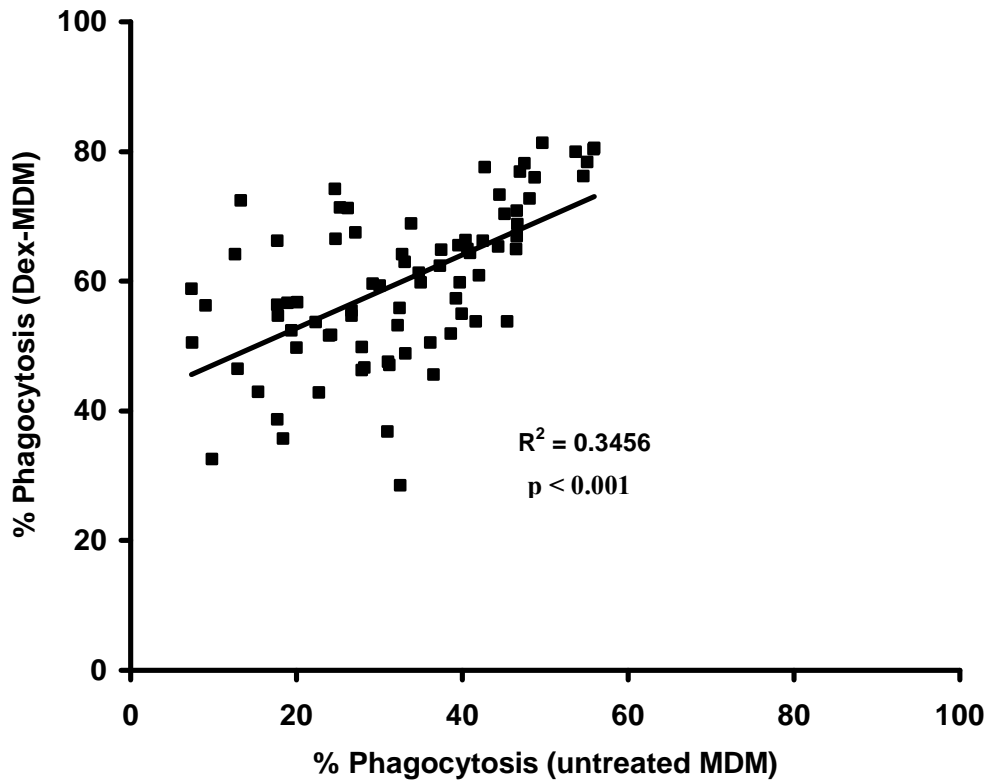
Figure 3.4: Levels of phagocytosis by untreated and Dex-MDMɸ in the presence of serum are highly variable

Phagocytosis assays were performed as described in Figure 3.2. Data distribution of untreated vs Dex-MDMɸ phagocytosis of heterologous apoptotic neutrophils in the presence of 10% fresh autologous serum. Data were analysed using the Pearson correlation test, *** $p < 0.001$, n=80 (where n = the number of phagocytosis assays performing using a pool of ~30 different macrophage donors).

3.3.3 *Effect of sera from different human donors or alternative species on phagocytosis of apoptotic neutrophils by Dex-MDMϕ*

Fresh autologous serum used for phagocytosis assays was derived from the neutrophil donor. This led us to perform a series of experiments to determine if the serum-dependent augmentation of Dex-MDMϕ phagocytosis of apoptotic neutrophils was a donor-specific phenomenon, i.e. required serum obtained from the neutrophil or macrophage donor, or if serum from any human donor could convey this effect. We found that serum enhanced phagocytosis of apoptotic neutrophils by Dex-MDMϕ irrespective of whether the serum was derived from the neutrophil donor (*fig. 3.2c*; *** $p < 0.001$, n=80), macrophage donor (*fig. 3.5a*; *** $p < 0.001$, n=4) or a non-autologous donor (*fig. 3.5b*; *** $p < 0.001$, n=6). Based upon the lack of donor restriction on the serum effect, we were also interested in investigating whether the pro-phagocytic effect was restricted to human serum, or if serum from alternative species could reproduce this enhancing effect on Dex-MDMϕ phagocytosis. Addition of 10% serum (frozen) from mice with a C57BL/6 background during the phagocytosis assay enhanced Dex-MDMϕ phagocytosis to comparable levels observed for 10% autologous serum (*fig. 3.5c*; ** $p < 0.01$, n=3). A similar pro-phagocytic effect was observed with addition of 10% FBS (frozen, and heat-inactivated) (*fig. 3.5d*; *** $p < 0.001$, n=6). This data suggests that a common component(s) in these distinct sera has a pro-phagocytic effect on Dex-MDMϕ. Interestingly, we found that 10% goat serum (frozen) did not confer augmentation of Dex-MDMϕ phagocytosis, and instead had a slight inhibitory effect (*fig 3.5e*; $p > 0.05$, n=5).
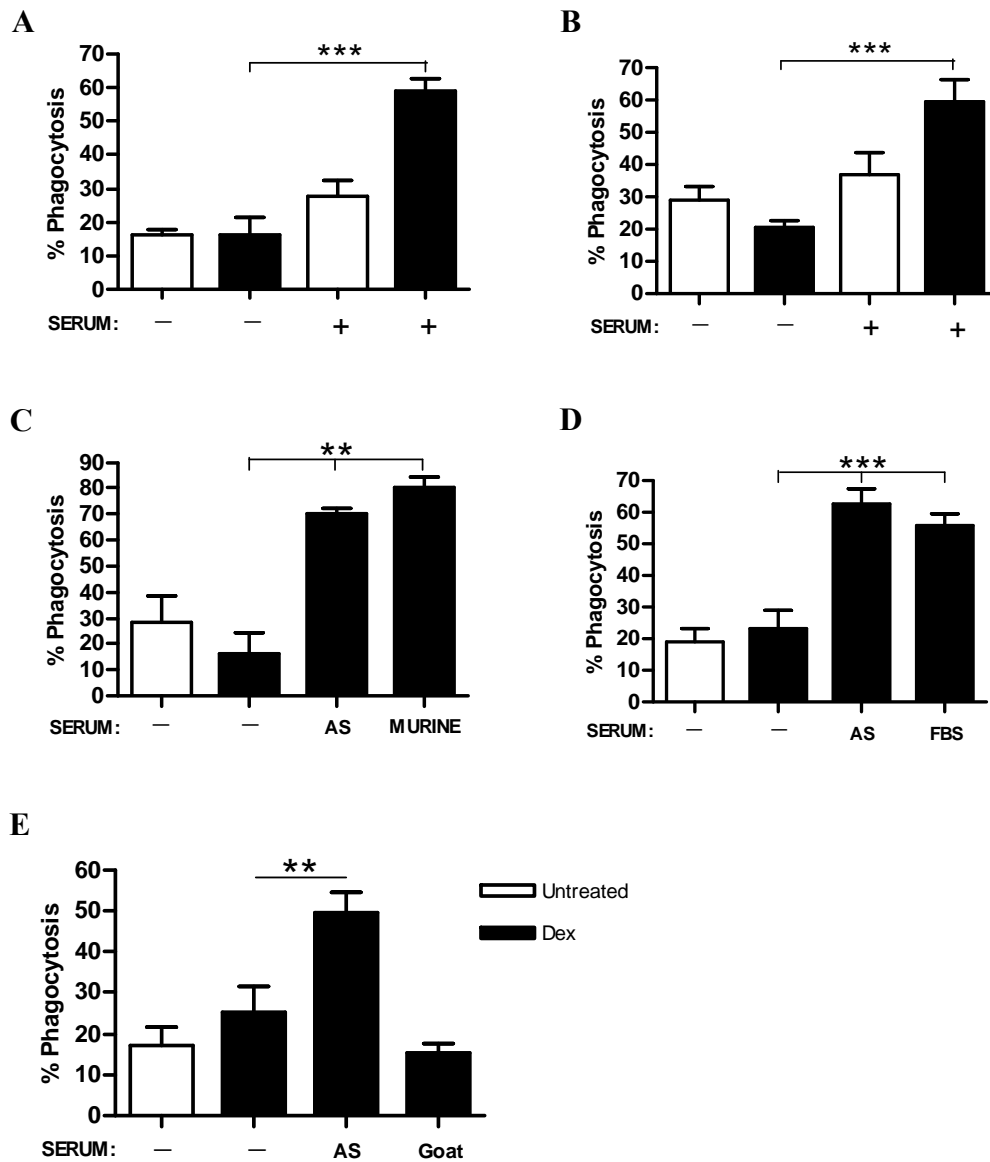
Figure 3.5: Effects of sera from different human donors or from different species on Dex-MDMφ phagocytosis of apoptotic neutrophils.

Phagocytosis assays were performed as described in Figure 3.2. Dex-MDMφ phagocytosis (black bars) of serum-free cultured heterologous apoptotic neutrophils was enhanced in the presence of 10% serum derived from the macrophage donor (fresh) (**A**, n=4) or a non-autologous human donor (fresh) (**B**, n=6), and serum from C57/BL6 mice (frozen) (**C**, n=3), and FBS (frozen, heat-inactivated) (**D**, n=6). In contrast, 10% goat serum (frozen) (**E**, n=6) had a slight inhibitory effect on Dex-MDMφ phagocytosis. Mean phagocytosis ± SEM (**, p<0.01; *** p<0.001 when compared to uptake by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test). Phagocytosis by untreated MDMφ (white bars) is shown for comparison. N = the number of different macrophage donors.

3.3.4 *Glucocorticoids promote swift uptake of apoptotic neutrophils by Dex-MDMφ*

When phagocytosis of <u>serum-free cultured</u> apoptotic neutrophils by Dex-MDMφ was observed in real-time by time-lapse video microscopy (see attached CD for videos 1 to 4), it became evident that phagocytic uptake by Dex-MDMφ was a quick process as several macrophages in the serum-containing chamber had already internalised apoptotic neutrophils in the 10min it took to set up the experiment and start acquisition. Indeed, macrophages treated with Dex are highly active and mobile in the presence of serum, and essentially searching for apoptotic prey though the continual extension and retraction of processes. Time-course analysis of MDMφ phagocytosis by flow cytometry showed that ~40% of Dex-MDMφ phagocytosed apoptotic neutrophils within 10min compared to ~18% of untreated MDMφ, reaching ~65% after 20min compared to ~32% for untreated MDMφ (*fig. 3.6*). Hence, macrophages treated with Dex are highly efficient phagocytes both in terms of phagocytic capacity and swiftness of action.

3.3.5 *Serum may act to promote internalisation of apoptotic neutrophils by Dex-MDMφ*

MDMφ phagocytosis of <u>serum-free cultured</u> apoptotic neutrophils was also assessed by confocal microscopy, as described in Chapter 2, 2.8.3 Confocal microscopy. Preliminary data showed that both untreated and Dex-MDMφ bound a significant number of apoptotic neutrophils in the absence of serum, with only a few MDMφ containing internalised neutrophils (*figs. 3.7a* and *b*). However, the differential phagocytic capacity of untreated and Dex-MDMφ was again evident in the presence of serum. Whilst most neutrophils remained bound to untreated MDMφ with very few internalised (*fig. 3.7c*), almost all Dex-MDMφ had internalised bound apoptotic neutrophils (*fig. 3.7d*), and the presence of more than one apoptotic neutrophil in Dex-MDMφ indicated the efficiency of this process. Our data suggests that serum acts to promote internalisation of bound neutrophils by Dex-MDMφ.
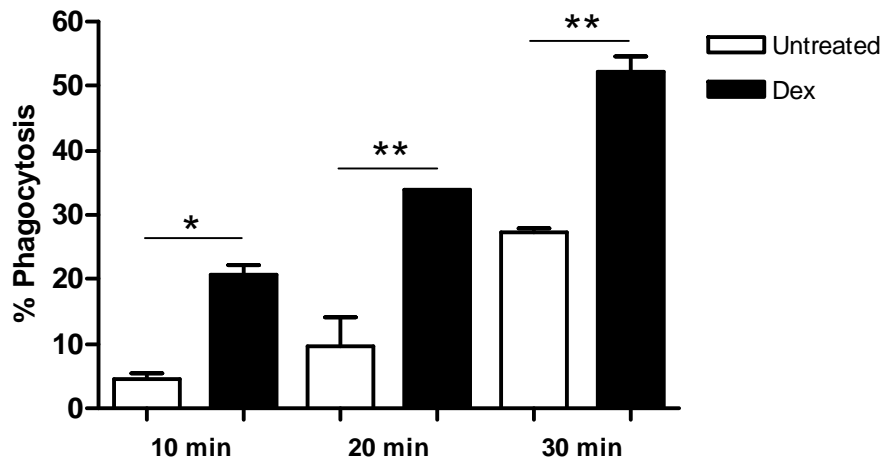
Figure 3.6: Time-course analysis of serum-dependent phagocytosis of apoptotic neutrophils by Dex-MDMφ.

MDMφ were cultured in the absence or presence of 1μM Dex for 5 days, and phagocytosis of serum-free cultured heterologous apoptotic neutrophils by untreated (white bars) and Dex-treated (black bars) MDMφ was assessed at 10min intervals by flow cytometry. 10% fresh autologous serum enhanced Dex-MDMφ phagocytosis of apoptotic neutrophils compared to untreated MDMφ at every time point. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (*, $p < 0.05$; **, $p < 0.01$ when compared to uptake by untreated MDMφ, one-way ANOVA with a Bonferroni post test).
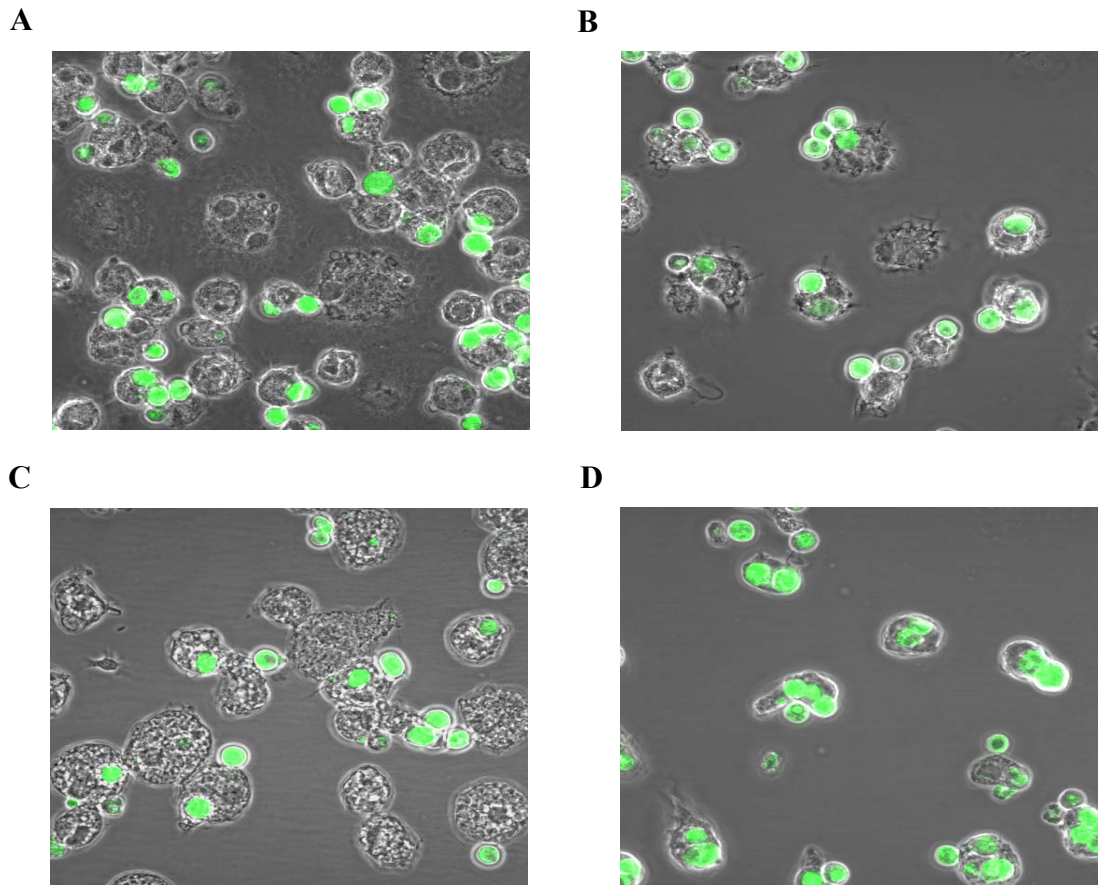
Figure 3.7: Analysis of Dex-MDMϕ phagocytosis of apoptotic neutrophils by confocal microscopy.

MDMϕ were cultured in the absence or presence of 1μM Dex for 5 days, and phagocytosis of serum-free cultured heterologous apoptotic neutrophils by untreated (panels **A** and **C**) and Dex-treated MDMϕ (panels **B** and **D**) was visualised by confocal microscopy. The representative micrographs (n=2) illustrate that 10% fresh autologous serum promotes internalisation of apoptotic neutrophils by Dex-MDMϕ (**D**), but not untreated MDMϕ (**C**). N = the number of different macrophage donors.

3.3.6 Glucocorticoid augmentation of macrophage phagocytosis requires serum opsonisation of apoptotic neutrophils

We next sought to investigate whether serum-enhanced phagocytosis by Dex-MDMφ required opsonisation of apoptotic neutrophils with a serum component(s). For this, serum-free cultured apoptotic neutrophils were pre-incubated in the absence or presence of 10% autologous serum for 1 hour, then centrifuged at 200 x g for 5min and resuspended in IMDM before assessment of MDMφ phagocytosis by flow cytometry. Maximal Dex-MDMφ phagocytic capacity was found to require pre-incubation of apoptotic neutrophils with serum (no serum: 24.7%; 10% serum 1 hour pre-incubation: 68.5%. *** $p<0.001$, n=10) (*fig. 3.8a*), generating equivalent levels of Dex-MDMφ phagocytosis to those observed when serum was added during the phagocytosis assay (no serum: 19.9%; 10% serum during: 59.9%. *** $p<0.001$, n=80) (*fig. 3.2c*). These data excluded the possibility that serum acts to directly regulate the phagocytic activity of macrophages, instead suggesting that enhanced phagocytosis by Dex-MDMφ required binding of a serum component(s) to apoptotic neutrophils. Interestingly, when apoptotic neutrophils pre-incubated with serum were washed in IMDM containing 5mM EDTA prior to phagocytosis, the phagocytic effect was significantly reduced, suggesting that a calcium-dependent serum opsonin was required (*fig. 3.8b*; *** $p<0.001$, n=3). However, EDTA did not completely reduce phagocytosis to untreated control levels. Furthermore, a slight decrease in Dex-MDMφ phagocytosis was observed when apoptotic neutrophils were pre-incubated in serum and subsequently washed in calcium-containing medium (IMDM) (*fig. 3.8b*; $p>0.05$, n=3), indicating that serum may also have a calcium-independent phagocytic effect.
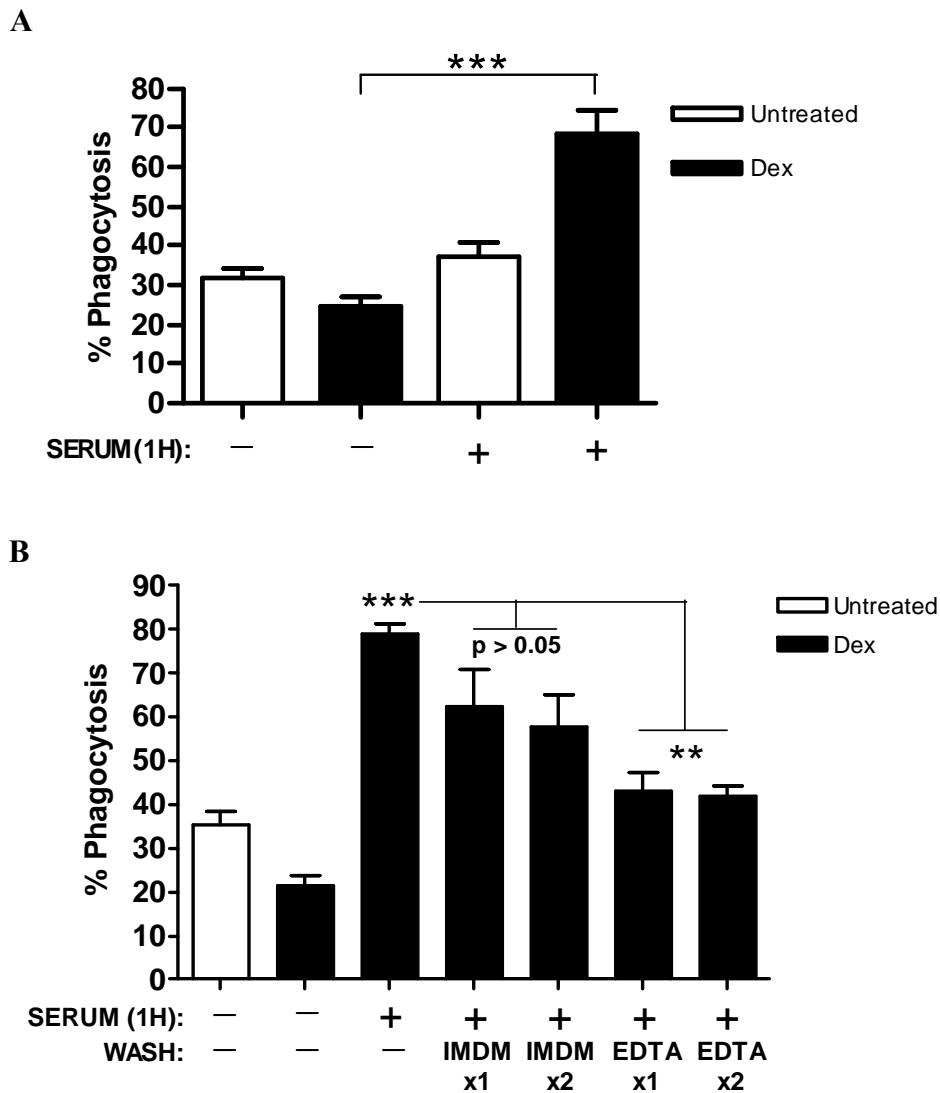
Figure 3.8: Phagocytosis of apoptotic neutrophils by Dex-MDMφ requires a serum opsonisation event.

MDMφ were cultured in the absence or presence of 1μM Dex for 5 days. Serum-free cultured heterologous apoptotic neutrophils were pre-incubated in the absence or presence of 10% fresh autologous serum for 1 hour prior to assessment of phagocytosis by untreated (white bars) and Dex-treated (black bars) MDMφ by flow cytometry. Serum preincubation of apoptotic neutrophils enhanced subsequent uptake by Dex-MDMφ (**A**, n=10), an effect that was significantly reduced by washing serum-preincubated apoptotic neutrophils with EDTA-containing medium prior to phagocytosis (**B**, n=3). Mean phagocytosis ± SEM (*** $p<0.001$ when compared to uptake by Dex-MDMφ in the absence of serum, ** $p<0.01$ when compared to uptake by Dex-MDMφ in the presence of autologous serum, one-way ANOVA with a Bonferroni post test). N = the number of different macrophage donors.

3.3.7 *Glucocorticoids augment phagocytosis of 'early' apoptotic neutrophils by macrophages*

We next carried out a temporal analysis of neutrophil apoptosis during serum-free culture and subsequent phagocytosis of these neutrophil cultures by Dex-MDMϕ. Preliminary evidence using freshly isolated neutrophils showed that there was almost negligible cell death in these cultures (<1% annexin V+ cells, n=2), and fresh neutrophils were not phagocytosed by Dex-MDMϕ (no serum: 4.8%; 10% serum: 3.78%, n=2), in support of previous studies that human MDMϕ phagocytosis requires apoptosis-related changes in the neutrophils (Savill et al., 1989b). 4-hour serum-free neutrophil cultures contained some apoptotic neutrophils (32–43% annexin V+/propidium iodide-, n=3. Results presented as 95% confidence limits), with little evidence of necrosis (1–3% annexin V+/propidium iodide+, n=3), and a significant percentage of Dex-MDMϕ phagocytosed apoptotic neutrophils obtained from 4-hour cultures (*fig. 3.9a*; * p<0.05, n=3). Maximal Dex-MDMϕ phagocytosis was observed when neutrophils populations were cultured in serum-free conditions for 12 hours (*fig. 3.9a*; ** p<0.01, n=3). 12-hour neutrophil cultures demonstrated higher levels of apoptosis (40-44% annexin V+/propidium iodide-, n=3), and very low levels of necrosis (<3% annexin V+/propidium iodide+, n=3). Importantly, Dex-MDMϕ ingested 12-hour cultured neutrophils to a comparable rate observed for 20-hour neutrophil cultures (** p<0.01, n=3), a time-point where levels of apoptosis are higher (63–70%, n=35), and a significant proportion of neutrophils have become secondarily necrotic (18–26%, n=35).

Neutrophils undergo apoptosis in a relatively heterogeneous manner during serum-free culture *in vitro* (see Chapter 2, *fig. 2.2b*), making it difficult to determine whether enhanced phagocytosis following opsonisation with serum depends upon the stage of cell death. To overcome the issue of heterogeneity of phagocytic target populations, neutrophil cell death was actively induced by culture for 4 hours in serum-free conditions in the absence or presence of either 20μM roscovitine, or 10pg/ml TNFα in combination with 0.1μg/ml gliotoxin. After 4 hours in serum-free culture without treatment, there was a small proportion of apoptotic cells present

(32–43% annexin V+/propidium iodide-, n=3) with negligible secondarily necrosis (1–3% annexin V+/propidium iodide+, n=3) (*fig. 3.9b*). Neutrophil cultures treated with roscovitine exhibit a high percentage of apotosis (80–90% annexin V+/propidium iodide-, n=3) with little evidence of secondary necrosis (1–3% annexin V+/propidium iodide+, n=3). Similarly, treatment with TNFα and gliotoxin generated high levels of apoptosis (79–91% annexin V+/propidium iodide-, n=3) with a slightly higher level of secondary necrosis (1–7% annexin V+/propidium iodide+, n=3). Thus, the target cells for use in phagocytosis assays will be at an early stage of apoptosis and allow determination of the effect of serum on uptake of apoptotic neutrophils in the absence of secondary necrosis.

When untreated 4-hour serum-free cultured neutrophils were used as targets for phagocytosis, there was a small but statistically significant enhancement of Dex-MDMϕ phagocytosis in the presence of serum (*fig. 3.9c*; * p<0.05, n=3). However, a significant serum-dependent augmentation of Dex-MDMϕ phagocytosis was observed for neutrophil cultures induced to undergo apoptosis by treatment with roscovitine or TNFα/gliotoxin (*fig. 3.9c*; *** p<0.001, n=3). These data suggest that serum promotes internalisation of "early" apoptotic neutrophils by Dex-MDMϕ and the presence of secondarily necrotic cells was not necessary for the serum effect.

A

% Phagocytosis (y-axis 0–70)

* ** **
Untreated / Dex

4h neutrophils 12h neutrophils 20h neutrophils

B

Untreated (4h)	TNFα + Gliotoxin (4h)	Roscovitine (4h)

Viable	56 – 65%
Apoptotic	32 – 43%
Necrotic	1 – 3%

Viable	2 – 19%
Apoptotic	79 – 91%
Necrotic	1 – 7%

Viable	2 – 15%
Apoptotic	80 – 90%
Necrotic	1 – 3%

C

% Phagocytosis (y-axis 0–80)

Serum-free   TNF/Gliotoxin   Roscovitine   Untreated / Dex

*   ***   ***
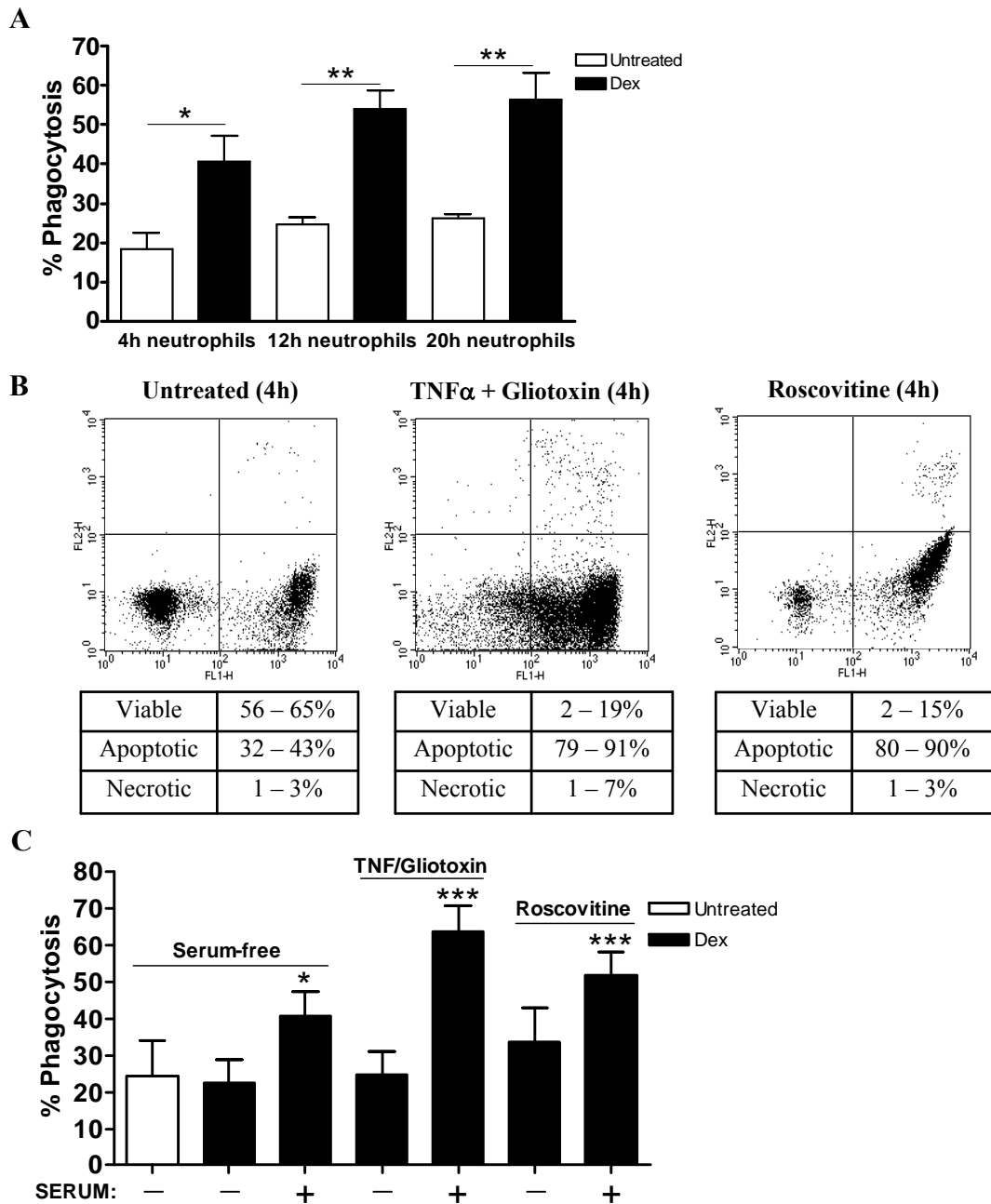
SERUM: — — + — + — +

Figure 3.9: Effect of serum on Dex-MDMφ phagocytosis of "early" apoptotic neutrophils

MDMφ were cultured in the absence or presence of 1μM Dex for 5 days. **A**, The effect of 10% fresh autologous serum on MDMφ phagocytosis of serum-free cultured heterologous neutrophils (4h, 12h or 20h cultures) was assessed by flow cytometry. **B** and **C**, Neutrophils were untreated or treated with either 20μM roscovitine, or 10pg/ml TNFα plus 0.1μg/ml gliotoxin for 4h in serum-free medium. Neutrophil viability was determined by annexin V-FITC binding on FL-1 and propidium iodide staining on FL-2 (**B**) and MDMφ phagocytosis of 4h apoptotic neutrophils was assessed by flow cytometry (**C**). Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (*, $p<0.05$; **, $p<0.01$; *** $p<0.001$, one-way ANOVA with a Bonferroni post test).

3.4   Discussion

3.4.1   The role of serum in MDMφ phagocytosis of apoptotic neutrophils

The environmental cues monocytes are exposed to during maturation has a profound influence on their phenotype and hence their capacity for phagocytosis. Under our standard culture conditions (IMDM containing 10% autologous serum), 5-day cultured untreated MDMφ were composed of a heterogeneous population of cells with different morphologies: multinucleated giant cells (MNGCs), spread cells that showed evidence of membrane ruffling, and more rounded cells that were weakly adherent (Giles et al., 2001). The morphological appearance of untreated MDMφ correlates with their phagocytic capability, where large multinucleated MDMφ were poorly phagocytic compared to efficient phagocytosis by smaller, more rounded macrophages (Heasman et al., 2004). Hence, the morphological and functional heterogeneity of MDMφ preparations may represent a complication when assessing the capacity for phagocytosis of apoptotic neutrophils and the role of serum in this process. Indeed, data obtained from studies of "serum-free" *in vitro* phagocytosis assays using such untreated MDMφ as phagocytes and serum-cultured neutrophils as apoptotic targets (Newman et al., 1982, Savill et al., 1989b) suggested that the presence of different concentrations of serum (0-75%) in the culture medium had no effect on the subsequent phagocytosis of apoptotic neutrophils by untreated MDMφ (Newman et al., 1982). However, Newman and colleagues assessed phagocytosis using 4-hour neutrophil cultures instead of the 20-hour cultures used in my study, therefore it is possible that maximal phagocytosis by untreated MDMφ was reached irrespective of serum. One possibility is that at this early time point of apoptosis, neutrophils may not have undergone the surface changes required for binding of serum opsonins that promote clearance by untreated MDMφ. Based upon these early reports, it has been widely assumed that serum is not required for apoptotic cell clearance. However, other studies have reported a serum requirement for untreated human MDMφ phagocytosis of apoptotic cells *in vitro* (Mevorach et al., 1998a, Bijl et al., 2003, Kask et al., 2004).

The *in vivo* importance of apoptotic cell clearance is well illustrated in the case of systemic lupus erythematosus (SLE), a systemic chronic inflammatory disease characterised by high titres of autoantibodies against nuclear constituents and deposition of immune complexes in kidney glomeruli (Haas, 1994, Reichlin, 1994) that may result from impaired clearance of apoptotic cells by macrophages before lysis and divulgence of cellular contents (Herrmann et al., 1998, Baumann et al., 2002, Roos et al., 2004, Tas et al., 2006, Gaipl et al., 2007). Bijl *et al* reported that reduced uptake of apoptotic Jurkat cells by untreated MDMϕ from human SLE patients was not an intrinsic macrophage defect, but was serum-dependent and could be reversed by addition of normal human serum (Bijl et al., 2006). This observation raised the possibility that the phagocytic capacity of MDMϕ is not entirely dependent on the inherent capability of macrophages, but in certain conditions serum components may also play an important role. The contrasting reports on the role of serum in apoptotic cell clearance by untreated MDMϕ may be a consequence of differences in methodology, semantics for definition of apoptosis/necrosis, the cell types used as apoptotic targets or the nature of the phagocyte population, and the conditions used for culture of phagocytic targets. Furthermore, it is unclear whether the "phagocytosis" signal in some of these studies may be a consequence of a failure to distinguish tethering of apoptotic cells from internalisation (Mevorach et al., 1998a). Using a carefully validated *in vitro* phagocytosis assay (Jersmann et al., 2003), we have shown that phagocytosis of <u>serum-cultured</u> neutrophils by untreated MDMϕ occurred at an equivalent level in the absence or presence of serum during the phagocytosis assay. However, serum had a small (<8%) but statistically significant effect on untreated MDMϕ phagocytosis of <u>serum-free cultured</u> neutrophils. When performing the EDTA washing experiment, we found that pre-incubation of serum-free cultured neutrophils with autologous serum for 1 hour prior to phagocytosis resulted in a slight increase in uptake by untreated MDMϕ, an effect that could be reversed by washing in EDTA-containing medium, but not calcium-containing medium (IMDM) (no serum: 35.4%; 10% serum (1h): 44%; 10% serum (1h) EDTA wash: 35.7%; 10% serum (1h) IMDM wash: 42.5%). The "serum-free" phagocytosis assays performed by others used human neutrophils and MDMϕ cultured in <u>serum-containing</u> medium, and both cell types were washed in calcium-

containing HBSS before assessment of phagocytosis in the absence of added serum (Savill et al., 1989a, Savill et al., 1989b, Savill et al., 1992). Thus, previous studies may have missed a role for supplementary serum.

3.4.2 Glucocorticoid augmentation of macrophage phagocytosis of apoptotic neutrophils is serum-dependent

The effects of glucocorticoids on apoptotic cell clearance was first described by Liu *et al*, who demonstrated that short term exposure (final 24 hours) of 5-day human or murine MDMφ to the synthetic glucocorticoids methylprednisolone, Dex and hydrocortisone, increased phagocytosis of apoptotic neutrophils (Liu et al., 1999). Giles *et al* expanded this observation by showing Dex to enhance phagocytosis in a time-dependent manner, with the greatest effect observed following prolonged exposure (5 days) during *in vitro* culture (Giles et al., 2001). In order to investigate the mechanism of glucocorticoid-augmented phagocytosis, we employed an *in vitro* model using primary human MDMφ as phagocytes and neutrophils as apoptotic prey. This system may reflect *in vivo* conditions following an acute inflammatory event, where large numbers of extravasated neutrophils must be efficiently removed by undergoing apoptosis and ingestion by macrophages. Indeed, monocytes differentiating *in vitro* acquire many of the characteristics of mature macrophages (Musson et al., 1980, Newman et al., 1980). An advantage of using neutrophils as apoptotic targets is that they can isolated from peripheral blood in large numbers and easily separated from other leukocytes, providing nearly pure cell suspensions (routinely >95% purity). By necessity of experimental design, these studies were performed with neutrophils and MDMφ obtained from different donors, i.e. allogeneic phagocytosis. However, my own work and previously published data suggest that this does not affect MDMφ phagocytic potential.

In this chapter, I demonstrated that enhanced phagocytosis by human MDMφ following long-term (5 days) exposure to 1μM Dex involved a serum-dependent mechanism. The influence of serum may have been overlooked in previous studies where neutrophils were rendered apoptotic following *in vitro* culture in serum-

containing media (Liu et al., 1999, Giles et al., 2001). In previous studies, MDMφ and apoptotic neutrophils were washed out of serum-containing medium before assessment of phagocytosis in the "absence" of serum. Based upon our findings, the presence of autologous serum during apoptosis induction was sufficient to confer augmentation of Dex-MDMφ phagocytosis, and no further enhancement was achieved following addition of serum during the phagocytosis assay. The precise role of serum in modulating the phagocytic process was only uncovered when apoptotic neutrophils were prepared in the absence of serum. When used in serum-free phagocytosis assays, <u>serum-free cultured</u> neutrophils were ingested by untreated and Dex-MDMφ at an equivalent rate. For Dex-MDMφ, this may represent the basal level of phagocytosis that is serum-independent. The addition of serum to serum-free neutrophil cultures immediately before phagocytosis restored Dex-MDMφ phagocytic capacity in a concentration-dependent manner, finally reaching levels observed for serum-cultured neutrophils at 10% serum. Similarly, Cortes-Hernandez *et al* demonstrated that Dex (200nM, 24h pre-treatment) significantly increased murine mesangial cell phagocytosis (2.5 fold) of apoptotic neutrophils in a serum-dependent manner (Cortes-Hernandez et al., 2002). A comparison of the effects of different sera revealed that the serum effect was not restricted to autologous serum, i.e. serum prepared from PRP obtained from the macrophage or neutrophil donor, as non-autologous human serum or serum derived from alternative species, including mouse and FBS, were also capable of enhancing Dex-MDMφ phagocytosis. An exception was found with goat serum, which surprisingly conferred a slight inhibitory effect on Dex-MDMφ phagocytosis, an effect that may be a consequence of the presence of thimerosal preservative (0.01%) in goat serum that has been shown to negatively affect the phagocytic potential of macrophages (Rampersad et al., 2005).

3.4.3   Glucocorticoid-treated macrophages are highly efficient phagocytes in terms of phagocytic capacity and swiftness of action

Preliminary experiments suggested that acquisition of MDMφ phagocytic capacity for apoptotic neutrophils was accelerated following exposure to Dex, with Dex-

MDMφ displaying enhanced levels of phagocytosis compared to untreated MDMφ as early as day 3 following isolation (data not shown). Temporal analysis of apoptotic neutrophil ingestion by 5-day Dex-MDMφ revealed serum-dependent uptake to be a rapid process, as ~40 and ~65% of Dex-MDMφ had internalised apoptotic neutrophils after only 10 and 20min, respectively, compared to ~18% and ~32% of untreated MDMφ at these time points. Although the percentage of untreated MDMφ that had internalised apoptotic neutrophils increased considerably after 30min of co-incubation, we found that phagocytosis by Dex-MDMφ was consistently more than two-fold higher at this time point. Glucocorticoids also improve phagocytic efficiency by increasing the capacity of individual MDMφ to ingest multiple apoptotic neutrophils, and by promoting removal of alternative apoptotic targets like eosinophils and Jurkat T cells (Liu et al., 1999, Giles et al., 2001). Cortes-Hernandez *et al* confirmed this using a murine model, where Dex-treated mesangial cells were efficient phagocytes irrespective of lineage of apoptotic cells used (Cortes-Hernandez et al., 2002).

Previous work in our lab gave an insight into the intracellular molecular events associated with glucocorticoid-induction of a highly phagocytic macrophage phenotype (Giles et al., 2001). Despite their rounded appearance, scanning electron microscopy revealed Dex-MDMφ to form attachments with tissue culture plastic, however there was decreased cell spreading when compared with untreated MDMφ (Giles et al., 2001). Altered adhesion status was also observed by Ehrchen *et al* following short-term treatment (24 hours) of human monocytes with 10nM of the glucocorticoid, fluticasone propionate (Ehrchen et al., 2007). Interestingly, expression of surface molecules involved in cell adhesion, including CD44, CD11a, CD36 and ficolin, were downregulated following glucocorticoid treatment (Giles et al., 2001, Ehrchen et al., 2007). Furthermore, Dex-MDMφ display ruffled membranes and filopodial processes (Giles et al., 2001). These distinct morphological changes were associated with alteration of signalling complexes that play an essential role in adhesion processes, and have also been strongly implicated in regulation of MDMφ phagocytic capability. This link between regulation of

cytoskeletal machinery and phagocytic capacity has also been demonstrated upon ligation of CD44 (Hart et al., 1997) or adhesion to fibronectin (McCutcheon et al., 1998), both of which significantly increased apoptotic neutrophil uptake by human untreated MDMϕ. In contrast, elevation of cAMP using dibutryl-cAMP has been shown to disrupt cytoskeletal and adhesion contacts and inhibit phagocytosis of apoptotic neutrophils (Rossi et al., 1998).

Despite these marked changes in cytoskeletal organisation and phosphorylation induced by Dex treatment, time-lapse video microscopy analysis of *in vitro* phagocytosis assays by Giles *et al* suggested that Dex-MDMϕ were extremely membrane active through rapid extension and retraction of cellular processes sufficient for phagocytosis of serum-cultured neutrophils, a finding that correlated with increased levels of active Rac in these cells (Giles et al., 2001). Efficient clearance of apoptotic neutrophils *in vivo* is likely to be highly dependent on cell motility as it is unlikely that phagocytic macrophages would be in the immediate vicinity of apoptotic neutrophils. Indeed, our examination of time-lapse video sequences revealed that Dex-MDMϕ continually contacted adjacent cells by sending out and retracting processes, and it is tempting to speculate that this allowed Dex-MDMϕ to detect signs of apoptosis. Once identified, apoptotic neutrophils were internalised swiftly. This active search for apoptotic prey required the presence of autologous serum, and may contribute to the swiftness of serum-dependent clearance by Dex-MDMϕ. In contrast to our findings, time-lapse microscopy analysis of *in vitro* phagocytosis assays by Giles *et al* suggested that Dex-MDMϕ did not appear actively motile during a 20-minute time-lapse sequence (Katherine Giles, unpublished data). It is important to mention that there were differences in the experimental methods used for time-lapse video microscopy in this study compared to that used in previous studies. Giles *et al* used serum-cultured neutrophils that were washed once prior to phagocytosis in the absence of serum. Based upon my data, I would predict that this was sufficient to trigger apoptotic neutrophil ingestion due to the presence of serum opsonins on the apoptotic sufarce. In contrast, we cultured neutrophils in the absence of serum and supplemented with autologous serum immediately prior to assessment of phagocytosis. Therefore, it is possible that the

motility observed in our Dex-MDMφ cultures is an additional serum-dependent process that is independent of the serum opsonisation phenomenon.

As well as enhanced motility in the presence of autologous serum, many Dex-MDMφ appeared more adherent and polarised in nature compared to the more rounded appearance of Dex-MDMφ in the absence of serum, possibly due to enhanced Rac activity in these cells. Intriguingly, a few of the Dex-MDMφ appeared to chase apoptotic neutrophils until they were bound and internalised. Interestingly, real-time analysis of Dex-MDMφ "wound" assays, performed by wounding MDMφ monolayers with a pipette tip followed by imaging of MDMφ migration into the wound, indicated that Dex-MDMφ had altered migratory potential (Sarah Heasman, unpublished data). When compared to untreated MDMφ, a polarised population of cells that showed formation of large lamellipodia and directed migration, Dex-MDMφ migrated much faster and randomly extended and retracted membrane processes. However, in the intitial 3 hours of imaging, Dex-MDMφ displayed occasional formation of lamellipodia, suggesting that these cells had the potential for directed migration. The capacity of Dex-MDMφ for directed migration could be tested experimentally by performing transwell assays to examine the migratory potential of Dex-MDMφ added to Boyden chambers in the absence or presence of 10% autologous serum, towards serum-free apoptotic neutrophil-conditioned medium or towards a defined macrophage chemoattractant like MCP-1. Interestingly, exposure to Dex may result in decreased phosphorylation and hence activity of extracellular signal-related kinase (ERK) (Katherine Giles, unpublished data), a signalling molecule thought to be important for directed migration (Klemke et al., 1998, Cheresh et al., 1999). ERK activity can be stimulated by serum factors, including the 'find me' signal lysophosphatidylcholine (Dikic et al., 1996, Lauber et al., 2003), however ERK activity in Dex-MDMφ was not recovered by serum replenishment (Katherine Giles, unpublished data). Intriguingly, glucocorticoid-treated monocytes display enhanced capacity for spontaneous migration (Ehrchen et al., 2007). Furthermore, microarray analysis and real-time PCR analysis of glucocorticoid-treated monocytes revealed increased expression of formyl peptide

receptor (FPR), the receptor for the potent chemoattractant fMLP (Ehrchen et al., 2007). Upregulation of FPR at the protein level was confirmed by flow cytometry and correlated with increased chemotactic capacity of glucocorticoid-treated macrophages toward fMLP. Moreover, glucocorticoid treatment upregulated expression of intracellular signalling molecules involved in cell motility and chemotaxis of monocytes, including Traf2 and NCK interacting kinase and metastasis suppressor (Ehrchen et al., 2007). Despite these advances in understanding glucocorticoid effects on monocyte/macrophage motility, no study has evaluated the role of serum in this process. Hence, further experiments are required to investigate the serum effect on Dex-MDMφ motility and whether this is important for the augmented phagocytic capability of these cells.

3.4.4 Glucocorticoids augment phagocytosis of 'early' apoptotic neutrophils by macrophages

One of the problems with biochemical analysis of neutrophil apoptosis is the relatively heterogeneous manner in which neutrophils enter apoptosis during *in vitro* culture. Differences in the methodology used for inducing cell death in phagocytic targets may influence the extent of necrosis and/or opsonisation with serum components and thus directly affect the molecular pathways that are engaged in phagocytosis. It has been previously shown that neutrophils can be easily maintained in serum-free media (Payne et al., 1994). However, we found that serum-free cultured neutrophils progressed through the apoptotic process quicker than their serum-cultured counterparts, as evident by a slightly higher proportion of secondarily necrotic cells, consistent with the presence of a survival factor(s) in serum. Serum-free culture also had a profound influence on the biochemical status of neutrophils, with surface expression of CD16, CD62L, CD11b and CD32 receptors significantly reduced after 20-24 hour culture in serum-free medium compared to neutrophil popuations maintained in serum-containing conditions (Stelios Bournazos, personal communication). The presence of a significant proportion of annexin V+/propidium iodide+ (secondarily necrotic) neutrophils within the phagocytic target population after induction of cell death in serum-free conditions may influence the effect of

serum on phagocytosis. In support of previous studies, freshly isolated neutrophils were not phagocytosed by Dex-MDMφ (Savill et al., 1989b). Instead, phagocytosis by Dex-MDMφ required neutrophils to undergo apoptotic-related processes during *in vitro* culture for at least 4 hours. Maximal Dex-MDMφ phagocytosis was observed with neutrophils cultured for 12 hours, where the proportion of apoptotic cells was much higher. Interestingly, a similar level of Dex-MDMφ were capable of serum-dependent phagocytosis of 20-hour cultured neutrophils, despite the presence of significantly high levels of secondarily necrotic neutrophils, suggesting that post-apoptotic changes were not required for the serum-enhancing effect.

To overcome the issue of heterogeneity of neutrophil targets, we used the pharmacological methods described in Chapter 3, 3.1 Introduction to actively induce neutrophil cell death and allow investigation of the role of serum in Dex-MDMφ phagocytosis of apoptotic neutrophils in the absence of necrosis. We used a 4-hour treatment period since this was reported to be optimal both for synergistic effects of TNFα and gliotoxin and for roscovitine, and we found that these treatments induced apoptosis in serum-free neutrophil cultures with little sign of secondary necrosis. These experiments showed that Dex-MDMφ still significantly ingested these apoptotic targets, indicating that serum promotes ingestion of "early" apoptotic neutrophils by Dex-MDMφ and the presence of secondarily necrotic neutrophils was not required. Our findings are in confirmation of work by Cortes-Hernandez *et al*, who reported that serum-dependent uptake by Dex-treated murine mesangial cells was specific for apoptotic neutrophils, and was not observed for viable or necrotic neutrophils (Cortes-Hernandez et al., 2002). The prompt clearance[1] of cells at an early stage of apoptosis is essential to avoid the deleterious consequences that occur upon leakage of cellular contents during the later stages of apoptosis, including stimulation of pro-inflammatory responses by macrophages (Searle et al., 1982) and autoimmune reactions (Mevorach et al., 1998b, Botto, 1998, Herrmann et al., 1998,

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[1] Apoptotic cell clearance is a multi-step process requiring binding, internalisation, degradation and metabolism of apoptotic cell contents within the macrophage. The pattern of apoptotic cell opsonisation, the condition of the apoptotic cell (membrane intact/lysed, viral infection), and the receptor pathway employed by macrophages can have significant inflammatory and immunological consequences.

Scott et al., 2001, Gaipl et al., 2006, Tas et al., 2006), and may be a prerequisite for effective resolution of inflammation.

3.4.5 *Levels of phagocytosis by human MDMφ are highly variable*

Macrophages play a central role in triggering, instructing and terminating immune responses, and their ability to do so depends on the functional phenotype they acquire in response to environmental signals. As a result of these signals, macrophages can be markedly heterogeneous in their phenotype, allowing specialisation of function within particular microenvironments (Taylor et al., 2005). Hence, variation in phagocytic potential may result from the conditions monocytes are exposed to during *in vitro* culture. After isolation from peripheral blood, monocytes were selected by adherence to tissue culture plastic for 1 hour, after which the non-adherent lymphocytes were removed by washing. However, a residual number of lymphocytes will persist for the duration of the culture, releasing a number of cytokines including IL-10, IFNγ and IL-4, which can affect monocyte maturation/differentiation processes, including macrophage phagocytic capacity (Ren and Savill, 1995, Erwig et al., 1998, Michlewska et al., 2009). Additionally, autologous serum likely contains a number of cytokines and other modulatory agents, which may be variable between donors depending on factors such as immunological status and stress levels that may alter the MDMφ phagocytic response. Untreated cultures contain a highly heterogeneous population of MDMφ, and variation in capacity for phagocytosis may result from different levels of these cytokines in the culture medium. Although Dex induces a more homogeneous macrophage phenotype, the phagocytic potential of these cells may also be regulated by culture conditions, including the presence of immunoregulatory cytokines such as IFNγ and TNFα (Heasman et al., 2004, Michlewska et al., 2009). It might be desirable to reduce lymphocyte contamination, with relatively pure monocyte populations achievable using the Macs system, or to use alternatives to autologous serum such as M-CSF.

Another important aspect to be considered is the proportion of apoptotic or secondarily necrotic cells in the target population. However, the phagocytic potential of Dex-MDMφ was not significantly altered by the percentage of secondary necrosis or by the number of apoptotic cells available in serum-free neutrophil cultures, as Dex-MDMφ phagocytosis was unaffected using neutrophil populations diluted to 50% (3:1 ratio of apoptotic prey to Dex-MDMφ) of the normal concentration. This is further evidence that Dex-MDMφ are highly efficient in seeking out apoptotic prey, even when present in low numbers.

3.4.6 Serum opsonises apoptotic neutrophils to promote their internalisation by Dex-MDMφ

Macrophage phagocytosis of apoptotic cells is a highly complex process involving macrophage receptors and serum components that seem to operate in a redundant manner. Furthermore, macrophage receptors have distinct functions for direct binding of apoptotic cells, indirect binding of serum-opsonised apoptotic cells, and for internalisation, and it is likely that coordinated engagement of these phagocytic pathways is required for efficient uptake of apoptotic cells. Analysis of *in* vitro phagocytosis assays by confocal microscopy revealed that Dex-MDMφ we capable of binding apoptotic neutrophils in the absence of serum, but that serum was required to promote internalisation of bound apoptotic neutrophils. Macrophage phagocytic capacity can be regulated by serum in two ways: direct stimulation of macrophages to promote ingestion, or opsonisation of apoptotic cells to promote efficient removal. From serum pre-incubation studies, we demonstrated that serum opsonises apoptotic neutrophils to promote their ingestion by Dex-MDMφ. Furthermore, phagocytosis of serum-opsonised apoptotic neutrophils by Dex-MDMφ involved both cation-dependent and cation-independent opsonisation events, suggesting that more than one serum-dependent pathway may participate in the opsonisation phenomenon and that cooperative action may be required for highly efficient clearance of apoptotic cells. Surprisingly, given the potential importance of apoptotic cell removal in so many diverse processes, there have been few studies that have compared molecular mechanisms and functional consequences of phagocytic clearance of early and late

apoptotic cells. Although reports on serum-dependent clearance have suggested that serum opsonins such as MBL, SAP and pentraxin-3 bind to late, membrane permeable (secondarily necrotic) cells to modulate their clearance by macrophages (Nauta et al., 2003c, van Rossum et al., 2004), our results suggest that serum opsonises "early" apoptotic cells for removal by Dex-MDMφ.

The data presented in this chapter led to my initial attempts to identify the serum component(s), where I employed an "add-back" approach to assess the involvement of individual serum components with a well-characterised role in apoptotic cell opsonisation.

CHAPTER 4: IDENTIFICATION OF A SERUM OPSONIN REQUIRED FOR THE GLUCOCORTICOID EFFECT: "ADD-BACK" APPROACH

4.1   Introduction

During *in vitro* culture, human neutrophils undergo spontaneous apoptosis and display a distinct surface molecular phenotype, including down-regulation of FcγRIII (CD16) and L-selectin expression, altered glycosylation of cell surface proteins, changes in surface charge, loss of CD31 detachment function, and exposure of PS (Dransfield et al., 1994, Dransfield et al., 1995, Martin et al., 1995, Hart et al., 2000, Brown et al., 2002). These changes distinguish them from viable neutrophils and signal their specific removal by human macrophages (Savill et al., 1989b). The phagocytic potential of macrophages may be enhanced by a number of soluble molecules that bind to or "opsonise" apoptotic neutrophils to promote their clearance through opsonin-specific receptors like integrins (Savill et al., 1992, Hanayama et al., 2002) or complement receptors (Mevorach et al., 1998a, Ogden et al., 2001, Vandivier et al., 2002). The importance of apoptotic cell opsonisation is evident in C1q-deficiency, which leads to the development of an SLE-like autoimmune disease in mouse models and in humans characterised by impaired clearance of apoptotic cells (Botto, 1998, Taylor et al., 2000). Interestingly, many of the soluble molecules with a putative function as apoptotic cell opsonins are present in human plasma/serum (Rimoldi et al., 1989, Sorensen et al., 1996, Mevorach et al., 1998a) and therefore could mediate the pro-phagocytic effect of serum on Dex-MDMϕ phagocytosis of apoptotic neutrophils.

Analysis of surface receptor expression through mAb binding and flow cytometry revealed that glucocorticoids induce homogeneity in MDMϕ surface receptor expression of HLA-DR, CD14, and CD44 (Giles et al., 2001). However, initial studies suggested that there was no apparent increase in the expression of putative phagocytic receptors that could account for the serum-dependent pro-phagocytic phenotype displayed by Dex-MDMϕ. Functional inhibitor studies of phagocytosis

using specific mAbs and soluble ligand inhibitors failed to identify a dominant phagocytic pathway employed by Dex-MDMϕ, with inhibition of CD36 or αvβ3 using SMϕ mAb (1:50 ascites) or 0.5mM RGDS peptide respectively, or 10mM glucosamine (thought to inhibit a charge sensitive mechanism employing CD36/αvβ3/TSP-1), or inhibition of PS-dependent recognition of apoptotic cells using 2mM phospho-L-serine, only partially suppressing Dex-MDMϕ phagocytosis of apoptotic neutrophils (Giles et al., 2001). Ligation of CD44 on untreated human MDMϕ induced a rapid increase in phagocytic uptake of apoptotic neutrophils (Hart et al., 1997), however CD44 was shown to be downregulated on Dex-MDMϕ and preliminary studies suggested that CD44 ligation did not convey augmented clearance of apoptotic neutrophils (Giles et al., 2001).

In the previous chapter, I demonstrated that augmented phagocytosis by Dex-MDMϕ required serum-dependent opsonisation of apoptotic neutrophils. In this chapter, I employed an "add-back" approach in order to evaluate whether molecules with a well-characterised role in apoptotic cell clearance demonstrated pro-phagocytic activity.

4.2 Materials and methods

4.2.1 *Proteins and sera*

Fresh human platelet-poor plasma (PPP) was obtained following centrifugation of PRP at 13,000 x *g* for 1 minute to deplete the majority of platelets and leave an upper PPP layer that was transferred to a fresh epindorph. To obtain platelet releasate, platelets purified from human blood were prepared by washing in EDTA-containing PBS with 50mM glucose, then resuspended in PBS with 50mM glucose at 20U/ml at 37°C. Thrombin was added and left for 10min at 37°C, after which a thrombin-inhibitor was added and the material was centrifuged at 200 x *g* to remove aggregated platelets and the supernatant was retained. Serum, from coagulated whole blood, was obtained by cardiac puncture from wild-type, annexin 1-deficient (Hannon et al., 2003) and C1q-deficient (Botto, 1998) mice on a C57BL/6 background. C1q-depleted human serum and purified human C1q were obtained from Merck. A soluble recombinant form of human complement receptor 1 (sCR1) (a kind donation from Paul Morgan) was used for inhibition of C3 activation in serum (Piddlesden et al., 1994). A dose of 250μg/ml completely blocks complement activity as assessed by haemolytic assays (Paul Morgan, personal communication). The FPRL1 antagonist was a kind donation from Peter Barlow.

4.2.2 *Induction of neutrophil apoptosis*

CMFDA-labelled neutrophils were cultured at 4 x 10^6/ml in <u>serum-free</u> medium (IMDM) at 37°C for 20-24 hours to render neutrophils apoptotic. Neutrophil populations were then centrifuged at 200 x *g* for 5min and resuspended in fresh IMDM prior to assessment of phagocytosis.

4.3 Results

4.3.1 *Ultrafiltration of serum into size-specific fractions: a serum factor(s) larger than 50-kDa enhances uptake of apoptotic neutrophils by Dex-MDMϕ*

From the results obtained in the previous chapter, it became clear that generation of apoptotic neutrophils in serum-containing medium was sufficient to confer augmentated clearance by Dex-MDMϕ. To obviate the difficulty associated with serum opsonisation of apoptotic neutrophils during *in vitro* culture on investigating phagocytic pathways, we continued to use a system where neutrophils were rendered apoptotic in <u>serum-free</u> medium in order to address the role of single serum components or MDMϕ receptor usage. Serum contains many different components that may mediate apoptotic neutrophil clearance, ranging from small molecules to very large protein complexes. In view of this, we sought to investigate the molecular weight of human serum components to narrow down the number of potential candidate molecules. To achieve this, autologous serum was fractionated using YM-50 and YM-100 membranes, as described in Chapter 2, 2.9.2 Ultrafiltration of serum. Addition of 10% YM-50 retentate, but not YM-50 filtrate, enhanced phagocytosis of apoptotic neutrophils by Dex-MDMϕ to similar levels conveyed by 10% unfiltered serum (*fig. 4.1*; *** $p < 0.001$, n=4), indicating that the pro-phagocytic serum component(s) was larger than 50-kDa in molecular mass. Furthermore, preliminary data obtained using serum ultrafiltered through YM-100 membranes suggested that the serum component(s) was larger than 100-kDa in molecular mass (Dex 5d, No serum: 23%; 10% serum: 66.4%, 10% YM-100 filtrate (<100kDa): 20.3%; 10% YM-100 retentate (>100kDa): 63.2%. n = 1). .

A
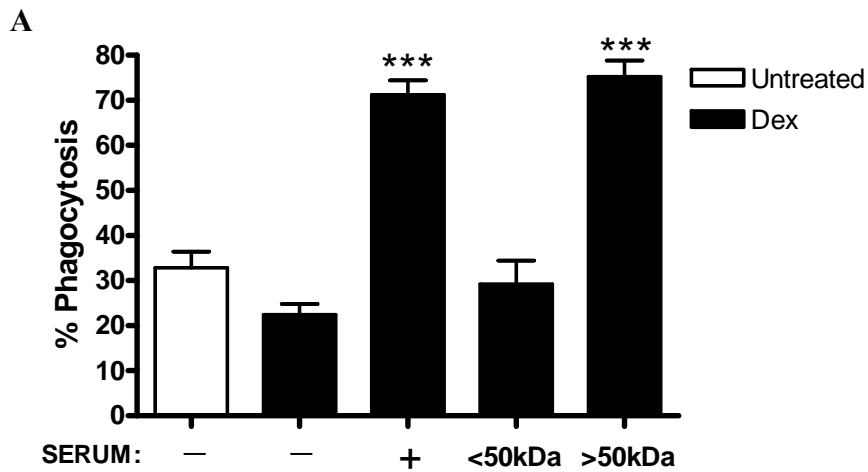
Figure 4.1: Effect of serum fractionation into size-specific fractions on uptake of apoptotic neutrophils by Dex-MDMφ.

MDMφ were cultured in the absence or presence of 1μM Dex for 5 days, and phagocytosis of <u>serum-free cultured</u> heterologous apoptotic neutrophils by untreated (white bars) and Dex-treated (black bars) MDMφ was assessed by flow cytometry. Dex-MDMφ phagocytosis of apoptotic neutrophils required a serum component larger than 50-kDa in molecular mass. Mean phagocytosis ± SEM, n=4 where n = the number of different macrophage donors. (*** p<0.001 when compared to uptake by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test). Phagocytosis by untreated MDMφ is shown for comparison.

4.3.2 Boiling, but not heat-inactivation, significantly reduced the ability of serum to augment Dex-MDMφ phagocytosis

Next, we wanted to investigate the biochemical nature of the serum component(s). For this, freshly prepared autologous serum was either boiled for 3min at 95°C, or heat-inactivated for 30min at 56°C, and then tested immediately for phagocytic activity. We found serum-dependent augmentation of Dex-MDMφ phagocytic capacity to be unaltered when the serum was heat-inactivated (*fig. 4.2*; p>0.05, n=3), suggesting that the component(s) was not heat-labile. However, boiling the serum significantly reduced the pro-phagocytic activity (*fig. 4.2*; ** p<0.01, n=3), suggesting that the required component(s) might be a protein.

4.3.3 Ultracentrifugation of serum into particle-specific fractions: protein aggregates are not required for augmentation of Dex-MDMφ phagocytosis

Due to the high molecular weight of the serum component indicated by serum fractionation (*fig. 4.1*), and the implication that the component was protein in nature (*fig. 4.2*), we decided to investigate whether serum-enhanced Dex-MDMφ phagocytosis involved binding of aggregated proteins to apoptotic neutrophils. Fresh autologous serum was ultracentrifuged to pellet out protein aggregates (see Chapter 2, 2.9.1 Ultracentrifugation of serum). The middle straw-coloured protein layer was carefully removed to avoid disturbing the pellet or mixing with the upper lipid layer, and reconstituted to the original volume (200μl) with IMDM. 10% of this ultracentrifuged fraction significantly enhanced Dex-MDMφ phagocytosis of apoptotic neutrophils to a similar extent as non-centrifuged serum (*fig. 4.3*; *** p<0.001, n=4), demonstrating that protein aggregates such as denatured proteins or immune complexes are not required.

After gaining some insight into the molecular weight and biochemical nature of the serum component(s), we decided to take a more systematic approach through 'add-back' of serum components with a well-characterised role in apoptotic cell phagocytosis.
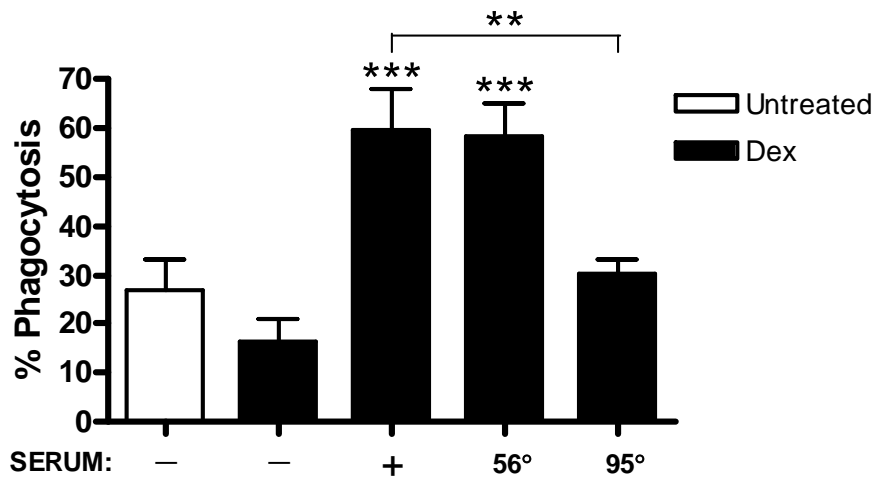
Figure 4.2: Effect of boiling and heat-inactivation on the pro-phagocytic effect of serum.

Phagocytosis assays were performed as described in Figure 4.1. Boiling, but not heat-inactivation, significantly reduced the pro-phagocytic effect of 10% fresh autologous serum on Dex-MDMφ (black bars) phagocytosis of heterologous apoptotic neutrophils. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (*** $p < 0.001$ when compared to uptake by Dex-MDMφ in the absence of serum, ** $p < 0.01$ when compared to Dex-MDMφ phagocytosis in the presence of serum, one-way ANOVA with a Bonferroni post test).
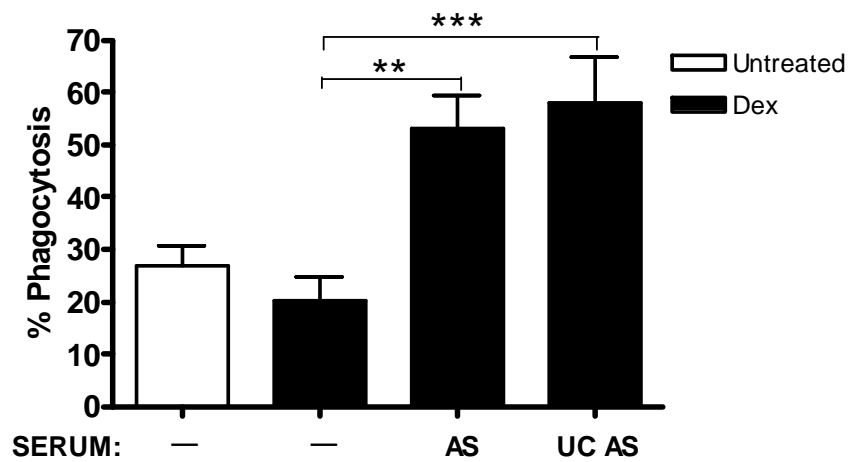
Figure 4.3: Effect of ultracentrifugation on the pro-phagocytic effect of serum.
Phagocytosis assays were performed as described in Figure 4.1. Ultracentrifugation
(US AS) did not alter the pro-phagocytic effect of 10% fresh autologous serum (AS)
on Dex-MDMφ (black bars) phagocytosis of heterologous apoptotic neutrophils.
Mean phagocytosis ± SEM, n=4 where n = the number of different macrophage
donors. (** $p<0.01$, *** $p<0.001$ when compared to uptake by Dex-MDMφ in the
absence of serum, one-way ANOVA with a Bonferroni post test).

4.3.4 High abundance human serum proteins, albumin and IgG, are not required for augmented phagocytosis by Dex-MDMφ

Our serial dilution experiments from the previous chapter indicated that augmentation of Dex-MDMφ phagocytosis was still significant at 1% final serum concentration, suggesting that the pro-phagocytic component(s) may be present at a high concentration in serum. Albumin is the most abundant protein in serum, constituting around 60% of protein content (Travis et al., 1976). However, human serum albumin (HSA) at 1% final concentration did not increase Dex-MDMφ phagocytosis, instead conferring a slight inhibition on uptake of serum-free cultured apoptotic neutrophils compared to that in the absence of serum (*fig. 4.4a*; p>0.05, n=4), demonstrating that albumin is not required. Furthermore, preliminary data indicated that 1% albumin could also significantly inhibit serum-dependent phagocytosis of apoptotic neutrophils by Dex-MDMφ (untreated MDMφ, 10% serum: 28.3%; Dex-MDMφ, 10% serum: 76.2%, 10% serum plus 1% albumin: 31.6%. n = 2). These data demonstrate that human serum albumin is not required for the serum effect.

IgG, another prominent protein in serum, has been reported to stimulate uptake of apoptotic targets through Fcγ receptors on macrophages (Hart et al., 2004). However, addition of 2mg/ml human IgG during the assay did not enhance Dex-MDMφ phagocytosis above untreated control levels (*fig. 4.4b*; p>0.05, n=3), a finding that suggests IgG is not the pro-phagocytic component in serum and confirms previous reports that phagocytosis by Dex-MDMφ does not engage a pro-inflammatory pathway (Liu et al., 1999).
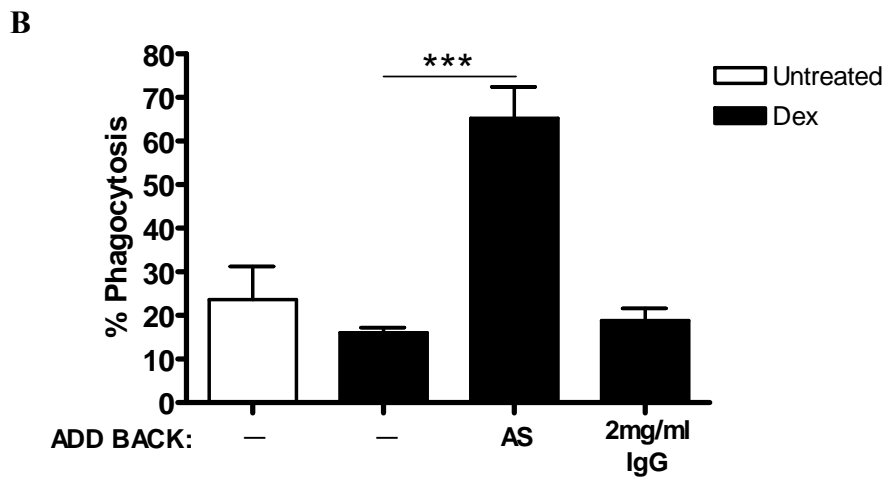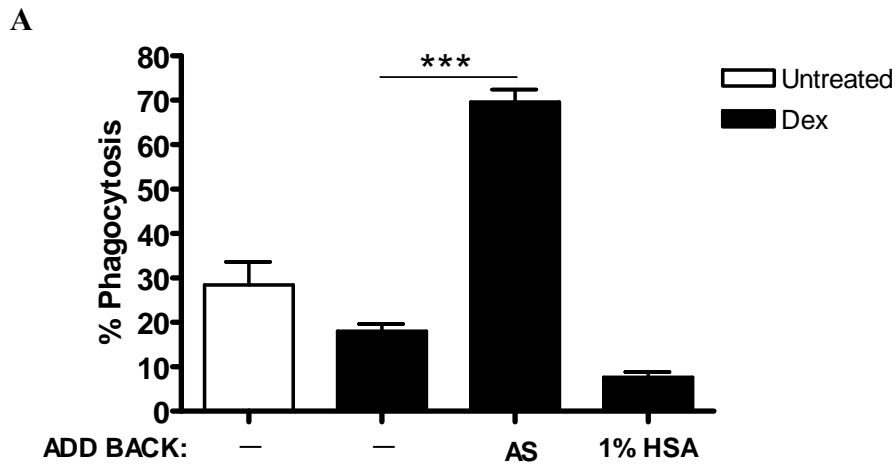
Figure 4.4: Effect of human albumin and IgG on uptake of apoptotic neutrophils by Dex-MDMφ.

Phagocytosis assays were performed as described in Figure 4.1. In contrast to 10% fresh autologous serum (AS), 1% HSA (**A**, n=4) and 2mg/ml human IgG (**B**, n=3) failed to enhance Dex-MDMφ (black bars) phagocytosis of heterologous apoptotic neutrophils. Mean phagocytosis ± SEM (*** $p < 0.001$ when compared to uptake by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test). N = the number of different macrophage donors.

4.3.5 *Serum dependent-enhancement of Dex-MDMɸ phagocytosis of apoptotic neutrophils does not require complement activation*

A number of complement proteins have been implicated in apoptotic cell opsonisation (Korb and Ahearn, 1997, Mevorach et al., 1998a). On this basis, we wanted to determine whether phagocytosis of <u>serum-free cultured</u> neutrophils by Dex-MDMɸ required complement-dependent opsonisation, focusing on C1q and C3b opsonins. To investigate whether enhanced Dex-MDMɸ phagocytic capacity for apoptotic neutrophils required opsonisation with C3b, autologous serum was pre-treated for 10min with 250µg/ml of a soluble recombinant form of human complement receptor 1 (sCR1) to inhibit C3 activation in serum prior to addition to apoptotic neutrophils (as described in 4.2.1 Proteins and sera). The pro-phagocytic effect of serum was still evident after inhibition of complement activation, with 10% C3-inhibitor-treated serum generating levels of Dex-MDMɸ phagocytosis indistinguishable from those observed for 10% control PBS-treated serum (*fig. 4.5a*; p>0.05, n=4) (McColl et al., 2009).

Initially, we assessed the phagocytic potential of complement C1q using commercially available C1q-depleted human serum and purified human C1q. Interestingly, 10% C1q-depleted human serum failed to enhance phagocytosis by Dex-MDMɸ above levels observed in the absence of serum (*fig. 4.5b*; p>0.05, n=3), suggesting that C1q was the serum opsonin binding to apoptotic neutrophils for enhanced clearance by Dex-MDMɸ. However, addition of 70µg/ml purified human C1q alone or to C1q-depleted serum did not restore phagocytosis by Dex-MDMɸ to levels observed in the presence of 10% autologous serum (*fig. 4.5b*; *** p<0.001, n=3). In order to further characterise the role of C1q, we took advantage of the observation that murine serum conferred augmentation of Dex-MDMɸ phagocytosis (see Chapter 3, *fig. 3.5c*) and assessed phagocytic activity of serum obtained from C1q-deficient mice. We found that serum derived from either male (♂) or female (♀) C1q-deficient mice was able to confer augmentation of Dex-MDMɸ phagocytosis of apoptotic neutrophils (*fig. 4.5c*; ** p<0.01, n=3) (McColl et al., 2009), demonstrating that C1q was in fact not the serum opsonin required.
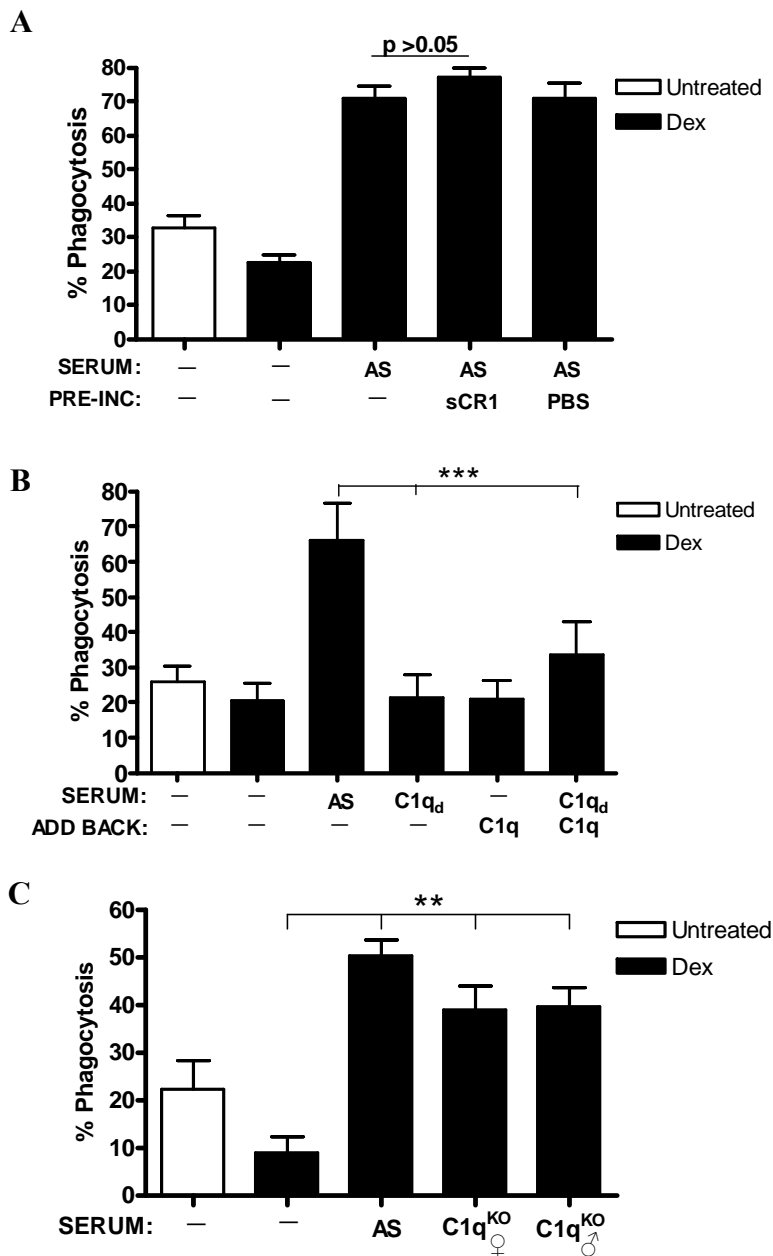
Figure 4.5: Effect of C1q and C3b complement proteins on Dex-MDMφ phagocytosis.

Phagocytosis assays were performed as described in Figure 4.1. **A**, Pre-treatment of 10% fresh autologous serum (AS) with 250μg/ml sCR1 to inhibit C3 activation had no effect on serum-enhanced phagocytosis by Dex-MDMφ (n=4). **B**, The pro-phagocytic effect of human serum (frozen) was lost upon depletion of C1q, an effect that was not restored by addition of 70μg/ml purified C1q (n=3). **C**, Serum from male (♂) or female (♀) C1q-deficient mice (frozen) enhanced Dex-MDMφ phagocytosis (n=3). Mean phagocytosis ± SEM (*** $p<0.001$ compared to uptake by Dex-MDMφ in the presence of serum, ** $p<0.01$ compared to uptake by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test). N = the number of different macrophage donors.

The inability of C1q-depleted human serum to confer augmentation of phagocytosis by Dex-MDMφ may be attributable to the addition of EDTA during commercial depletion. Our data suggests that EDTA can inhibit binding of the serum opsonin(s) to apoptotic neutrophils and reduce ingestion by Dex-MDMφ. However, we did not observe a calcium-independent opsonising effect of C1q-depleted serum that was evident from autologous serum pre-incubation studies, and it is possible that contaminating EDTA in the assay medium may also directly inhibit Dex-MDMφ phagocytic capacity by chelating calcium required for initiating phagocytosis (Gronski et al., 2009). An alternative possibility is that through the method of C1q-depletion, using anti-C1q bound to a column, C1q-binding proteins may have been inadvertently depleted. Interestingly, fibronectin and pentraxin-3, both proteins with an ability to bind C1q, may modulate human untreated MDMφ phagocytosis of apoptotic neutrophils *in vitro* (McCutcheon et al., 1998, van Rossum et al., 2004). However, addition of 50µg/ml of human fibronectin (*fig. 4.6a*) or 5µg/ml of human pentraxin-3 (*fig. 4.6b*) alone, or in combination with 70µg/ml human C1q (data not shown), did not confer augmentation of Dex-MDMφ phagocytosis.

Finally, we were surprised by preliminary data obtained using two independent sources of C1q, which inhibited serum-dependent phagocytosis by Dex-MDMφ (Dex 5d, 10% serum: 67.8% phagocytosis; 10% serum plus 70µg/ml C1q (Merck): 36.6% phagocytosis; 10% serum plus 70µg/ml C1q (kindly supplied by Simon Hart): 40.9% phagocytosis. n=2, duplicate wells). Preliminary data suggested this inhibitory effect was specific to C1q, as serum-dependent augmentation of Dex-MDMφ phagocytosis was unaltered by addition of 20µg/ml alpha-2 macroglobulin (10% serum: 67.8%; 10% serum plus alpha-2 macroglobulin (Sigma): 68.1%. n=2, duplicate wells).

This series of experiments demonstrating a lack of requirement of complement C3b and C1q for serum-enhanced Dex-MDMφ phagocytosis of apoptotic neutrophils was in confirmation of the heat-inactivation data (*fig. 4.3*), as activation of the complement system requires proteins that are heat-labile. Further investigations are required to assess the precise effect of C1q on Dex-MDMφ phagocytic capacity.
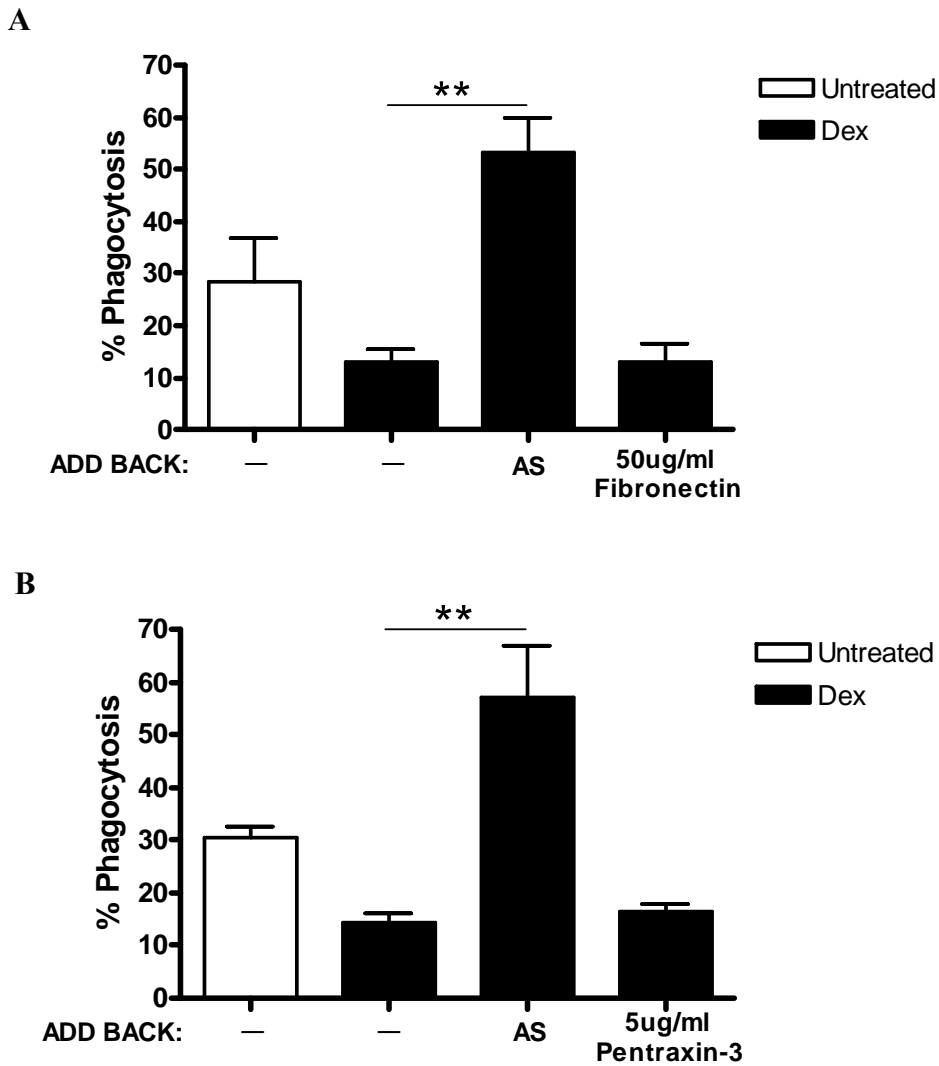
Figure 4.6: Effect of the C1q-binding proteins, fibronectin and pentraxin-3, on Dex-MDMφ phagocytosis.

Phagocytosis assays were performed as described in Figure 4.1. In contrast to 10% fresh autologous serum (AS), 50μg/ml human fibronectin (**A**) and 5μg/ml human pentraxin-3 (**B**) failed to enhance Dex-MDMφ (black bars) phagocytosis of heterologous apoptotic neutrophils. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (** $p < 0.01$ when compared to uptake by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test).

4.3.6 *The annexin-1/FPRL1 pathway is not utilised by Dex-MDMφ during removal of apoptotic neutrophils*

Annexin-1 is a glucocorticoid-inducible protein that exerts anti-inflammatory effects by signalling through members of the FPR family, in particular FPRL1 (Walther et al., 2000, Perretti et al., 2001, Perretti et al., 2002, Ernst et al., 2004, Hayhoe et al., 2006, John et al., 2007). Annexin-1 and annexin-1 derived peptides have been reported to stimulate FPRL1-dependent phagocytosis of apoptotic neutrophils by human untreated MDMφ *in vitro* (Maderna et al., 2005, Scannell et al., 2007). To evaluate whether this pathway was utilised by Dex-MDMφ for phagocytosis of apoptotic neutrophils, Dex-MDMφ were pre-treated with 10μM WRW4, an FPRL1 antagonist, for 1 hour prior to assessment of phagocytosis. However, we found that inhibition of the FRPL1 pathway did not abolish serum-dependent phagocytosis by Dex-MDMφ (*fig. 4.7a*; p>0.05, n=3). Despite a slight enhancing effect of WRW4 pre-treatment on serum-dependent uptake by Dex-MDMφ (p>0.05, n=3), pre-treatment with WRW4 did not augment Dex-MDMφ phagocytosis in the absence of serum (18.5% phagoctyosis, n=2), nor did it have any effect on phagocytosis by untreated MDMφ in the presence of serum (23.6% phagocytosis, n=3).

We decided to further characterise the phagocytic potential of annexin-1 using annexin-1-deficient mouse serum, and found the effect of 10% annexin-1-deficient serum to be indistinguishable from control matched annexin-1 wild type serum in enhancement of Dex-MDMφ phagocytosis of apoptotic neutrophils (*fig. 4.7b*; p>0.05, n=3). Interestingly, both wild type and annexin-1-deficient mouse sera conferred an even more significant enhancement of Dex-MDMφ phagocytic capacity than observed for autologous serum (*fig. 4.7b*; ** p<0.01, *** p<0.001, n=3).

Together, these data suggest that the annexin-1/FPRL1-dependent pathway is not utilised by Dex-MDMφ.
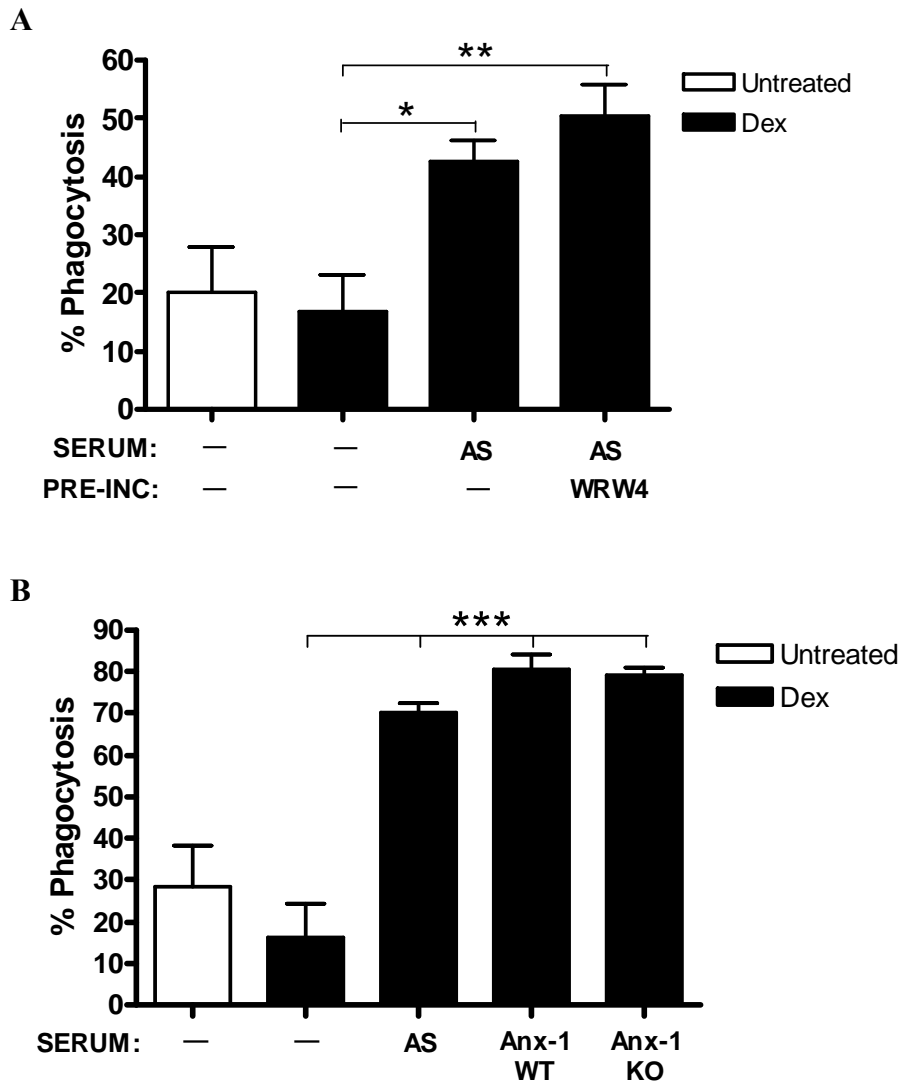
Figure 4.7: <u>Effect of the annexin-1 on phagocytosis by Dex-MDMφ.</u>
Phagocytosis assays were performed as described in Figure 4.1. **A**, Pre-treatment of Dex-MDMφ (black bars) with 10μM WRW4, an FPRL-1 antagonist, failed to inhibit the uptake of heterologous apoptotic neutrophils in the presence of 10% fresh autologous serum (AS). **B**, Annexin-1 deficiency did not reduce the ability of mouse sera (frozen) to enhance Dex-MDMφ phagocytosis. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ when compared to uptake by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test).

4.3.7 *Dex-MDMφ phagocytosis of apoptotic neutrophils does not require a platelet-derived factor*

Fresh autologous serum was prepared by recalcification of human plasma, a process which induces platelet aggregation and stimulates the abundant release of platelet factors. It was therefore imperative to determine whether a platelet-derived product such as TSP-1 was responsible for enhancement of Dex-MDMφ phagocytosis of apoptotic neutrophils. We initially evaluated Dex-MDMφ phagocytic capacity in the presence of human platelet-poor plasma (PPP), prepared by centrifugation of whole plasma to deplete platelets (as described in 4.2.1 Proteins and sera). Addition of 10% PPP to the culture medium resulted in formation of a "jelly-like" material, presumably due to activation of the coagulation cascade. As discussed in the previous chapter, serial dilution experiments showed that 1% serum was sufficient to significantly enhance Dex-MDMφ phagocytosis (see Chapter 3, *fig. 3.3*). Accordingly, the use of 1% PPP avoided the complication of coagulation during the assay and demonstrated that platelet depletion had no significant effect on the ability of human plasma to augment Dex-MDMφ phagocytosis of apoptotic neutrophils (*fig. 4.8a*; $p > 0.05$ when compared to uptake in the presence of 1% autologous serum, n=3). To further investigate the phagocytic potential of platelet-derived factors, we assessed phagocytosis in the presence of platelet releasate, prepared as described in 4.2.1 Proteins and sera. However, we found that 50μg/ml platelet releasate did not enhance apoptotic neutrophil uptake by Dex-MDMφ (*fig. 4.8b*). These data suggested that Dex-MDMφ phagocytosis does not require a platelet-derived factor.
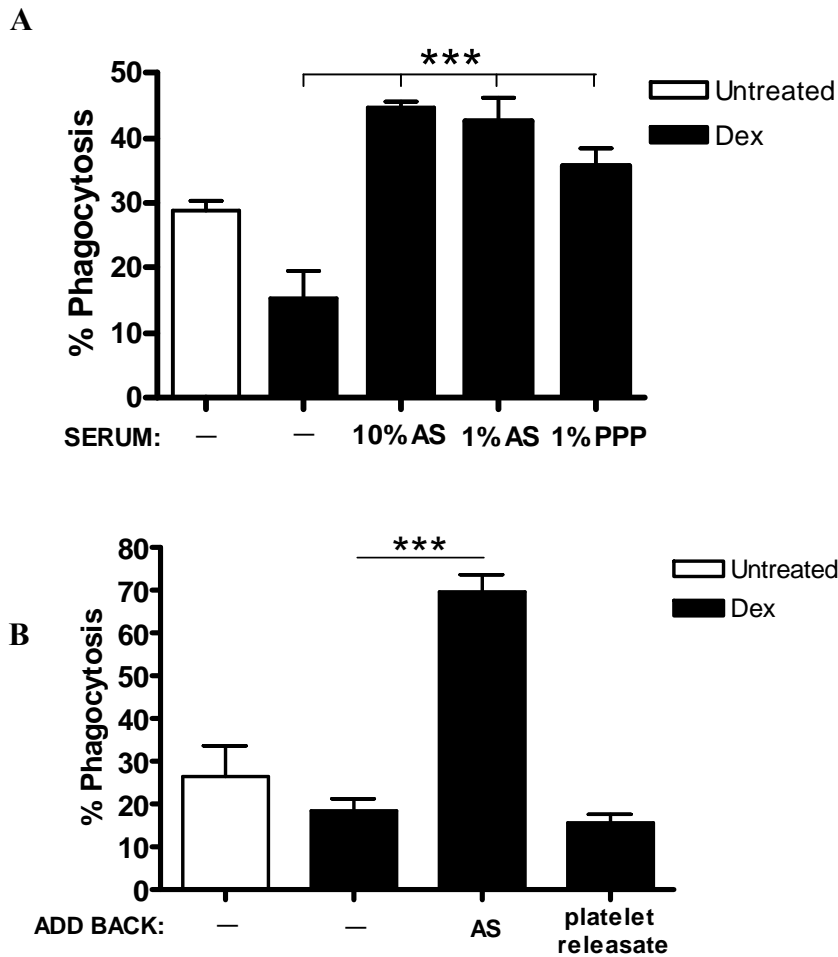
Figure 4.8: Effect of human platelet-poor plasma and platelet releasate on phagocytosis by Dex-MDMφ.

Phagocytosis assays were performed as described in Figure 4.1. **A**, 1% fresh human platelet poor plasma (PPP) was equivalent to 1% fresh autologous serum (AS) in enhancement of Dex-MDMφ phagocytosis of heterologous apoptotic neutrophils. **B**, 50μg/ml platelet releasate did not confer augmentation of Dex-MDMφ phagocytosis. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (*** $p < 0.001$ when compared to uptake by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test).

4.4   Discussion

4.4.1   *Opsonisation plays an essential role in apoptotic cell clearance: effect on macrophage phagocytic potential and immunological consequences*

The uptake of apoptotic cells by macrophages involves two stages; firstly, the recognition and "tethering" of the apoptotic cell by the macrophage, and secondly, its ingestion and destruction within the macrophage (Henson et al., 2001). Macrophages express a multitude of surface receptors that bestow the capacity for apoptotic cell recognition. Although some of these receptors have been suggested to mediate direct recognition and tethering of apoptotic cells prior to engulfment, including scavenger receptors, CD31, CD14 and CD36 (Rigotti et al., 1995, Platt et al., 1996, Devitt et al., 1998, Vernon-Wilson et al., 2006), most macrophage receptors do not directly bind to apoptotic cells. Rather, efficient clearance by macrophages requires soluble host proteins that may "opsonise" the altered apoptotic cell surface and provide a mechanism for phagocytic removal that does not require general "apoptotic cell" receptors, allowing diversity of phagocyte responses both in terms of the apoptotic cell type recognised and the phagocytic cell that is capable of recognition based on opsonin receptor expression. As demonstrated in the previous chapter, Dex-MDMϕ efficiently recognised and ingested neutrophils that had undergone spontaneous apoptosis during culture in 10% serum, but did not display phagocytic potential for serum-free cultured neutrophils (Chapter 3, *figs. 3.1* and *3.2*). Ingestion of serum-free cultured apoptotic neutrophils instead required opsonisation of apoptotic neutrophils with serum proteins prior to phagocytic interaction (Chapter 3, *figs. 3.8*). Many plasma/serum components have been accredited with an ability to function as apoptotic cell opsonins including pentraxins (SAP, CRP and pentraxin-3), collectins (MBL, SP-A and SP-D), complement proteins, antibody, TSP-1, β2-GPI, MFG-E8, properdin and galectin-3 (Savill et al., 1992, Balasubramanian et al., 1997, Mevorach et al., 1998a, Bickerstaff et al., 1999, Gershov et al., 2000, Rovere et al., 2000, Gaipl et al., 2001, Schagat et al., 2001, Ogden et al., 2001, Mold et al., 2002, Hanayama et al., 2002, Nauta et al., 2003c, Zwart et al., 2004, Xu et al., 2008, Karlsson et al., 2009), promoting apoptotic cell clearance through opsonin-specific receptors (Savill et al., 1992, Mevorach et al., 1998a, Finnemann and Rodriguez-Boulan, 1999, Ogden

et al., 2001, Vandivier et al., 2002, Hanayama et al., 2002). Interestingly, many of these soluble proteins are important effectors in innate recognition of microbial products. How similar opsonin-dependent receptor mechanisms can be used to generate both pro- and anti-inflammatory outcomes to foreign and self-antigens, respectively, is unclear, but is likely to require complex pathways of integrated signalling to generate these diverse responses. Furthermore, the pattern of opsonisation may have significant consequences in terms of the cellular response to apoptotic cells, both with respect to the phagocyte population engaged and the inflammatory outcome of this interaction. For example, opsonisation with of apoptotic neutrophils with immune complexes may induce a pro-inflammatory macrophage phenotype upon ingestion, inducing the release of TNFα and IL-6 presumably as the result of ligation of macrophage FcγRs (Hart et al., 2004), whereas ingestion of CRP-opsonised cells is associated with release of anti-inflammatory mediators by macrophages (Gershov et al., 2000). Many of these soluble proteins may be produced locally by the macrophages themselves (Ezekowitz et al., 1984, Hanayama et al., 2002, Faust et al., 2002, Maderna et al., 2005), allowing macrophages to influence the pattern of apoptotic cell opsonisation through opsonin availability and subsequently the clearance mechanism(s) engaged based on the different environmental signals received by macrophages during normal tissue homeostasis, inflammation and chronic disease. Interestingly, glucocorticoids upregulate monocyte/macrophage opsonin and scavenging mechanisms known to be associated with apoptotic cell recognition and uptake, including MFG-E8, C1q, collectins (MBL, SP-A and SP-D), SLPI and annexin I opsonins, and FPR1 and Mertk receptors (Abbinante-Nissen et al., 1995, de Coupade et al., 2001, Faust et al., 2002, Maderna et al., 2005, Ehrchen et al., 2007), which would be consistent with involvement of an opsonisation mechanism for apoptotic neutrophil clearance by glucocorticoid-treated macrophages.

There are discrepancies in reports of the stage of cell death necessary for binding of such recognition molecules, which may partially be explained by differences in semantics, experimental design and phagocytic readout. Several reports have suggested opsonisation with serum proteins like IgG, TSP-1, collectins and

pentraxins to be a relatively late event in apoptosis, proceeding PS exposure and loss of mitochrondrial membrane potential and instead occurring when cells have become membrane-permeable (i.e. secondary necrotic cells), possibly as a mechanism for regulating removal of these potentially damaging cells and their contents (Hart et al., 2000, Rovere et al., 2000, Gershov et al., 2000, Gaipl et al., 2001, Navratil et al., 2001, Vandivier et al., 2002, Nauta et al., 2003c, Bijl et al., 2003, van Rossum et al., 2004, Zwart et al., 2004, Hart et al., 2005). The role of serum opsonisation in the uptake of "early" apoptotic cells has not yet been fully established. In order to assess the role of serum opsonisation in uptake of "early" apoptotic neutrophils by Dex-treated macrophages, we initally employed an "add-back" approach using well-defined apoptotic cell serum opsonins.

4.4.2 *Serum fractionation identified a fraction larger than 50-kDa with phagocytic activity that was not due high abundance serum proteins or protein aggregates*

Due to the abundance of factors in serum with the ability to modulate apoptotic cell clearance, we decided to minimise the number of potential components through serum fractionation techniques. The pro-phagocytic effect of serum was only evident in serum fractions containing components larger than 50-kDa in molecular weight, allowing us to rule out stimulation of Dex-MDMϕ phagocytosis by cytokines or small lipid mediators such as prostaglandins that have previously demonstrated to modulate untreated MDMϕ phagocytosis of apoptotic neutrophils (Ren and Savill, 1995, Rossi et al., 1998). The abolishment of phagocytic activity upon boiling serum (95° for 3min) indicated that the serum effect likely required a protein component. A small number of proteins represent a high percentage of the total protein content in serum, including albumin and IgG, which have a molecular weight of >50-kDa (Steel et al., 2003, Best et al., 2006). However, these high abundance proteins did not confer augmentation of Dex-MDMϕ phagocytosis for apoptotic neutrophils. We were surprised to find that HSA actually exerted a slight inhibitory effect on basal levels of phagocytosis and significantly abrogated serum-enhanced phagocytosis by Dex-MDMϕ. Decreased spreading of neutrophils in the presence of HSA (Nathan et

al., 1993), and possibly a similar effect on Dex-MDMϕ, may have impacted on the phagocytic interaction and subsequent serum-dependent internalisation of apoptotic neutrophils. Visualisation of the effect of albumin on Dex-MDMϕ phagocytosis by time-lapse video microscopy might provide a valuable insight into the characteristics required for highly efficient phagocytosis by these cells in the presence of serum.

Many human inflammatory diseases like SLE are associated with the production, circulation and tissue deposition of immune complexes (Gaipl et al., 2007, Yung and Chan, 2008), and inflammatory processes can be mimicked by administration of immune complexes in animal models (Yamaguchi et al., 1975, Guo and Ward, 2002). In diseases such as antiphospholipid syndrome (APLS), autoantibodies may be produced against phospholipid-associated serum proteins such as β2-GPI, which binds to apoptotic cells via PS (Price et al., 1996, Subang et al., 2000). Previous work in our lab demonstrated specific opsonisation of apoptotic, but not viable human neutrophils with human IgG-containing immune complexes in an FcγRIIA-dependent manner (Hart et al., 2004). Although surface expression of FcγRIIA was reduced on apoptotic neutrophils, the receptor may have been functionally "enabled" during apoptosis to generate increased avidity for immune complex binding. We have shown that removal of immune complexes and other aggregated proteins by ultracentrifugation had no effect on the pro-phagocytic activity of serum. Furthermore, monomeric IgG does not bind significantly to apoptotic neutrophils (Hart et al., 2004) or enhance their removal by Dex-MDMϕ. The data presented here indicate that serum-dependent augmentation of Dex-MDMϕ phagocytic capacity for apoptotic neutrophils does not require monomeric or immune-complexed immunoglobulin.

4.4.3 *The role of the complement system in apoptotic cell clearance*

The complement system is an integral part of innate immune defence against invading pathogens. One important consequence of activation of the complement cascade is the cleavage of complement C3 to generate large amounts of C3b product

(Volanakis, 1990), which exerts a major effector function of complement via opsonisation of pathogens or infected target cells (Yuste et al., 2008). Most of this surface-bound C3b is rapidly converted by the serum protease factor I to C3bi, a more stable product that retains opsonic activity (Nagasawa and Stroud, 1977). Complement receptors mediate binding and ingestion of target cells opsonised with complement proteins or their degradation products, including CR1 (CD35), which binds C3b, C4b and C1q (Fearon, 1980, Klickstein et al., 1997), and the relatively C3bi-specific receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (Brown, 1991, Sengelov, 1995, Carroll, 1998). Human monocytes/macrophages express all three of these receptors (Mevorach et al., 1998a), highlighting their essential role in phagocytic removal of complement-opsonised targets.

Apoptotic cells have also been shown to trigger complement activation to become opsonised with C3b/C3bi (Tsuji et al., 1994, Matsui et al., 1994, Takizawa et al., 1996). Neutrophils undergoing apoptosis down-regulate surface expression of complement regulatory molecules DAF (CD55), MCP (CD46), and CR1 (CD35) (Jones and Morgan, 1995) that may allow complement activation to proceed on the cell surface and lead to opsonisation with these complement factors. Furthermore, exposure of PS on apoptotic cells has been suggested to contribute to this opsonisation phenomenon (Mevorach et al., 1998a). Using an entirely autologous system of MDMϕ, apoptotic neutrophils and serum from the same human donor, Mevorach *et al* showed that phagocytic interaction between untreated MDMϕ and apoptotic neutrophils *in vitro* could be enhanced ~4-fold by the presence of 10-15% serum, with little association of apoptotic cells with macrophages in the absence of serum (Mevorach et al., 1998a). Serum-dependent augmentation of phagocytosis required C3bi opsonisation of apoptotic neutrophils for removal through CR3 and CR4 on untreated MDMϕ. C3bi-dependent uptake of apoptotic cells via CR3 and CR4 has also been demonstrated by Takizawa *et al* using alternative apoptotic targets and phagocytes (Takizawa et al., 1996). This requirement for complement activation in the clearance of apoptotic cells is contrary to the common perception that apoptotic cell clearance is a non-inflammatory process, as it imposes the risk of assembly of membrane-attack complexes on the apoptotic cell surface and

inflammatory consequences following cell lysis. However, CR3-mediated phagocytosis by macrophages does not trigger release of leukotrienes (Aderem et al., 1985) or generate a respiratory burst (Wright and Silverstein, 1983), as well as suppressing production of pro-inflammatory cytokines like IL-12 (Marth and Kelsall, 1997). Interestingly, Mevorach *et al* rendered neutrophils apoptotic (20-30% annexin V+) following incubation in medium in the absence of serum for 6 hours, a time point when very low levels of necrosis were obtained (<5% propidium iodide-positive), indicating that the presence of necrosis was not required for C3bi-dependent phagocytosis (Mevorach et al., 1998a). However, untreated MDMϕ phagocytosis required significantly higher ratios of apoptotic targets to macrophages (40:1), and the pro-phagocytic effect of serum was significantly diminished following heat-inactivation of serum (56° for 30min) or depletion of C3b (Mevorach et al., 1998a). Of note, levels of phagocytosis in these studies were low and efficient uptake required longer interaction times of up to 3 or 4 hours (Takizawa et al., 1996). Furthermore, the methods used by Mevorach *et al* to measure phagocytic ability did not distinguish between binding and internalisation of apoptotic cells by MDMϕ, and indeed there appeared to be a significant amount of binding in their microscopy images of phagocytosis (Mevorach et al., 1998a). Through pre-treatment of serum with sCR1 to inhibit complement C3 activation, we have shown that serum enhanced apoptotic neutrophil uptake by Dex-MDMϕ independently of complement activation, suggesting that CR3 and CR4 receptor pathways are not utilised by Dex-MDMϕ. Although a dose of 250μg/ml sCR1 completely blocks complement activity as assessed by haemolytic assays (Paul Morgan, personal communication), experiments to confirm inhibition of C3bi opsonisation of apoptotic neutrophils were not performed in this study and could be assessed by immunofluorescence *via* flow cytometry or microscopy using specific mAbs. The lack of requirement for C3bi opsonisation in MDMϕ clearance of apoptotic neutrophils has been previously demonstrated *in vitro*, where blocking with mAb to macrophage CR3 and CR4 failed to affect uptake by untreated MDMϕ (Savill et al., 1989a), and also *in vivo* using CD11b- and CD18-deficient mice, which lack the CR3 receptor (Ren et al., 2001). Furthermore, macrophages from patients with leukocyte adhesion deficiency, a condition caused by deficiency of CD18, display normal phagocytic capacity for

apoptotic neutrophils (Davies et al., 1991). However, the consequence of complement receptor deficiency for serum-dependent phagocytosis by glucocorticoid-treated MDMϕ remains to be determined.

C1q, a ~440-kDa protein comprising six heterotrimeric chains arranged to form a C-terminal globular head region (GHR) and an N-terminal collagen-like tail region, is an important subcomponent of complement C1 that activates the classical pathway upon binding to immune complexes or CRP via the GHR recognition domain (Reid and Porter, 1976, Gaboriaud et al., 2003, Kishore et al., 2004). In addition to this function in activation of the complement cascade, C1q also interacts with cells to modulate their function. C1q binds specifically to the surface blebs of human apoptotic cells *in vitro* via the GHR domain (Korb and Ahearn, 1997, Navratil et al., 2001). Although surface blebbing during apoptosis in neutrophils is a rare process (Savill et al., 1989b, Payne et al., 1994), apoptotic neutrophils have been shown to bind C1q (Ogden et al., 2001, Donnelly et al., 2006). Interestingly, pre-incubation of apoptotic neutrophils with C1q increased subsequent ingestion by untreated human MDMϕ upon stimulation of a calreticulin/CD91 complex (Ogden et al., 2001), suggesting that C1q can function as an apoptotic cell opsonin. C1q binding occurs at an early stage of apoptosis, concurrent with exposure of PS on the surface of the apoptotic cell (Paidassi et al., 2008). The importance of C1q in apoptotic cell opsonisation has been highlighted by *in vivo* studies of C1q-deficiency (Botto, 1998, Taylor et al., 2000), where impaired clearance of apoptotic cells is thought to contribute to the development of an SLE-like autoimmune disease characterised by high titres of autoantibodies and accumulation of apoptotic cells in glomeruli of C1q-deficient mice. Interestingly, defective clearance of apoptotic cells is also observed in human SLE patients (Herrmann et al., 1998, Gaipl et al., 2006, Tas et al., 2006) and may contribute to disease pathogenesis (Mevorach et al., 1998b). Additionally, human C1q deficiency leads to SLE in ~90% of cases (Petry et al., 1997), suggesting that C1q may protect against development of SLE by targeting early apoptotic cells for clearance before they become membrane-permeable and expose self-antigenic material to the immune system (Paidassi et al., 2008). Indeed, C1q-deficient untreated human MDMϕ cultured in autologous serum show impaired phagocytosis

of apoptotic cells, and phagocytic ability can be restored with purified human C1q (Taylor et al., 2000). Furthermore, normal human serum depleted of C1q lost its pro-phagocytic activity on apoptotic cell clearance by untreated MDMϕ (Mevorach et al., 1998a, Kask et al., 2004). Interestingly, glucocorticoids increase C1q expression in human monocytes/macrophages (Armbrust et al., 1997, Faust et al., 2002, Ehrchen et al., 2007), suggesting that this innate protein may convey an anti-inflammatory function of glucocorticoids. We initially obtained encouraging data using commercially available C1q-depleted human serum, which failed to confer augmentation of apoptotic neutrophil uptake by Dex-MDMϕ. However, addition of purified human C1q during the phagocytosis assay in the absence of serum, or even when added along with the corresponding C1q-depleted human serum, did not restore phagocytosis by Dex-MDMϕ to the levels observed in the presence of autologous serum. One possible explaination is that the commercially obtained C1q was functionally inactive, and one approach to investigating this would be to perform a complement fixation test to assess the ability of C1q to activate complement-induced lysis of antibody-coated sheep red blood cells in the presence of C1q-depleted human serum. Binding of C1q to apoptotic neutrophils could also be evaluated by immunofluorescence *via* flow cytometry or microscopy using specific mAbs.

The lack of pro-phagocytic role for purified human C1q when added alone during phagocytosis was also observed by Mevorach *et al*, who suggested that instead of acting as a direct opsonin for apoptotic neutrophil clearance, C1q in the presence of serum may activate the complement system to generate C3b/iC3b opsonins for enhanced phagocytosis by untreated MDMϕ (Mevorach et al., 1998a). However, as discussed above, inhibition of C3 activation and subsequent generation of C3b/iC3b opsonins did not abrogate the serum-enhancing effect on Dex-MDMϕ. Moreover, C1q-deficient mouse serum was able to confer phagocytic activity, demonstrating that C1q is not involved. Alteratively, the method used to prepare commercially available C1q-depleted serum, where normal human serum was passed through an anti-C1q column, may have removed other serum proteins involved in the recognition of apoptotic neutrophils. Fibronectin, a high molecular weight

glycoprotein present in soluble form in plasma, is a functional C1q-binding protein (Pearlstein et al., 1982, Sorvillo et al., 1983) that can also bind to the surface of neutrophils (Hoffstein et al., 1981) and function as an opsonin *in vivo* and *in vitro* (Saba and Jaffe, 1980). Although adhesion of untreated human MDMφ to immobilised fibronectin rapidly augments uptake of apoptotic neutrophils (McCutcheon et al., 1998), soluble human plasma-derived fibronectin did not exert this effect (Vernon-Wilson et al., 2006). We did not find a pro-phagocytic effect for soluble fibronectin in apoptotic neutrophil uptake by Dex-MDMφ, even when used in combination with C1q. Several members of the pentraxin family have been demonstrated to bind C1q, including C-reactive protein, serum amyloid protein and pentraxin-3 (Hicks et al., 1992, Nauta et al., 2003a, Baruah et al., 2006). Interestingly, opsonisation with C1q has been suggested to target apoptotic cells to dendritic cells (Nauta et al., 2004), and pentraxin-3 binding to C1q in the fluid phase can inhibit C1q-mediated uptake of apoptotic cells by these phagocytes (Baruah et al., 2006). This suggests that binding to other serum opsonins may regulate C1q function in apoptotic cell clearance, both in terms of the phagocyte targeted and inflammatory outcome. In terms of macrophage phagocytic potential, pentraxin-3 has been suggested to inhibit phagocytosis of late apoptotic neutrophils (van Rossum et al., 2004). However, the phagocytic potential of Dex-MDMφ for apoptotic neutrophils was unaltered in the presence of pentraxin-3. IgM represents an additional C1q binding protein (Zwart et al., 2004) that functions as an apoptotic cell opsonin (Kim et al., 2003). However, a role for IgM in promoting phagocytosis by Dex-MDMφ has been disproved previously in our lab (data not shown).

Finally, examination of Dex-MDMφ phagocytosis of apoptotic neutrophils by time-lapse video microscopy indicated that phagocytosis was an active process mediated by extension of membrane process around the particle followed by rapid internalisation (Giles et al., 2001). This is not indicative of complement receptor-dependent phagocytosis, where complement-opsonised particles instead sink into the phagocyte surface and are engulfed without the extension of pseudopodia (Kaplan, 1977). Dex-enhanced phagocytosis of apoptotic neutrophils in a serum-dependent

but complement-independent manner has also been reported in a murine model of phagocytosis using Dex-treated mesangial cells (Cortes-Hernandez et al., 2002).

4.4.4 *The role of the annexin-1/FPR-L1 pathway in apoptotic cell clearance*

Annexin-1 is a glucocorticoid-inducible protein thought to mediate many of the anti-inflammatory actions of glucocorticoids *in vivo*, as demonstrated by altered expression of annexins, COX-2, and cytoplasmic phospholipase A2 (cPLA2); exaggerated responses to carrageenin- or zymosan-induced inflammation; and partial resistance to the anti-inflammatory effects of glucocortocoids in annexin-1-deficient mice (Hannon et al., 2003, Yang et al., 2004, Yang et al., 2006). Administration of exogenous annexin-1 conferred anti-inflammatory activity in some models of inflammatory disease (Arur et al., 2003), being particularly effective in the carrageenin paw oedema model where an intense accumulation of neutrophils is observed (Cirino et al., 1989). Conversely, infusion of annexin-1 antibodies neutralises the effect of annexin-1 and abrogates the anti-inflammatory activities of glucocorticoids (Duncan et al., 1993, Yang et al., 1999). It has been suggested that autoantibodies to annexin-1 may contribute to glucocorticoid resistance and the pathogenesis found in inflammatory diseases where glucocorticoids may be used as a treatment, such as rheumatoid arthritis and SLE (Goulding et al., 1989, Podgorski et al., 1992).

Although undetectable in plasma, annexin-1 is expressed in many leukocytes and tissues (Morand et al., 1995, Voermans et al., 1997, Solito et al., 1998, de Coupade et al., 2000) and has been suggested to have a multifactoral role in modulating apoptotic neutrophil clearance. Predominately localised in the cytosol of resting neutrophils (Perretti, 1998), annexin-1 may undergo caspase-dependent recruitment to the outer plasma membrane during apoptosis and colocalise with surface-exposed PS to facilitate recognition by macrophages (Arur et al., 2003). Additionally, annexin-1 released by macrophages (Maderna et al., 2005) or apoptotic neutrophils (Scannell et al., 2007) may function as a pro-phagocytic factor, actively promoting FPRL1-dependent clearance of apoptotic neutrophils by human MDMϕ in a non-

phlogistic and concentration-dependent manner, and bone marrow-derived macrophages from annexin-1-deficient mice show defective phagocytosis of apoptotic neutrophils compared to wild-type mice (Maderna et al., 2005). Interestingly, expression and release of annexin-1 by murine neutrophils (Perretti and Flower, 1996) and human monocytes/macrophages (Comera and Russo-Marie, 1995, Maderna et al., 2005), and FPRL1 expression by human monocytes (Sawmynaden and Perretti, 2006) are increased following glucocorticoid treatment. Furthermore, soluble annexin-1 binds in a PS-dependent manner to promote tethering and internalisation of apoptotic cells, suggesting an opsonic function (Arur et al., 2003). However, experiments using a FPRL1 antagonist, or using annexin-1-deficient mouse serum demonstrated that this pathway is not utilised by Dex-MDMϕ for recognition of serum-opsonised apoptotic neutrophils. This may also rule out a role for LXA4, another FPRL1 ligand known to stimulate non-phlogistic phagocytosis of apoptotic neutrophils by human MDMϕ *in vitro* (Godson et al., 2000) and *in vivo* (Mitchell et al., 2002), however additional experiments using a purified form of lipoxin A4 are required in order to evaluate the precise role of this molecule with respect to Dex-MDMϕ phagocytic potential. Annexin-1 is a relatively small molecule of 37-kDa (Hannon et al., 2003), hence the lack of requirement for annexin-1 opsonisation is consistant with data obtained from serum fractionation experiments, where phagocytic activity was only present in serum fractions containing components larger than 50-kDa in molecular mass.

4.4.5 *Platelet-derived factors are not required for phagocytosis of apoptotic neutrophils by Dex-MDMϕ*

Autologous serum in our system was prepared by recalcification of human PRP, a process that induces platelet aggregation and the release of platelet factors (Browder et al., 2000). TSP-1 is a platelet-derived product that has been reported to enhance clearance of apoptotic neutrophils through a CD36/αvβ3 receptor complex on the surface of untreated human MDMϕ (Savill et al., 1992). Enhanced macrophage recognition was still evident if apoptotic neutrophils were pre-incubated with TSP-1, indicating that TSP-1 may function as an opsonin of apoptotic neutrophils (Savill et

al., 1992). The role of CD36 class B scavenger receptor and $\alpha v\beta3$ vitronectin receptor in the uptake of serum-opsonised apoptotic neutrophils has been demonstrated in blocking studies using specific mAbs and the tetrapeptide arg-gly-asp-ser (RGDS) to block CD36 and $\alpha v\beta3$ integrin receptor function, respectively (Savill et al., 1990, Savill et al., 1992). Mevorach *et al* also observed inhibition of serum-dependent uptake of apoptotic cells by untreated human MDMϕ in the presence of RGDS peptide, albeit the inhibition was only partial (20%) (Mevorach et al., 1998a). Although Cortes-Hernandez *et al* demonstrated serum-dependent phagocytosis of murine apoptotic cells by Dex-treated murine glomerular mesangial cells to be entirely inhibitable by RGDS tetrapeptide (Cortes-Hernandez et al., 2002), inhibitors of CD36 (smϕ mAb 1:50 ascites) and $\alpha v\beta3$ (0.5mM RGDS peptide) did not prevent serum-dependent phagocytosis of apoptotic neutrophils by human Dex-MDMϕ (Giles et al., 2001). It is possible that augmentation of phagocytosis following treatment with glucocorticoids may require different receptor mechanisms based on phagocytic cell type or species origin. I have demonstrated in this chapter that PPP still contains phagocytic activity whereas platelet releasate displayed no enhancing effect on Dex-MDMϕ. Taken together, these data indicate that the CD36/$\alpha v\beta3$/TSP-1 pathway does not play a major role in apoptotic neutrophil uptake by Dex-MDMϕ in an entirely human system. Furthermore, this may rule out a role for MFG-E8, a bridging molecule that binds to PS on apoptotic cells to promote their removal through $\alpha v\beta3$ integrin on macrophages (Hanayama et al., 2002). However, future experiments are required to test the phagocytic potential of human purified MFG-E8 on apoptotic neutrophil ingestion by Dex-MDMϕ.

In this chapter, I employed an "add back" approach to assess the contribution of well-defined opsonins in clearance of apoptotic neutrophils by Dex-MDMϕ. We have shown phagocytosis to be serum-dependent, requiring a protein opsonin larger than 50-kDa in molecular weight that is not an antibody, complement protein, annexin I, or a platelet-derived factor. To identify the component, we next undertook a more investigative approach to obtain a serum fraction with pro-phagocytic activity using chromatography techniques.

CHAPTER 5: PURIFICATION OF THE PRO-PHAGOCYTIC PROTEIN USING CHROMATOGRAPHY TECHNIQUES

5.1   Introduction

Based upon the data presented in chapters 3 and 4, I have demonstrated that highly efficient clearance of apoptotic neutrophils by Dex-MDMϕ involves a serum-dependent mechanism, and using an "add-back" approach during *in vitro* phagocytosis assays I was unable to demonstrate a requirement for several well-characterised apoptotic cell opsonins. A limitation to using whole serum for identification of our apoptotic cell opsonin is that serum represents a highly complex mixture of proteins, and while many of these proteins such as SP-A, SAP and complement proteins have been suggested to promote apoptotic neutrophil clearance by untreated macrophages when added individually to *in vitro* "serum-free" phagocytosis assays (Mevorach et al., 1998a, Schagat et al., 2001, Mold et al., 2002), only deficiency in C1q or MFG-E8 appears to have significant *in vivo* consequences in terms of impaired clearance of apoptotic cells (Botto, 1998, Taylor et al., 2000, Hanayama et al., 2004), suggesting possible redundancy in serum-dependent apoptotic cell clearance mechanisms. It was therefore unlikely that the serum opsonin would be discovered using a "scouting" approach. Proteins can be purified from complex sources using chromatography techniques that separate them according to differences in specific properties. We hypothesised that our serum opsonin could be isolated from human serum using a multi-step purification scheme involving anion exchange and gel filtration chromatography to fractionate serum proteins based on surface charge and molecular weight, respectively. Importantly, these chromatography methods offer high binding capacities and high-resolution separation, make them ideal for purification of proteins from serum. Indeed, ion exchange and/or gel filtration chromatography techniques have been applied in several studies for protein purification from human plasma/serum, including isolation of ferritin (Worwood et al., 1976), IgA (Loomes et al., 1991) and complement proteins (Hammer et al., 1981), and more specifically for isolation of proteins

involved in apoptotic cell clearance, such as human C-reactive protein (Mold et al., 2001), human C1q (Kishore et al., 1998) and human fibronectin (Buck et al., 1992).

5.1.1 Purification strategy

In order to test our hypothesis that the pro-phagocytic factor could be isolated from a complex protein mixture in human serum, we firstly decided to fractionate serum proteins according to their net surface charge using anion exchange chromatography. This technique is recommended as a first step in purification (i.e. protein capture) when little is known about the target molecule. We chose to perform anion exchange using positively charged mono Q Sepharose that associates only with negatively charged proteins or "anions". The net charge on a given protein and hence its ability to bind to mono Q Sepharose can be significantly influenced by altering the buffering conditions, especially pH. The isoelectric point (pI) of a protein, the pH at which a particular protein has an overall neutral charge and will not bind to either a cationic (negative) or anionic (positive) exchanger, can be used to determine protein binding capabilities at a particular pH. Only proteins with a pI below the pH of the buffering solution will have a net negative charge and hence bind to the anion exchanger. Bound proteins are eluted from mono Q Sepharose by increasing the ionic strength of the buffering solution. To achieve better separation of crude protein fractions containing the pro-phagocytic activity of serum obtained following optimisation of anion exchange chromatography, we decided to further fractionate proteins according to molecular weight *via* gel filtration. Since preliminary experiments indicated the serum opsonin to be larger than 100-kDa in molecular mass (see Chapter 4, *fig. 4.1b*), we decided to use Sephacryl S-300 gel. Fractionation by gel filtration is based on differential diffusion of proteins into the gel; high molecular weight proteins (HMW) do not enter the gel pores and pass through the column quicker than low molecular weight proteins (LMW). Thus, proteins elute from the column in order of decreasing molecular weight, allowing size-specific fractions to be tested for pro-phagocytic activity.

5.2   Materials and methods

5.2.1   *Serum proteins and antibodies*

Protein S, obtained from Enzyme Research Laboratories, was isolated from human plasma and supplied as lyophilized powder (>95% purity by SDS PAGE analysis) that was reconstituted at 1mg/ml in dH$_2$O.

5.2.2   *Induction of neutrophil apoptosis*

CMFDA-labelled neutrophils were cultured at 4 x 10^6/ml in serum-free medium (IMDM) at 37°C for 20-24 hours to render neutrophils apoptotic. Neutrophil populations were then centrifuged at 200 x *g* for 5min and resuspended in fresh IMDM prior to assessment of phagocytosis.

5.2.3   *Flow cytometry*

Neutrophils cultured for 20-24 hours in serum-free conditions were pre-incubated for 1 hour with 2.5μg/ml human protein S or 10% autologous serum as a source of protein S, then incubated for 30min with 10μg/ml of unlabelled polyclonal rabbit anti-human protein S antibody or rabbit IgG as a control, washed twice then incubated for 30min with a secondary FITC-conjugated anti-rabbit antibody. Neutrophil cultures were washed twice before assessment of protein S binding by flow cytometry. IMDM was used throughout for incubations and washes. Duplicate wells were used for each experiment, and the experiment was repeated twice more using different neutrophil donors.

5.3   Results

5.3.1   *Serum fractionation by anion exchange chromatography using variations in pH: the serum factor binds at pH7*

Many serum proteins have a pI of around 5 – 8, some of which are indicated in *Table 1*. We hypothesised that altering the pH of the buffering solution used for anion exchange chromatography would allow binding and hence purification of different proteins from serum. In order to investigate this, we prepared buffering solutions at pH6 (50mM MES and 0.14M NaCl), pH7 and pH8 (50mM HEPES and 0.14M NaCl) and fractionation of serum proteins by anion exchange was performed as previously described (see Chapter 2, 2.10.1.1 Optimisation of pH). As shown in Figure 5.1, the pro-phagocytic component in fresh human serum was bound to mono Q Sepharose at pH7 or pH8 at an ionic strength of 0.14M NaCl, and was eluted with 0.5M NaCl. Furthermore, the component bound very efficiently at pH7 and pH8, as there was no phagocytic activity in the flow through at these pH values (*fig. 5.1*). No significant phagocytic activity was found in either the flow through or eluted fractions when ion exchange was performed using pH6 buffer (50mM MES and 0.14M NaCl). One possibility is that slight acidification may have resulted in irreversible loss of the serum factor required for enhanced Dex-MDMϕ phagocytosis, an effect that could be tested *via* acidification of human serum prior to addition to phagocytosis assays. Alternatively, the serum factor may only be stable above pH6, resulting in reduced binding to mono Q Sepharose at pH6.

Thus, the pro-phagocytic component(s) of human serum bound to mono Q Sepharose at pH7, indicating that the component(s) has a pI value below 7. We chose to perform subsequent anion exchange chromatography experiments at pH7 since we anticipated that fewer proteins would bind at this pH and thus facilitate identification of the component.

Protein	pI value	Reference
Albumin	4.7	www.sigmaaldrich.com
Thrombospondin	4.7	(Lawler et al., 1978)
Alpha-2 macroglobulin	5.2	(Barrett et al., 1979)
Protein S	5 – 5.5	(DiScipio and Davie, 1979)
IgM	5.5 – 6.7	(Prin et al., 1995)
C4BP	6.7	(Nagasawa and Stroud, 1980)

Table 1: <u>Approximate pI values of proteins in human plasma/serum.</u>
Abbreviations: C4BP, C4-binding protein; pI, isoelectric point.
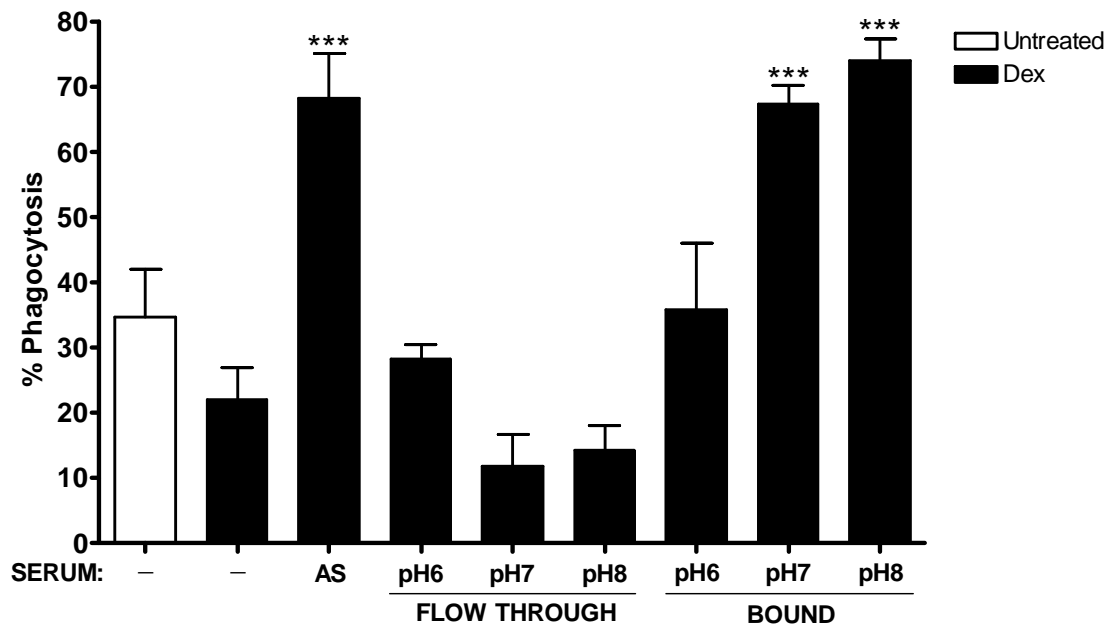
Figure 5.1: <u>Isolation of a fraction displaying the pro-phagocytic activity of serum by anion exchange chromatography.</u>
MDMϕ were cultured in the absence or presence of 1μM Dex for 5 days, and phagocytosis of <u>serum-free cultured</u> heterologous apoptotic neutrophils by untreated (white bars) and Dex-treated (black bars) MDMϕ was assessed by flow cytometry. Fresh human serum proteins bound to mono Q Sepharose at pH7 or pH8 (50mM HEPES and 0.14M NaCl) enhanced uptake of apoptotic neutrophils by Dex-MDMϕ, with no pro-phagocytic activity observed in the flow through at these pH values. No pro-phagocytic activity was observed in either bound or flow through fractions obtained from anion exchange of human serum at pH6 (50mM MES and 0.14M NaCl). Mean phagocytosis $\pm$ SEM, n=3 where n = the number of different macrophage donors. (*** $p<0.001$ when compared to uptake by Dex-MDMϕ in the absence of serum, one-way ANOVA with a Bonferroni post test). Phagocytosis by untreated MDMϕ is shown for comparison.

5.3.2 *Serum fractionation by anion exchange chromatography using a salt gradient: 0.2M fractions contain the pro-phagocytic activity*

Proteins display different binding capacities that can influence how/when they are eluted from mono Q Sepharose. We hypothesised that altering the ionic strength of the buffering solution used during the elution step would allow further separation of proteins according to strength of binding. In order to investigate this, fresh serum proteins bound to mono Q Sepharose at pH7 were eluted using a linear salt gradient of 0.2M to 0.5M NaCl prepared in pH7 buffer (50mM HEPES) as described in Chapter 2, 2.10.1.2 Optimisation of ionic strength, and elution of bound proteins was determined spectrophotometrically at 280nm (*fig. 5.2a*). Fractions were resolved by SDS PAGE on 9% gels under reducing conditions, as described in Chapter 2, 2.13 SDS polyacrylamide gel electrophoresis (*fig. 5.2b*). Most serum proteins bound to mono Q Sepharose at pH7 were eluted with 0.2M NaCl, with less protein evident in 0.3M NaCl elutions and negligible protein in 0.4M and 0.5M NaCl elutions (*figs. 5.2a and b*). Protein concentration in 0.2M and 0.3M fractions, estimated by the Pierce BCA method, was routinely found to be 15mg/ml and 1.5mg/ml, respectively. When added at a final concentration of 1.5mg/ml, we found that 0.2M NaCl, but not 0.3M NaCl elutions contained the pro-phagocytic activity of serum (*fig. 5.2c and d*). The effect on Dex-MDMϕ phagocytosis of apoptotic neutrophils was found to be dependent on the protein concentration of the 0.2M fraction and reached significance at 100µg/ml (*fig. 5.3*), suggesting that the pro-phagocytic component may be a prominent protein in 0.2M fractions.

Thus, we have shown that the pro-phagocytic component(s) of serum binds to mono Q Sepharose at pH7 at an ionic strength of 0.14M NaCl, and can be eluted with 0.2M NaCl, suggesting that the component(s) binds loosely at this pH.
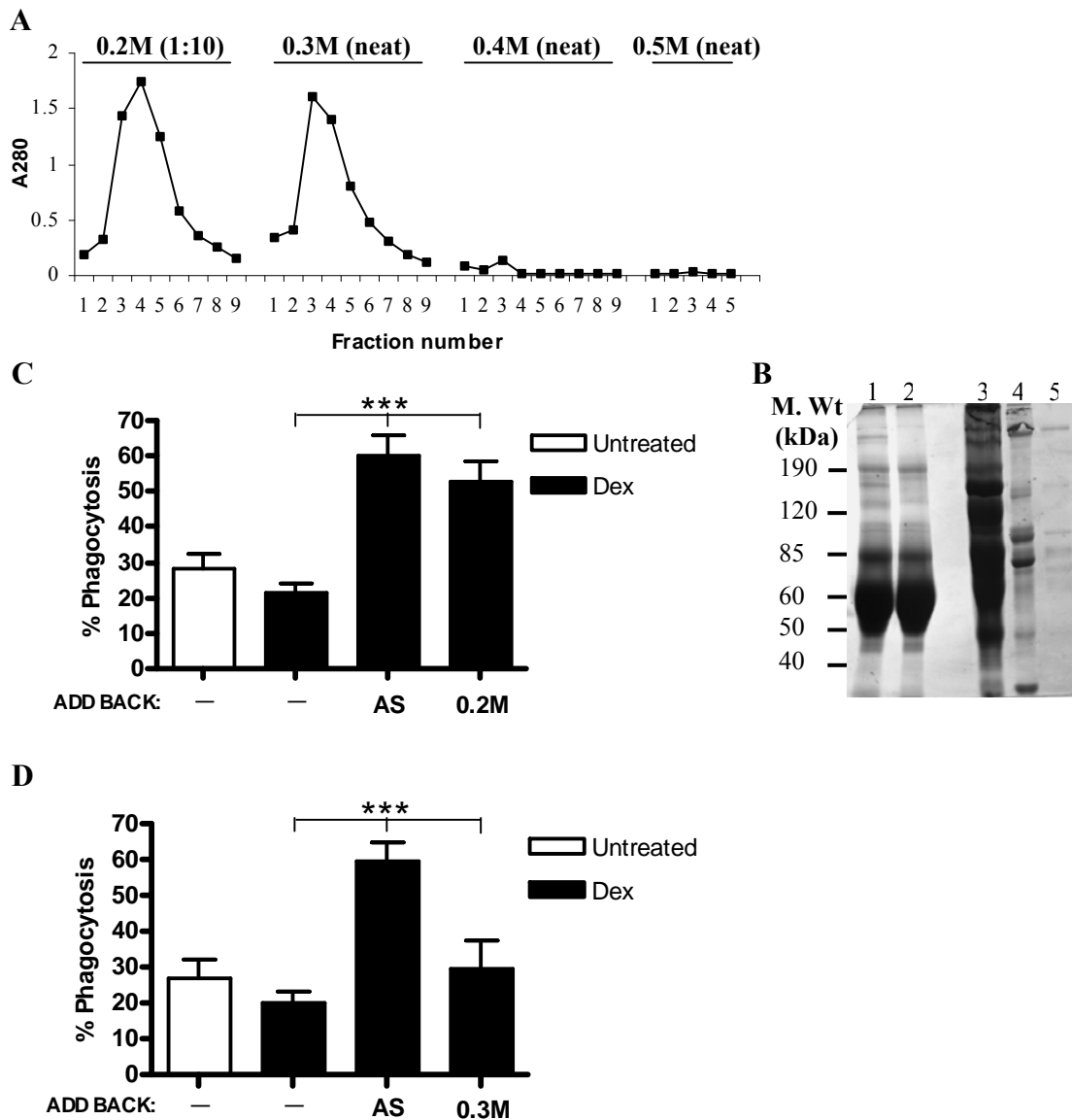
Figure 5.2: <u>0.2M anion exchange fractions contain the pro-phagocytic activity of serum.</u>

A, A280 measurements of anion exchange fractions eluted from mono Q Sepharose at pH7 using a salt gradient. **B**, Anion exchange fractions were resolved by SDS PAGE on 9% gels under reducing conditions then stained with Coombassie blue. Lane 1, 1:10 pH7-equilibrated serum; lane 2, 1:10 pH7 anion exchange flow through; lane 3, 0.2M elution; lane 4, 0.3M elution; lane 5, 0.4M elution. Anion exchange fractions were not standardised, showing that bound protein was mostly eluted with 0.2M NaCl. **C** and **D**, Serum proteins bound to mono Q Sepharose at pH7 and eluted with 0.2M NaCl (**D**, n=6), but not 0.3M NaCl (**C**, n=3) (final concentration of 1.5mg/ml used for each) enhanced uptake of heterologous apoptotic neutrophils by Dex-MDMϕ to a similar extent as unfractionated human serum. Mean phagocytosis ± SEM (*** $p < 0.001$ when compared to uptake by Dex-MDMϕ in the absence of serum, one-way ANOVA with a Bonferroni post test). N = the number of different macrophage donors.
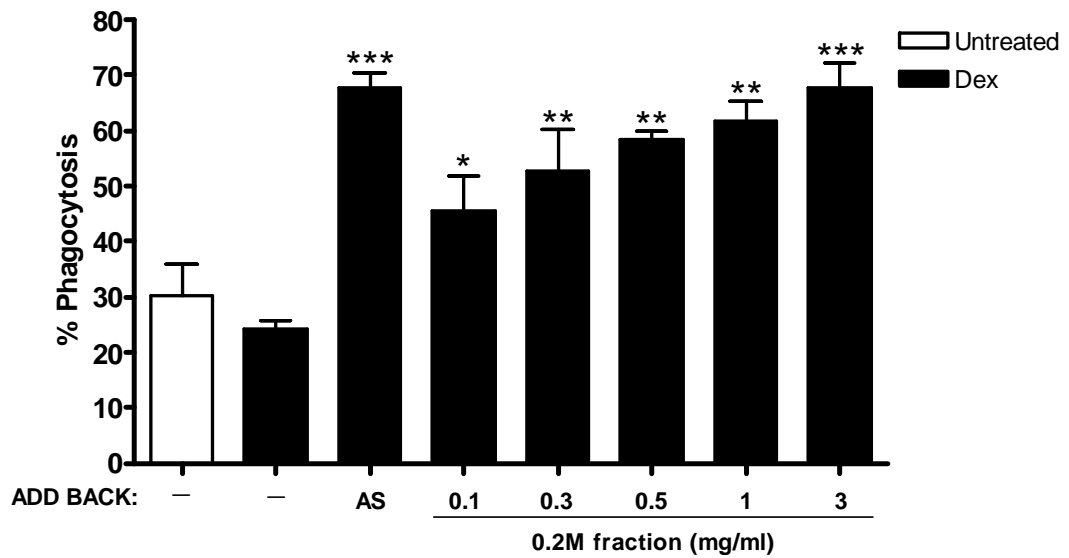
Figure 5.3: Effect of altering the protein concentration of 0.2M anion exchange fractions on uptake of apoptotic neutrophils by Dex-MDMφ.

Phagocytosis assays were performed as described in Figure 5.1. 0.2M anion exchange fractions enhanced Dex-MDMφ phagocytosis of heterologous apoptotic neutrophils in a concentration-dependent manner, reaching half-maximal and maximal activity at a final concentration of 0.1mg/ml and 3mg/ml, respectively. Mean phagocytosis $\pm$ SEM, n=3 where n = the number of different macrophage donors. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ when compared to uptake by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test).

5.3.3 *Identification of a high molecular weight fraction with phagocytic activity by gel filtration*

Selection of the appropriate buffering pH for protein binding and ionic strength of buffer for protein elution allowed optimal isolation of the pro-phagocytic protein from fresh human serum during anion exchange chromatography. However, a considerable number of protein contaminants were also evident when 0.2M fractions were resolved by SDS PAGE followed by staining with Coomassie blue (*fig. 5.2b*). In order to acheive better resolution, we decided to further separate proteins in 0.2M anion exchange elutions according to molecular weight by gel filtration.

A Sephacryl S-300 gel filtration column was calibrated with protein standards using the same sample volume (250μl), flow rate (2ml/min) and fraction volume (1.6ml/tube) throughout (as described in Chapter 2, 2.10.2.1 Calibration of the gel filtration column) and elution of proteins was determined spectrophotometrically at 280nm (*fig. 5.4a*). The volume of 1 x TBS buffer required to elute protein standards was calculated by measuring the volume collected from the point of protein application to the centre of the elution peak (*Table 2*). Two partially overlapping peaks of protein with descending molecular weight were consistently obtained following fractionation of 0.2M anion exchange samples (*figs. 5.4a and b*) (McColl et al., 2009). We have shown the serum opsonin to be calcium-dependent, so gel filtration fractions were reconstituted with 1mM $CaCl_2$ prior to addition to phagocytosis assays. Fractions A and B from the leading edge of the first peak, representing HMW proteins (>300-kDa) were found to contain the pro-phagocytic activity of serum, whereas LMW protein fractions were devoid of phagocytic activity (*fig. 5.4c*) (McColl et al., 2009).
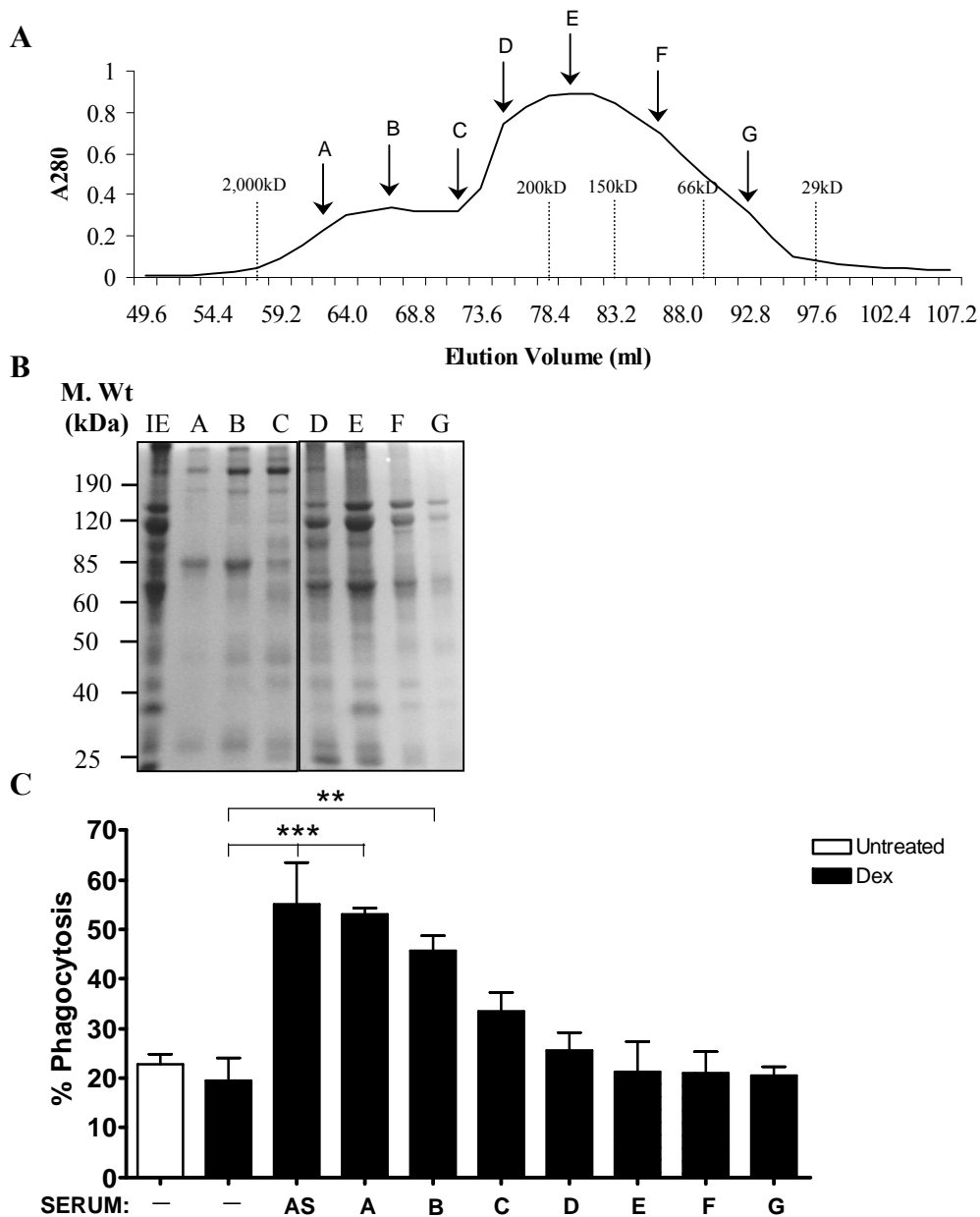
Figure 5.4: High molecular weight gel filtration fractions contain the pro-phagocytic activity of serum.

Serum proteins were fractionated by anion exchange followed by gel filtration of 0.2M elutions. **A**, Protein elution profile of a typical gel filtration separation (of 4 that were performed) determined by measurement of absorbance at 280 nm (A280) reveals two partially overlapping peaks of protein. **B**, Gel filtration samples (labelled A-G) were separated on a 9% acrylamide gel under reducing conditions and bands visualised with 0.5% Coomassie blue (IE: 0.2M ion exchange fraction). **C**, Gel filtration fractions (labelled A-G) were standardised for protein content (final concentration of 50μg/ml). HMW fractions A and B significantly augmented Dex-MDMϕ (black bars) phagocytosis of heterologous apoptotic neutrophils. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (** $p < 0.01$, *** $p < 0.001$ when compared to uptake by Dex-MDMϕ in the absence of serum, one-way ANOVA with a Bonferroni post test).

Protein standard (molecular weight)	Ve
Blue dextran (2,000-kDa)	57.6ml
β-amylase (200-kDa)	78.4ml
Alcohol dehydrogenase (150-kDa)	83.2ml
Albumin (66-kDa)	89.6ml
Carbonic anhydrase (29-kDa)	97.6ml
Cytochrome c (12.4-kDa)	110.4ml

TABLE 2: Determination of the elution volume (Ve) for proteins standards.
To calculate the elution volume, the volume of effluent collected from the point of sample application to the centre of the effluent peak was measured.

5.3.4 *Analysis of high molecular weight gel filtration fractions by Mass Spectrometry: Identification of C4-binding protein, IgM and alpha-2 macroglobulin as candidate proteins*

HMW proteins in gel filtration fraction A were resolved by SDS PAGE using 12% gels under reducing conditions (*fig. 5.5*). Analysis of protein bands by mass spectrometry revealed three of the principle proteins present in this active fraction as high molecular mass IgM, α2-macroglobulin and C4-binding protein (C4BP) (*fig.5. 5*). Previous work has eliminated a role for IgM in the augmentation of Dex-MDMϕ phagocytosis of apoptotic cells (data not shown). Addition of 20μg/ml α2-macroglobulin failed to restore augmented phagocytosis by Dex-MDMϕ, suggesting that α2-macroglobulin was not involved (*fig. 5.6a*; p>0.05, n=3) (McColl et al., 2009). C4BP acts to prevent excessive complement activation and cell lysis occurring as a consequence of assembly of the membrane attack complex. In order to investigate whether C4BP contributed to the prophagocytic effect of serum on Dex-MDMϕ, we attempted to immunodeplete C4BP from 0.2M anion exchange fractions using a mouse anti-human C4BP mAb (see Chapter 2, 2.12 Protein immunodepletion from human serum). However, 0.2M fractions resolved on 12% gels and immunoblotted for C4BP demonstrated that C4-binding protein (~75-kDa) could not be effectively depleted using this mAb (*fig. 5.6b*). Interestingly, the multiple C4BP bands observed under reducing conditions correlate well with the protein bands observed on 12% reduced gels stained with Coombassie blue (*fig. 5.5* and *6b*). The slight decrease in phagocytosis observed for C4BP- and control-depleted fractions may be a consequence of dilution of the phagocytic activity upon addition of a large volume of anti-C4BP in attempt to successfully deplete C4BP, or mouse IgG as a control (20μl per 100μl 0.2M fractions for each of three rounds of depletion) (*fig. 5.6b* and *c*).
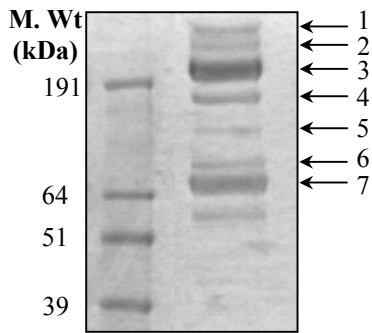
Band	Hypothetical Protein (s)	MW (kDa)
1	Lipoprotein	~233
2	Fibronectin 1 isoform 6 preproprotein	~ 243
3	IgM heavy chain	~ 50
4	C4-binding protein alpha chain Alpha2 macroglobulin	~ 69 ~ 162
5	C4-binding protein alpha chain	~ 69
6	Cullin 4A isoform 1	~ 88
7	C4-binding protein alpha chain IgM heavy chain	~ 69 ~ 50

Figure 5.5: Analysis of high molecular weight fractions by mass spectrometry. Proteins in HMW gel filtration fraction A were resolved by SDS PAGE using 12% gels under reducing conditions. Bands selected for analysis by mass spectrometry are numbered according to decreasing molecular weight. The accompanying table shows the putative proteins present in each band. Abbreviations: MW, molecular weight.
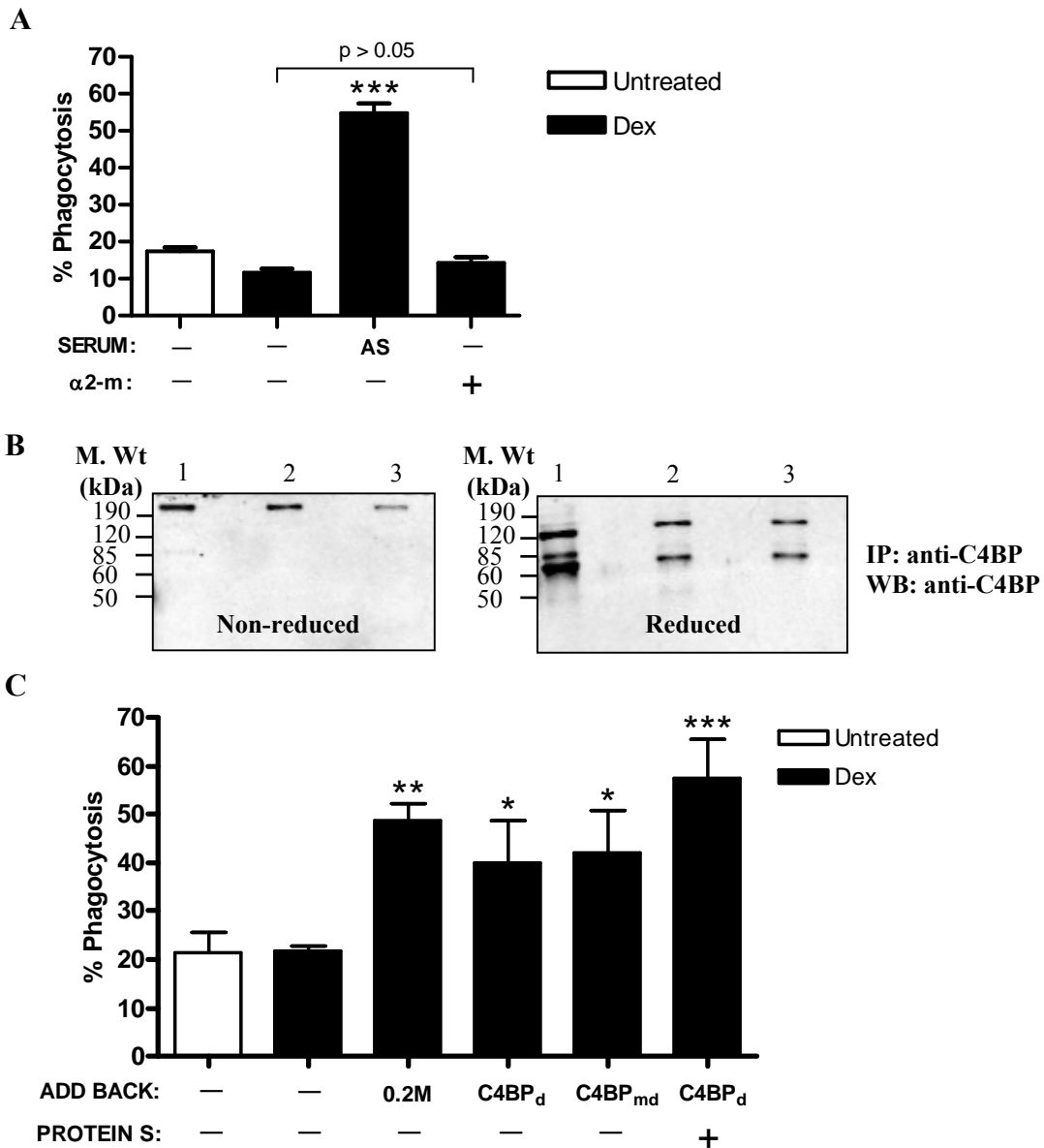
Figure 5.6: Effect of alpha-2 macroglobulin and C4BP on uptake of apoptotic neutrophils by Dex-MDMφ.

A, 20μg/ml α2-macroglobulin failed to enhance uptake of heterologous apoptotic neutrophils by Dex-MDMφ (black bars). **B**, 0.2M anion exchange fractions were separated on a 9% gel under reducing or non-reducing conditions and immunoblotted for C4BP using 1:500 anti-C4BP and 1:2500 HRP-conjugated goat anti-mouse Ig. Lane 1, 0.2M fraction; lane 2, C4BP-depleted 0.2M fraction; lane 3, mock control-depleted 0.2M fraction. Immunodepletion experiments using a mouse anti-human C4BP mAb failed to successfully deplete C4BP (**B**) or reduce the pro-phagocytic effect of 0.2M fractions when compared to a mouse IgG mock control depletion (**C**). (0.2M, 0.2M eluate; C4BP$_d$, 0.2M C4BP-depleted; C4BP$_{md}$, 0.2M mock C4BP-depleted; +, 250 ng/ml purified protein S). Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ when compared to uptake by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test).

5.3.5 *Protein S promotes phagocytosis of apoptotic neutrophils by Dex-MDMϕ*

C4BP exists as a >570-kDa high affinity complex with protein S (Dahlback et al., 1983). Whilst the C4BP-protein S complex has been suggested to inhibit phagocytosis of apoptotic cells, free protein S has been reported to stimulate uptake of early, membrane-intact (annexin V+/propidium iodide-) apoptotic cells by untreated human MDMϕ (Anderson et al., 2003, Kask et al., 2004). Protein S exists in human plasma at ~25μg/ml, and addition of 25μg/ml human purified protein S to the assay medium fully restored Dex-MDMϕ phagocytosis of apoptotic neutrophils to levels conferred by 10% autologous seurm (*fig. 5.7*). To determine the effect of protein S concentration on Dex-MDMϕ phagocytosis of apoptotic neutrophils, we altered the final concentration added to apoptotic neutrophils before use in a phagocytosis assay. The effect of protein S on Dex-MDMϕ phagocytosis was found to be dose-dependent, reaching half-maximal activity at 75ng/ml (*fig. 5.7*; * $p<0.05$, n=3) with full stimulatory effects observed at concentrations of 250ng/ml or above (*fig. 5.7*; *** $p<0.001$, n=3) (McColl et al., 2009). The pro-phagocytic effect of protein S was lost at 25ng/ml (equivalent to 1:1000 concentration in serum). As the most significant augmentation of Dex-MDMϕ phagocytosis was observed using 2.5μg/ml protein S, a concentration found in 10% human serum, we used this concentration for all subsequent experiments.
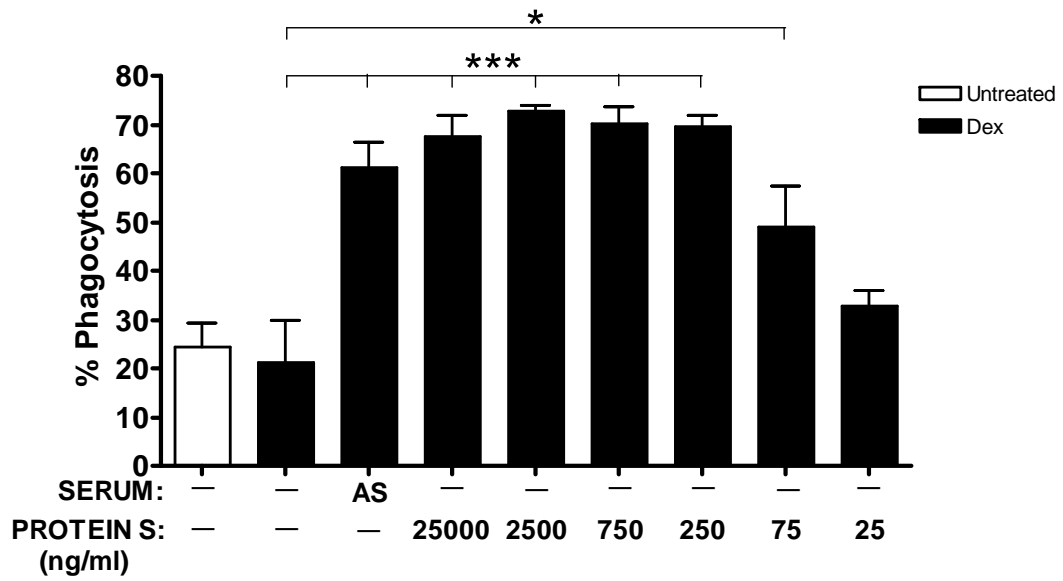
Figure 5.7: Effect of protein S on uptake of apoptotic neutrophils by Dex-MDMϕ.
Phagocytosis assays were performed as described in Figure 5.1. Purified human
protein S enhanced phagocytosis of serum-free cultured heterologous apoptotic
neutrophils by Dex-MDMϕ (black bars) in a concentration-dependent manner. Mean
phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (*,
$p < 0.05$; *** $p < 0.001$ when compared to uptake by Dex-MDMϕ in the absence of
serum, one-way ANOVA with a Bonferroni post test).

5.3.6 *Pro-phagocytic activity in 0.2M anion exchange fractions was lost upon depletion of protein S*

To find the best concentration of anti-protein S antibody for immunoblotting, several wells of 15μl of 2.5μg/ml purified human protein S were resolved by SDS PAGE under non-reducing conditions using 9% gels. Following transfer to PVDF (80V for 50min), the membrane was cut and immunoblotting performed using different dilutions of anti-protein S (*fig. 5.8a*). A dilution of 1:5000 was deemed to produce the most satisfactory results and was used for subsequent protein S immunoblotting experiments.

Although protein S was not identified by mass spectrometry, we confirmed the presence of protein S in 0.2M fractions eluted from mono Q Sepharose at pH7 and in HMW gel filtration fractions, presumably in complex with C4BP (*figs. 5.8b* and *c*). To investigate whether protein S was the protein in human serum required for augmented phagocytosis by Dex-MDMϕ, we immunodepleted protein S from 0.2M anion exchange fractions using a polyclonal rabbit anti-human protein S antibody, and rabbit IgG was added to the same volume of 0.2M fractions as a control (see Chapter 2, 2.12 Protein immunodepletion from human serum). 0.2M samples were resolved on 9% agarose gels under non-reducing conditions and immunoblotting with anti-protein S antibody provided a single band for protein S at ~80-kDa that was still evident in control-depleted 0.2M samples, but absent from protein S-depleted 0.2M samples (*fig. 5.8c*). The molecular weight reported here for human protein S is similar to that found in previous studies (Lu et al., 1997). Protein S-depleted 0.2M fractions were devoid of phagocytic activity, while control preparations retained the ability to stimulate Dex-MDMϕ phagocytosis of apoptotic neutrophils (*fig. 5.8d*) (McColl et al., 2009). Addition of 250ng/ml human protein S to protein S-depleted 0.2M fractions restored augmented phagocytosis by Dex-MDMϕ (*fig. 5.8d*). Loss of pro-phagocytic activity upon specific immunodepletion of protein S from 0.2M fractions implies that protein S is an important pro-phagocytic component in serum. Together with data presented in Figure 5.3c, these data suggested that protein S, possibly in complex with C4BP, was required to confer full phagocytic capacity of Dex-MDMϕ.
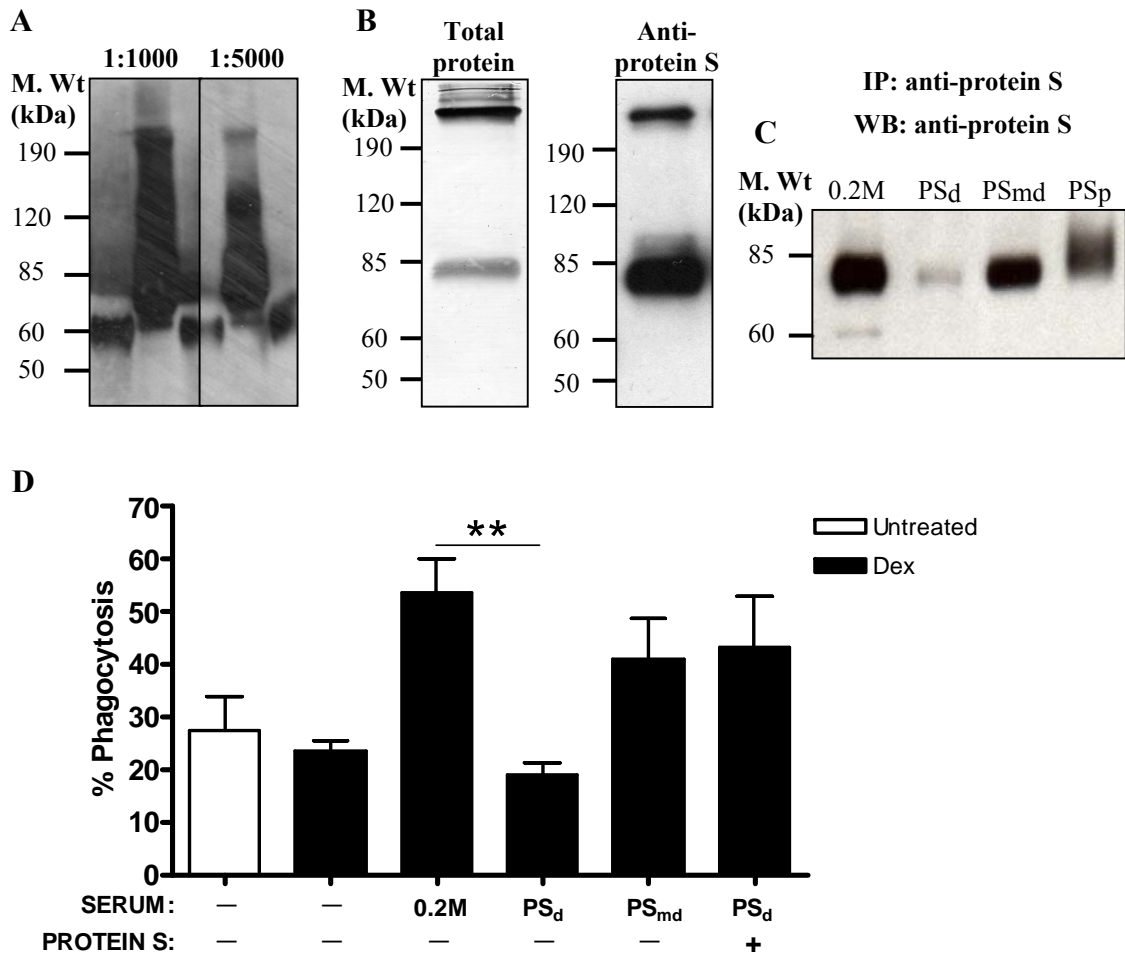
Figure 5.8: <u>Effect of protein S depletion on the pro-phagocytic activity of 0.2M anion exchange fractions.</u>

A, B and **C**, Protein samples were resolved by SDS PAGE on 9% gels under non-reducing conditions. **A**, Protein S immunoblot, performed with 2.5µg/ml purified protein S, 1:1000 and 1:5000 dilutions of an anti-protein S polyclonal antibody and 1:2500 HRP-conjugated goat anti-rabbit Ig. **B**, Immunoblotting with 1:5000 anti-protein S showed that protein S is present in HMW gel filtration fraction A. Blots were also stained with colloidal gold to show total protein content. **C**, The presence of protein S (~80-kDa monomer) in 0.2M anion exchange fractions was confirmed by immunoblotting. Protein S was effectively depleted from 0.2M fractions using a polyclonal rabbit anti-protein S Ab, but not control rabbit IgG. (0.2M, 0.2M eluate; PSd, 0.2M protein S-depleted; PSmd, 0.2M mock protein S-depleted; PSp, 250 ng/ml purified protein S). **D**, Protein S depletion significantly reduced the pro-phagocytic effect of 0.2M fractions (protein concentration ~150µg/ml) when compared to mock control depletions, an effect that was restored upon addition of 250ng/ml protein S to depleted sera. Mean phagocytosis ± SEM, n=4 where n = the number of different macrophage donors. (** $p < 0.01$, when compared to uptake by Dex-MDMϕ in the presence of 0.2M fractions, one-way ANOVA with a Bonferroni post test).

5.3.7 *Reduction and alkylation of gel filtration fractions to irreversibly dissociate disulfide-bonded proteins: effect on pro-phagocytic activity*

Although immunodepletion of protein S from 0.2M anion exchange fractions resulted in loss of pro-phagocytic activity that could be restored upon "add-back" of commercially purified human protein S (*fig. 5.8d*), it was unclear whether serum-derived human protein S in free form or in complex with C4BP, or indeed both, was required for the serum augmentation effect on Dex-MDMϕ phagocytosis. Based upon data obtained from gel filtration experiments, which strongly indicated that fractions conferring Dex-MDMϕ phagocytic capacity were of HMW (>300-kDa) (*fig. 5.4c*), we hypothesised that protein S in complex with C4BP also enhanced phagocytosis. In order to address the role of the C4BP-protein S complex, we attempted to irreversibly dissociate protein complexes into single polypeptide chains through reduction and alkylation using 5mM DTT and 25mM IOA, respectively (see Chapter 2, 2.11 Reduction and alkylation). Samples were resolved by SDS PAGE on 9% acrylamide gels followed by staining with Coombassie blue to visualise protein bands (as described in Chapter 2, 2.13 SDS polyacrylamide gel electrophoresis). DTT- and IOA-treatment of HMW gel filtration fractions (>300-kDa) resulted in the appearance of lower molecular weight protein bands, suggesting that disulfide bonds had been successfully dissociated (*fig. 5.9a*). LMW gel filtration fractions (~80-kDa) were treated with DTT and IOA as a control (*figs. 5.9a* and *b*). Reduction and alkylation of HMW fractions resulted in a complete loss of pro-phagocytic activity (*fig. 5.9b*), an effect that could not be reversed upon removal of the reducing agent by overnight dialysis prior to addition to phagocytosis assays, suggesting that DTT-induced reduction may have irreversibly altered the phagocytic potential of these protein fractions (*fig. 5.9b*). Interestingly, protein S in DTT- and IOA-treated HMW fractions appeared as a doublet of ~60-kDa and ~70-kDa when compared to a single band of ~80-kDa for PBS control-treated fractions, and a lower molecular weight protein S band was still evident upon removal of DTT by dialysis (*fig. 5.9a*), suggesting that protein S structure may have been altered by DTT treatment.
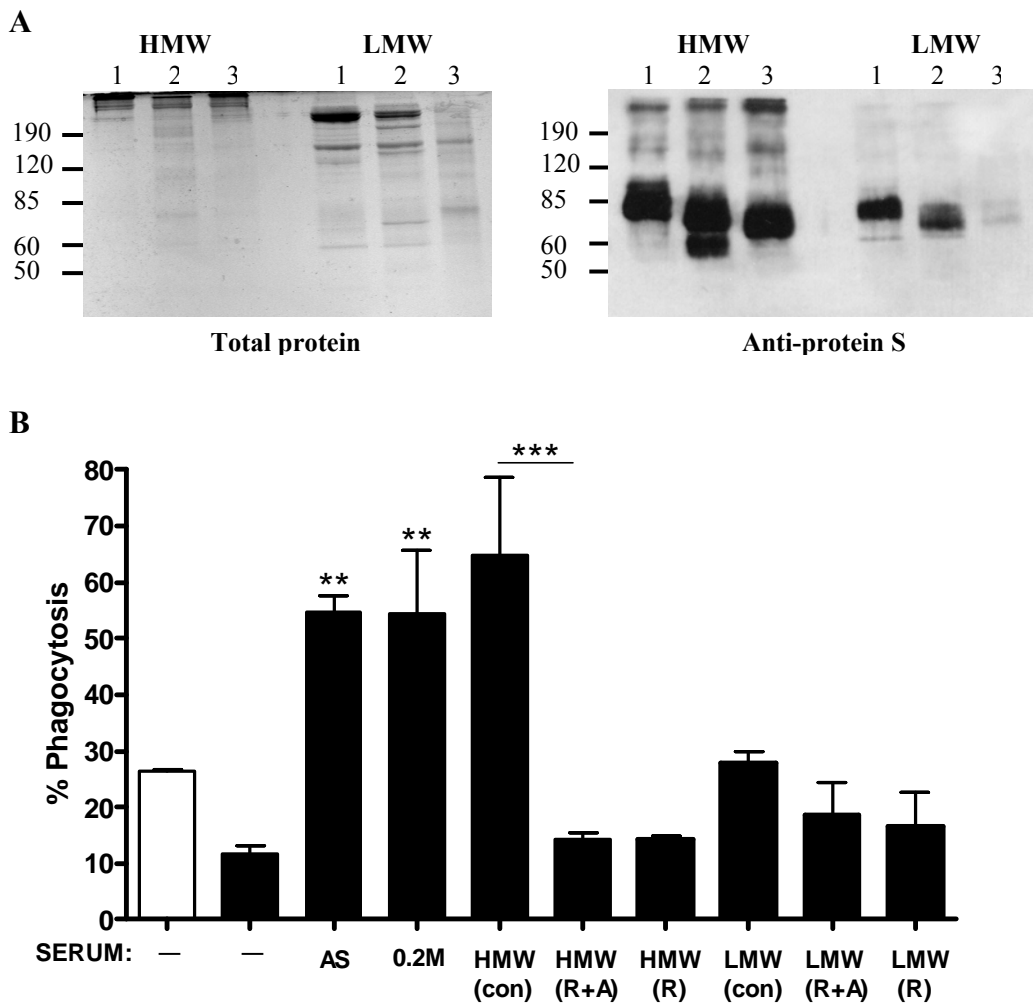
Figure 5.9: Effect of reduction and alkylation on the pro-phagocytic activity of gel filtration fractions.

A, HMW and LMW gel filtration fractions were treated with 5mM DTT followed by 25mM IOA to irreversibly dissociate protein disulfide bonds. Proteins were resolved by SDS PAGE on 9% gels under non-reducing conditions followed by staining with Coombassie blue. Alternatively, proteins were transferred to PVDF membrane and immunoblotted for protein S. Lane 1, PBS control; lane 2, DTT- and IOA-treated; lane 3, DTT-treated. **B**, Reduction (R) and alkylation (A) abolished the pro-phagocytic effect of HMW gel filtration fractions on Dex-MDMφ (black bars), an effect that could not be reversed upon removal of the reducing agent by overnight dialysis. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors.. (** $p<0.01$ when compared to uptake by Dex-MDMφ in the absence of serum, *** $p<0.001$ when compared to uptake by Dex-MDMφ in the presence of HMW fractions, one-way ANOVA with a Bonferroni post test).

5.3.8 *Protein S binds to apoptotic neutrophils in a calcium-dependent and phosphatidylserine-independent manner*

We next sought to investigate whether protein S could function as an opsonin of apoptotic neutrophils. Since protein S has the ability to bind multivalent ions and has been suggested to associate with membranes in a calcium-dependent manner (Nelsestuen et al., 1978, Schwalbe et al., 1990, Stenflo, 1999), we performed binding experiments using the same calcium-containing medium used for phagocytosis (IMDM) for incubations and washes (see 5.2.3 Flow cytometry). Protein S binding to serum-free cultured neutrophils (82.8% of gated neutrophils) correlated with annexin V-APC binding (81.6% of gated neutrophils), suggesting that protein S bound specifically to apoptotic neutrophils (*figs. 5.10a, b* and *c*). However, we were unable to demonstrate inhibition of protein S binding to apoptotic neutrophils with annexin V-APC, irrespective of whether annexin V-APC was added prior to or subsequent to protein S, indicating that the protein S binding site on apoptotic neutrophils may be distinct from PS (*figs. 5.10c* and *d*). Indeed, preliminary results obtained from phagocytosis assays using serum-free cultured apoptotic neutrophils pre-incubated for 10min with 1:50 annexin V-APC prior to phagocytosis in the absence or presence of 10% fresh autologous serum or 2.5µg/ml protein S, supports the suggestion that protein S enhances Dex-MDMφ phagocytosis of apoptotic neutrophils in a PS-independent manner (Dex 5d. No Serum: 14.5%; No Serum + Annexin V-APC: 15.11%; Serum: 55.8%; Serum + Annexin V-APC: 57.5%; Protein S: 72.3%; Protein S + Annexin V-APC: 66.3%; n=2).
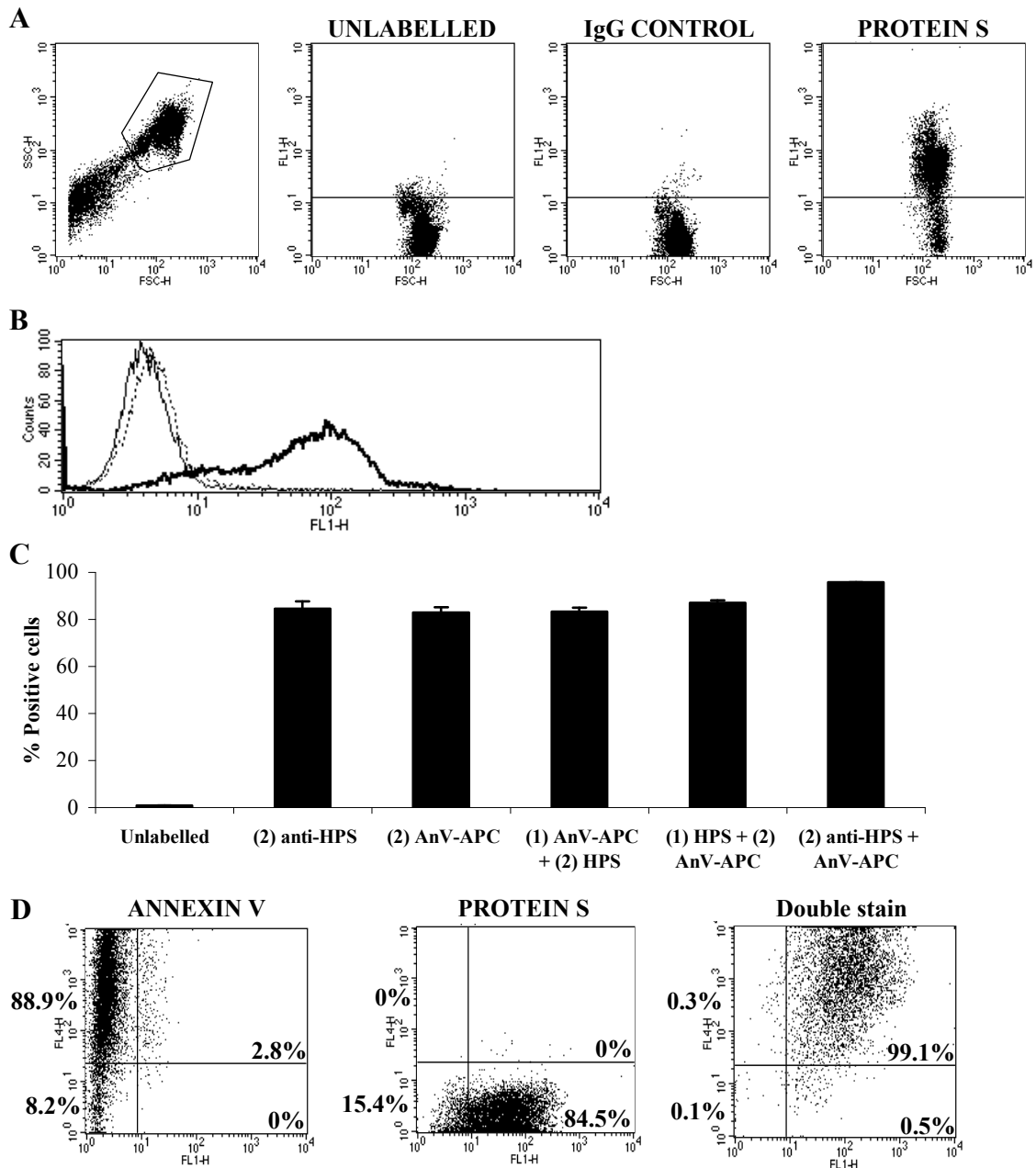
Figure 5.10: Protein S binds to apoptotic neutrophils in a phosphatidylserine-independent manner.

Analysis of protein S binding to serum-free cultured apoptotic neutrophils by flow cytometry using a FACS Calibur. **A**, Dot plots showing apoptotic neutrophils gated out from necrotic cells and cellular debris, and protein S binding on FL-1. **B**, Histogram of protein S binding (black line) compared to untreated neutrophils (dashed line) and IgG control (thin line). **C**, Human protein S (HPS) binding to apoptotic neutrophils (82.8% of gated neutrophils) correlated with AnV-APC binding (81.6% of gated neutrophils). However, protein S failed to reduce the percentage of AnV-APC positive neutrophils. (1) 1 hour pre-incubation, followed by (2) 15min incubation. Data are presented as Mean ± SEM, n=3 where n = the number of different neutrophil donors.. **D**, Dot plots showing protein S (FL-1) and annexin V-APC binding (FL-4) to neutrophils, resulting in a population of double-positive cells. Percentages shown are representative of 1 of three experiments.

5.3.9 *Glucocorticoid augmentation of MDMφ phagocytosis requires protein S opsonisation of apoptotic neutrophils*

I have demonstrated that the enhancing effect of human serum on Dex-MDMφ phagocytosis required calcium-dependent binding of a serum factor(s) to apoptotic neutrophils (see Chapter 3, *fig. 3.8*). To investigate whether protein S could perform this function, neutrophils were rendered apoptotic by culture for 20-24 hours in serum-free conditions then pre-incubated in the absence or presence of 2.5μg/ml protein S for 1 hour, centrifuged at 200 x *g* for 5min and then resuspended in IMDM before assessment of phagocytosis by flow cytometry. Enhanced Dex-MDMφ phagocytic capacity was found to require pre-incubation of apoptotic neutrophils with protein S (*fig. 5.11*) (McColl et al., 2009), generating levels of phagocytosis equivalent to those observed when the same concentration of protein S was added during phagocytosis assays (*fig. 5.7*). The pro-phagocytic effect of protein S pre-incubation was significantly reduced when the opsonised apoptotic neutrophils were washed once in calcium-free medium (EDTA) prior to phagocytosis assays, but maintained following washing in calcium-containing medium (IMDM), consistent with protein S opsonisation being a calcium-dependent event that is required for enhanced uptake of apoptotic neutrophils by Dex-MDMφ (*fig. 5.11*). However, our findings from the EDTA washing experiments performed using serum pre-incubated neutrophils indicated that there was also a calcium-independent effect of serum (Chapter 3, *fig. 3.8*), suggesting that an additional calcium-independent serum opsonin may contribute to serum-enhanced phagocytosis by Dex-MDMφ.
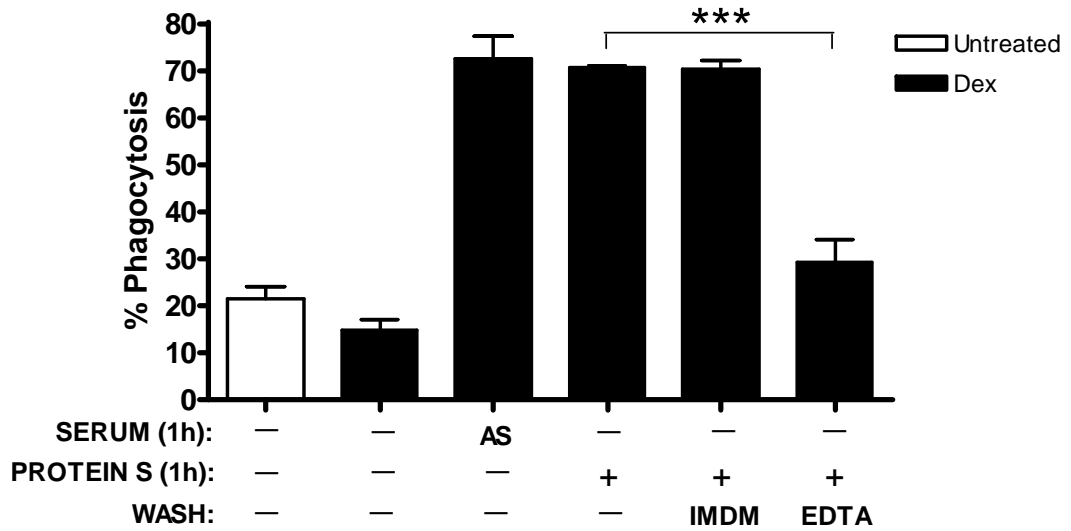
Figure 5.11: Effect of protein S pre-incubation of apoptotic neutrophils on phagocytosis by Dex-MDMφ.

Pre-incubation of serum-free cultured heterologous apoptotic neutrophils with 2.5μg/ml human protein S enhanced subsequent uptake by Dex-MDMφ (black bars), an effect that was reduced by washing pre-incubated neutrophils in calcium-free medium (EDTA), but not calcium-containing medium (IMDM) prior to phagocytosis. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (*** $p<0.001$ when compared to uptake of unwashed protein S-preincubated apoptotic neutrophils by Dex-MDMφ, one-way ANOVA with a Bonferroni post test).

5.3.10 *Anti-protein S antibody does not inhibit the pro-phagocytic effect of human serum on Dex-MDMφ*

Based upon our observation that protein S binding to apoptotic neutrophils could be detected by indirect immunofluorescence using an anti-protein S antibody (*fig. 5.10*), we hypothesised that pre-incubation of protein S-coated neutrophils with anti-protein S would be sufficient to block the pro-phagocytic effect of protein S on Dex-MDMφ. For this, 20-hour serum-free cultured neutrophils were pre-incubated for 1 hour with fresh autologous serum as a source of protein S, and 10μg/ml anti-protein S was added to neutrophils for 10min prior to assessment of phagocytosis. Due to the relatively high concentration of protein S in human serum, we chose to use 1% autologous serum for this experiment, a concentration that significantly augments Dex-MDMφ phagocytosis of apoptotic neutrophils (see Chapter 3, *fig. 3.3*). We were surprised to find that pre-incubation of protein S-opsonised apoptotic neutrophil cultures with anti-protein S did not reduce their uptake by Dex-MDMφ, instead stimulating slightly higher levels of Dex-MDMφ phagocytosis than achieved by protein S-opsonisation alone (*fig. 5.12*). Relating to studies of phagocytosis, it must be taken into consideration that cross-linking of Fc receptors on macrophages by "blocking" antibody may activate intracellular signalling pathways that may modulate phagocytic potential independently of the specific macrophage receptor engaged by protein S. One way of avoiding non-specific stimulation of macrophage Fc receptors is to use Fab' fragments of antibody, an approach taken by Flora *et al* to investigate the role of CD14 in untreated MDMφ phagocytosis of apoptotic neutrophils (Flora and Gregory, 1994). However, we decided to block macrophage FcγRII (CD32) by pre-incubating Dex-MDMφ with 20μg/ml IV.3 antibody for 10min prior to overlaying anti-protein S pre-incubated apoptotic neutrophils, an approach that reduced the slight stimulatory effect of anti-protein S on Dex-MDMφ phagocytosis (*fig. 5.12*). However, treatment with IV.3 did not influence protein S-dependent phagocytosis, possibly indicating the involvement of other Fc receptors (FcγRIII and/or FcγRI).
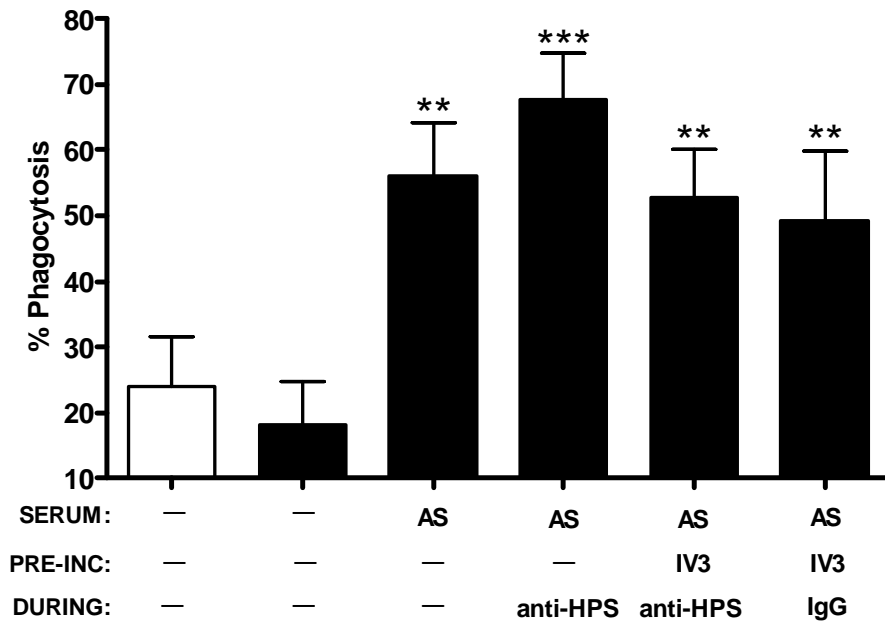
Comparison of Dex-MDMφ phagocytosis		P value
No Serum	1% AS	P < 0.01
No Serum	1% AS + anti-HPS	P < 0.001
No Serum	1% AS + IV.3 + anti-HPS	P < 0.01
No Serum	1% AS + IV.3 + IgG	P< 0.01
1% AS	1% AS + anti-HPS	P > 0.05
1% AS + anti-HPS	1% AS + IV.3 + anti-HPS	P > 0.05

Figure 5.12: Effect of anti-protein S on protein S-enhanced Dex-MDMφ phagocytosis of apoptotic neutrophils.
Pre-incubation of serum-free cultured heterologous apoptotic neutrophils with 1% fresh autologous serum (AS) enhanced subsequent uptake by Dex-MDMφ (black bars), an effect that was not reduced by pre-incubation of serum-opsonised apoptotic neutrophils with an anti-human protein S (HPS) Ab. Dex-MDMφ were pre-incubated with an anti-FcγRII Ab (clone IV.3) to block Fc receptors, and rabbit IgG added to serum-preincubated apoptotic neutrophils was used as a control for anti-protein S. Mean phagocytosis $\pm$ SEM, n=3 where n = the number of different macrophage donors. (** p<0.01, *** p<0.001 when compared to uptake of apoptotic neutrophils by Dex-MDMφ in the absence of serum, one-way ANOVA with a Bonferroni post test). Table indicates comparison of Dex-MDMφ phagocytosis in the presence of different add-back treatments, with the p value shown.

5.3.11 *Short-term treatment of human MDMφ with dexamethasone induces acquisition of a protein S-dependent pro-phagocytic pathway*

Previous studies in our lab reported that the Dex effect on macrophage phagocytic potential appeared to be mediated early during the monocyte to macrophage differentiation process (Giles et al., 2001). Treatment of monocytes with Dex during the first 24 hours of culture was found to confer maximal phagocytosis of apoptotic neutrophils when phagocytic capacity of MDMφ was tested at 5 days. However, the study by Giles *et al* did not demonstrate a rapid induction of capacity for phagocytosis of apoptotic neutrophils in monocytes following Dex treatment. Thus, we wanted to evaluate the effects of short-term treatment of human MDMφ with Dex upon the protein S dependency of phagocytosis of apoptotic neutrophils. For this, MDMφ that had been cultured in the absence of Dex for 96 hours were then exposed to Dex for the final 24 hours of culture, a time-point that has previously been shown to enhance human MDMφ phagocytosis of serum-cultured apoptotic neutrophils (Liu et al., 1999). Compared to untreated MDMφ, 96-120hour Dex-treated MDMφ had slightly higher basal levels of phagocytosis of apoptotic neutrophils, but exhibited a protein S-dependent increase in phagocytosis (*fig. 5.13*). Thus, addition of Dex to the culture medium at later time points during monocyte differentiation also revealed a requirement for a protein S-dependent clearance mechanism, suggesting that Dex treatment induces acquisition of a protein S-mediated phagocytic pathway irrespective of the stage of monocyte differentiation (*fig. 5.13*).
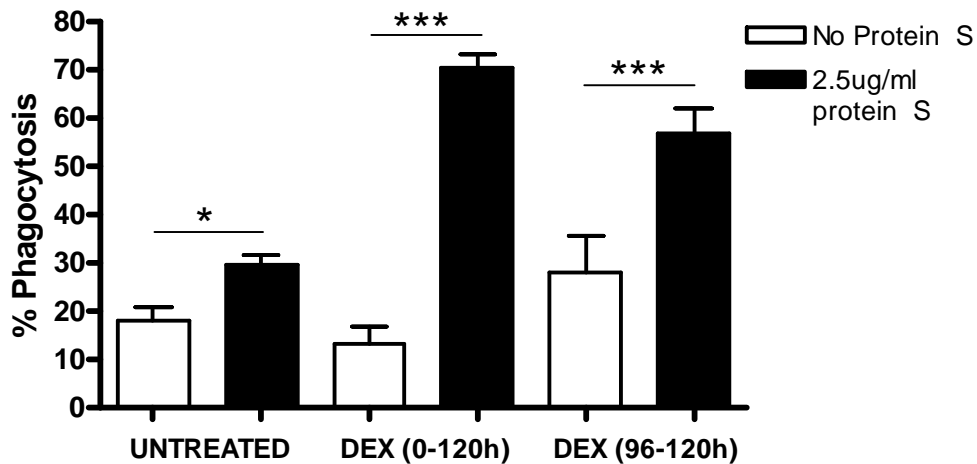
Figure 5.13: Effect of short-term treatment of human MDMφ with Dex on phagocytic capacity for apoptotic neutrophils.

MDMφ were cultured in the absence or presence of 1μM Dex for 5 days (0-120h) or for the final day of culture (96-120h), and MDMφ phagocytosis of serum-free cultured heterologous apoptotic neutrophils was determined in the absence (white bars) or presence (black bars) of 2.5μg/ml purified protein S. Short term-treatment of human MDMφ with Dex resulted in acquisition of a protein S-dependent pathway for enhanced ingestion of apoptotic neutrophils. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (*** p<0.001 when compared to uptake by MDMφ in the absence of protein S, one-way ANOVA with a Bonferroni post test).

5.4   Discussion

5.4.1   *Isolation of a high molecular weight protein fraction that contains the pro-phagocytic activity of human serum*

In this chapter, I designed a multi-step purification scheme in order to isolate the pro-phagocyic component from fresh human serum. Protein capture during anion exchange chromatography was optimised by applying human serum to mono Q Sepharose using buffering conditions that allowed the protein of interest to bind while minimising binding of protein contaminants. By performing step-wise elution of bound proteins, achieved by increasing the buffering ionic strength from 0.2M to 0.5M NaCl by 0.1M increments, we found that many of the proteins could be eluted from the column with 0.2M NaCl. The pro-phagocytic serum component was found to bind to mono Q Sepharose at pH7 (50mM HEPES and 0.14M NaCl) and could be eluted with 0.2M NaCl (pH7, 50mM HEPES), indicating that the serum factor has a pI<7 and associates with mono Q sepharose weakly at pH7. Importantly, no enhancing activity was observed in the flow through, demonstrating that the pro-phagocytic component could be efficiently isolated from large volumes of serum that contain a highly complex mixture of proteins. Although great care was taken with the gel filtration column, only partial purification of proteins from 0.2M anion exchange fractions could be achieved. It is unlikely that high-resolution separation so that a protein could be confined to one single fraction would be achievable, as 0.2M anion exchange samples still contained a significant number of proteins. However, chromatographic patterns obtained were quite reproducible, consistently producing two partially overlapping peaks of protein that corresponded to high- and low-molecular weight proteins, and HMW fractions with an estimated molecular weight of >300-kDa were found to contain the pro-phagocytic activity of serum. Among the several candidate proteins identified by mass spectrometry, we were interested in assessing the phagocytic effect of alpha-2 macroglobulin, IgM and C4BP. Interestingly, several reports have demonstrated alpha-2 macroglobulin, a ~720-kDa human plasma protein that inhibits the function of proteases like trypsin and thrombin (Hall and Roberts, 1978), to be a Dex-inducible protein (Lerner et al., 2003). Furthermore, ligation of the alpha-2 macroglobulin receptor, CD91, on

macrophages by calreticulin on the surface of apoptotic neutrophils triggers Rac activation and membrane ruffling in macrophages, and internalisation of apoptotic neutrophils (Gardai et al., 2005). However, we were unable to demonstrate a role for alpha-2 macroglobulin in enhancement of Dex-MDMϕ phagocytic capacity. IgM, a pentameric molecule of five subunits joined by disulphide bridges with a very high molecular weight (900-kDa), has been suggested to indirectly promote phagocytosis of apoptotic cells by untreated murine MDMϕ by increasing the deposition of complement opsonins on the apoptotic cell surface (Ogden et al., 2005). However, previous studies in our lab have shown IgM to be devoid of pro-phagocytic activity (data not shown). Interestingly, IgM was identified as a contaminant following C4BP purification from human plasma using the method of barium citrate adsorption followed by ion exchange and affinity chromatography, suggesting that co-purification of IgM with C4BP may be a common occurrence during application of chromatography techniques for protein purification from plasma/serum, possibly due to the high affinity of C4BP for IgM (Villiers et al., 1981, Dahlback et al., 1983).

C4BP, a macromolecular weight glycoprotein (~570-kDa) composed of seven identical 70-kDa α-chains, each of which contain a binding site for activated complement C4b, and a single 45-kDa β-chain, is synthesised in the liver and circulates in human plasma at a concentration of ~200μg/ml (Dahlback, 1983). C4BP functions as an important regulator of complement activation by enhancing the proteolytic inactivation of C4b by factor I, thereby preventing assembly of the classical pathway C3 convertase (C4bC2a) and limiting amplification of the complement cascade (Gigli et al., 1979). Interestingly, Moffat *et al* reported that treatment of the mouse liver cell line, NmuLi with 1μM Dex rapidly and dramatically increased C4BP mRNA and protein levels, suggesting that regulation of complement activation may represent an additional anti-inflammatory effect of glucocorticoids (Moffat et al., 1992). From our mass spectrometry results, multiple hits for C4BP were obtained from the several protein bands that were analysed, which we propose may be due to the presence of residual dimers/trimers as a result of incomplete reduction of α-chain linked disulphide bonds prior to gel

electrophoresis (*fig. 5.6b*). To assess whether C4BP was the high molecular weight protein that conferred augmentation of Dex-MDMφ phagocytic capacity for apoptotic neutrophils, we attempted to immunodeplete C4BP from 0.2M anion exchange fractions. However, depletion experiments using a very high concentration of anti-C4BP antibody failed to have an effect on the pro-phagocytic activity of serum. Furthermore, when the anti-C4BP mAb was tested out for immunoblotting, high concentrations and significantly long exposure times were required before C4BP bands were detected, and immunoblotting for C4BP in 0.2M anion exchange fractions demonstrated that that this antibody did not significantly deplete C4BP. C4BP free from protein S was not commercially available when these experiments were performed, and immunodepletion experiments using a different anti-C4BP antibody may allow further investigation into the contribution of C4BP to Dex-MDMφ phagocytosis.

5.4.2 *Dex-MDMφ utilise a protein S-dependent pathway for enhanced removal of apoptotic neutrophils*

In human plasma, the most common form of C4BP exists in a high molecular weight complex with protein S (Dahlback, 1983). Protein S is a vitamin K-dependent glycoprotein (DiScipio and Davie, 1979) composed of an N-terminal Gla domain, a thrombin-sensitive region, four epidermal growth factor (EGF)-like domains, and a C-terminal sex hormone-binding globulin (SHBG)-like domain (Hoskins et al., 1987, Gershagen et al., 1987), which is involved in high affinity binding to the β-chain of C4BP (Evenas et al., 1999). In humans, protein S is mainly synthesised by liver hepatocytes, but also by megakaryocytes, leydig cells of the testis and endothelial cells (Fair and Marlar, 1986, Malm et al., 1994), and is constitutively present in plasma at a relatively high concentration of 25μg/ml where is can exist either as a free form or in complex with C4BP (Dahlback, 1983). Free protein S (~40%; 10μg/ml) acts as a cofactor for the anti-coagulant activity of activated protein C in the inactivation of factor VIIIa (Dahlback, 1983, Walker et al., 1987). The remaining 60% of protein S (15μg/ml) circulates in plasma bound to C4BP, and displays no anticoagulant function (Dahlback, 1983, Dahlback, 1986).

By performing direct immunofluorescent labelling of apoptotic BL-41 cells with biotinylated human protein S followed by co-staining with FITC-avidin and PE–labelled annexin V, Anderson *et al* demonstrated that protein S bound exclusively to annexin V-positive apoptotic cells (Anderson et al., 2003). Furthermore, Trouw and co-workers found that protein S bound to both "early" and "late" apoptotic Jurkat T cells, with the kinetics of protein S binding similar to that of annexin V-binding, suggesting that PS exposure was sufficient for binding of protein S (Trouw et al., 2005). Webb and co-workers have shown protein S to bind apoptotic neutrophils in a PS-dependent manner, an effect that is inhibitable with a monoclonal antibody directed against the Gla domain of protein S (Webb et al., 2003). We performed indirect protein S labelling of 20-24 hour serum-free cultured apoptotic neutrophils, requiring detection of bound unlabelled protein S with a FITC-labelled secondary antibody. There are potential drawbacks to indirect labelling of protein binding, and our experiment was complicated by the requirement for three binding steps (human protein S, rabbit anti-human protein S Ab, anti-rabbit FITC-conjugated Ab). However, this experimental approach was found to be sufficient for detecting protein S binding to apoptotic neutrophils. We have demonstrated human protein S to opsonise apoptotic neutrophils in a calcium-dependent manner, consistent with findings by Anderson *et al* using apoptotic BL-41 cells (Anderson et al., 2003). While we found that the pattern of protein S binding to serum-free cultured apoptotic neutrophils strongly resembled binding of annexin V-APC, double staining of apoptotic neutrophils resulted in a large population of double-positive cells. The lack of inhibition of protein S binding by annexin V-APC is in contrast to findings of Anderson *et al*, where binding of protein S to apoptotic BL-41 cells could be inhibited by a 20-fold molar excess of annexin V (Anderson et al., 2003). However, the potential of a high molar excess of annexin V to deplete calcium from the binding buffer may inadvertently affect protein S binding, and supplementary calcium may be required to prevent this. Whether higher concentrations of annexin V-APC are required to inhibit protein S binding, or protein S binds to a site on the surface of apoptotic neutrophils distinct from PS, remains to be determined.

Free human protein S has been reported to stimulate uptake of "early", membrane-intact (annexin V+/propidium iodide-) apoptotic targets (BL-41 human Burkitt's lymphoma cells and Jurkat T cells) by untreated human MDMϕ to a similar extent as human serum and FBS (Anderson et al., 2003, Kask et al., 2004). Based upon our findings in Chapter 3, where serum was found to augment Dex-MDMϕ phagocytosis of "early" apoptotic neutrophils (Chapter 3, *fig. 3.9*), we were interested in assessing whether protein S was required. Using a highly purified commercial preparation (free form of protein S, >95% purity as judged by SDS PAGE), we found that human protein S augmented Dex-MDMϕ phagocytosis of apoptotic neutrophils in a dose-dependent manner, with maximal levels of phagocytosis equivalent to that conferred by human serum achieved in the presence of 250ng/ml protein S, a much lower concentration than was required for phagocytosis by untreated macrophages, where the maximal pro-phagocytic effect required 13μg/ml of protein S (52-fold higher concentration) (Anderson et al., 2003). Furthermore, the concentration we used for the remaining experiments (2.5μg/ml) only produced half-maximal phagocytic activity for untreated macrophages (Anderson et al., 2003), whereas the half-maximal effect on Dex-MDMϕ phagocytosis was observed at only 75ng/ml protein S. Intriguingly, our data suggest that the efficiency of protein S-dependent clearance of apoptotic neutrophils by untreated MDMϕ could be significantly increased following short-term exposure to Dex, suggesting that glucocorticoids have the capability to induce phagocytic potential in already differentiated MDMϕ by promoting the acquisition of a protein S-dependent clearance mechanism, an observation that is consistent with the ability of macrophages to functionally adapt to changing environmental signals (Stout and Suttles, 2004). The efficiency of this pathway for uptake of apoptotic neutrophils by 5-day Dex-MDMϕ was confirmed by the total loss of pro-phagocytic activity of 0.2M anion exchange fractions following immunodepletion of protein S with a polyclonal antibody. Importantly, phagocytic activity could be restored upon "add back" of purified protein S, indicating that protein S is an important modulator of apoptotic neutrophil clearance by Dex-MDMϕ.

5.4.3 *Protein S augments Dex-MDMφ phagocytosis of apoptotic neutrophils: role of free form versus complex*

Based upon the pI values for protein S (5–5.5) and C4BP (6.7) (as shown in *table 1*), it would be reasonable to hypothesise that both proteins would be negatively charged at pH7 and hence bind to the positively charged mono Q Sepharose, resulting in isolation of both free and C4BP-complexed forms of protein S. Interestingly, we found that only HMW fractions contained the pro-phagocytic activity of serum, and the presence of protein S in these fractions, presumably present in complex with C4BP, was confirmed by immunoblotting experiments. The C4BP-protein S complex has a 1:1 stoichiometry, and the interaction is non-covalent and of very high affinity ($Kd \sim 10^{-10}M$) (Dahlback, 1983, Schwalbe et al., 1990). Although protein S was not identified as a candidate protein by mass spectrometry, it is noteworthy to mention that our sample was run under reducing conditions, and C4BP appeared as a wide ~75-kDa band due to reduction of disulphide-linked polypeptide chains. It is therefore possible that protein S, a monomeric protein which has a molecular weight of ~80-kDa, would conjugate with C4BP under reducing conditions. Indeed, it has been reported that the C4BP-protein S complex appears as a single band on reduced gels because of the predominance of the C4BP α-chains in the complex (Dahlback et al., 1983, Kask et al., 2004), which therefore could have masked the signal from the lower abundance protein S during mass spectrometry analysis.

Although we have shown that the pro-phagocytic activity of 0.2M anion exchange fractions was lost entirely upon immunodepletion of protein S, it was impossible to determine whether the phagocytic activity was conferred by free protein S or C4BP-protein S complexes, or indeed both. Restoration of Dex-MDMφ phagocytic capacity by a HMW serum fraction raised the possibility that the C4BP-protein S complex can augment uptake of apoptotic neutrophils by Dex-MDMφ in some circumstances, and supports of preliminary data achieved using fresh ultrafiltered serum that indicated the serum component was larger than 100-kDa in molecular mass (Chapter 4, *fig. 4.1*). This would be in contrast to findings by Kask and co-workers, who reported that C4BP-protein S complexes strongly inhibited untreated MDMφ phagocytosis of apoptotic lymphocyte cell lines (Kask et al., 2004). Furthermore, an anti-phagocytic

role for C4BP has been reported for alternative phagocytic targets, including pathogenic bateria (Berggard et al., 2001). C4BP is a large protein complex with seven α-chains of 33nm in length which form an octopus-like structure when surface-bound (Dahlback, 1983), causing speculation that the size of the C4BP-protein S complex, which projects out of the membrane by 56nm (Schwalbe et al., 1990), could steroically block interaction between apoptotic cell ligands or surface-bound serum modulators and their phagocytic counterparts on untreated MDMϕ. Alternatively, binding of C4BP to protein S may specifically inhibit phagocytosis by untreated MDMϕ by masking the SHBG domain required for interaction with macrophage receptors such as the tyrosine kinase receptor, Tyro-3 (Evenas et al., 2000). Furthermore, C4BP may indirectly inhibit MDMϕ phagocytosis *via* inhibition of complement activation and deposition of C3b opsonin on the surface of apoptotic cells that may be important for apoptotic cell clearance by untreated macrophages (Mevorach et al., 1998a), but not Dex-MDMϕ (Chapter 4, *fig. 4.5a*).

To address the anomaly of the pro-phagocytic effect of free protein S and C4BP-protein S complexes when using commercially purified protein S and our chromatography samples, respectively, HMW fractions were treated with DTT to dissociate C4BP disulfide-linked alpha chains followed by irreversible alkylation to prevent re-association, and we hypothesised that C4BP-complexed protein S would become "free" protein S. In these experiments, we found that reduction of HMW fractions resulted in complete irreversible loss of phagocytic activity, with several possible interpretations for this. Firstly, the C4BP-protein S complex may be required for augmentation of Dex-MDMϕ phagocytosis. Although pro-phagocytic activity was not recoverable upon dialysis to remove excess DTT, the reducing agent may have irreversibly altered protein structure to prevent reassociation of protein S and C4BP into the HMW complex. However, the absence of C4BP-protein S complexes from bovine serum/plasma, despite the ability of bovine protein S to form a complex with human C4BP (Dahlback, 1986), together with the observation that FBS confers augmentation of Dex-MDMϕ phagocytosis of apoptotic neutrophils (Chapter 3, *fig. 3.5d*) suggests that in fact the complex is not required. Furthermore,

the inability to indentify a β-chain homologue in murine C4BP, which consists solely of seven α-chains (Moffat et al., 1992), indicates that murine serum enhances Dex-MDMφ phagocytosis (Chapter 3, *fig. 3.5c*) in the absence of C4BP-protein S complexes. Additionally, fresh autologous serum augments Dex-MDMφ phagocytic capacity for "early" apoptotic neutrophils (see Chapter 3, *fig. 3.9*), despite the absence of C4BP-protein S complex binding at this stage of apoptosis (Trouw et al., 2005). Since protein S has a pI value of 5–5.5, it would be expected to bind to mono Q Sepharose at pH6. However, we found that pH6 bound elutions were devoid of phagocytic activity, suggesting that the serum component had a pI value of >6. Based upon these findings, we would hypothesise that efficient isolation of protein S by anion exchange chromatography at pH7 occurred by means of its high affinity interaction with C4BP, which in turn bound specifically to the column, and this may explain the absence of pro-phagocytic activity in LMW fractions (see *fig 5.4*, fractions D – G). One way of testing this hypothesis would be to perform anion exchange and gel filtration chromatography under the same conditions using purified protein S.

Our data obtained using purified protein S suggested that the free form of protein S was sufficient to confer augmentation of Dex-MDMφ phagocytosis. Interestingly, the LMW fraction used for reduction and alkylation experiments had an estimated molecular weight of 80-kDa and protein S in the free form was detected in this fraction by immunoblotting, albeit at a much lower level than observed in HMW fractions (*fig. 5.9a*). Furthermore, the free protein S-containing fraction increased Dex-MDMφ phagocytosis of apoptotic neutrophils from 11.7% to 27.8%, consistent with a pro-phagocytic role for free protein S (*fig. 5.9b*). When proteins in HMW fractions were resolved by SDS PAGE and immunoblotted with anti-protein S, we were surprised to find that protein S in reduced and alkylated samples appeared as a lower molecular weight band than in untreated samples, and this was not recoverable by removal of the reducing agent by dialysis. Hence, the loss of phagocytic activity for both HMW and LMW fractions raises the possibility that DTT-induced reduction may have irreversibly altered protein S structure and/or phagocytic function. Calcium plays an essential role in protein S conformation and function, and binding to C4BP

and membranous surfaces (Nelsestuen et al., 1978, Dahlback et al., 1990, Schwalbe et al., 1990, Stenflo, 1999), hence modification of the calcium-binding sites in the EGF-like domains of protein S (Handford et al., 1991) may affect the phagocytic potential of protein S. One approach to testing this would be to examine the effect of DTT-treatment of purified protein S, followed by dialysis to remove excess reducing agent, on the pro-phagocytic activity of this molecule.

Since protein S binding can be demonstrated following incubation of serum-free cultured apoptotic neutrophils with either the HMW fraction or purified protein S, one possibility is that under certain conditions, protein S can dissociate from C4BP on the apoptotic neutrophil surface to promote clearance by Dex-MDMφ. Interestingly, the rate of dissociation of protein S from C4BP in solution can be influenced by altering the temperature and calcium content of buffering conditions (Dahlback, 1983, Schwalbe et al., 1990, Pauls et al., 2000). When added to phagocytosis assays at 37°C, the rate of dissociation would be significantly enhanced compared to when HMW fractions were stored at 4°C, so that a significant proportion of protein S would be free during the 30min phagocytosis assay (Dahlback, 1983). Although Dahlback *et al* estimated the dissociation rate in human plasma to be 40-fold higher at 37°C, they examined C4BP-protein S interaction in calcium-free buffer (Dahlback, 1983), whereas calcium-containing medium (IMDM; ~1.5mM) was consistently used in our study based on the requirement of calcium for protein S opsonisation of apoptotic neutrophils. In this regard, Schwalbe *et al* demonstrated that C4BP-protein S complexes assemble rapidly on phospholipid vesicles in the presence of calcium, and ~50% of C4BP dissociated from membrane-bound protein S at 37°C in buffer containing 0.4mM calcium after only ~8min. Plasma membranes can accommodate a high molar density of free protein S than the C4BP-protein S complex due to the bulky structure of the complex preventing tight packing (Schwalbe et al., 1990). Release of C4BP from protein S may be triggered by a conformational change induced upon binding of the protein S portion of the complex to the apoptotic neutrophil surface, which may occur when a certain threshold of binding is reached and can no longer accommodate the sheer size of the complex (Schwalbe et al., 1990). Alternatively, the binding of C4BP-protein S to the

apoptotic cell surface may represent an equilibrium reaction requiring the release of only a few molecules of C4BP to allow access of the protein S receptor on Dex-MDMϕ. Furthermore, the "free" protein S in our reduced and alkylated samples likely remains bound to the β-chain of C4BP by way of non-covalent bonds that, as mentioned above, may inhibit the pro-phagocytic effect of protein S on macrophages by masking the SHBG domain recognised by macrophage receptors (Evenas et al., 2000). The dissociation of protein S from C4BP to allow association of free protein S on "early" apoptotic neutrophils is consistent with the observation by Trouw *et al* that "early" apoptotic cells were capable of binding free protein S, with little evidence of C4BP-protein S complex binding (Trouw et al., 2005). Furthermore, a recent report by Uehara *et al* suggested that human protein S can form multiple layers on the apoptotic cell surface, an effect that was required for protein S enhancement of phagocytosis by untreated macrophages (Uehara and Shacter, 2008). Interestingly, protein S oligomerisation on the apoptotic cell surface required formation of disulfide bonds between protein S monomers, an effect that we would predict to be absent on apoptotic neutrophils cultured with DTT-treated HMW fractions. Furthermore, human protein S may be prone to self-aggregation and formation of multimers *in vitro* (Pauls et al., 2000, Sere et al., 2006), resulting in binding of protein S multimers to apoptotic neutrophils that may show decreased ability to bind C4BP (Sere et al., 2006). Indeed, the concentration of purified protein S consistently used in phagocytosis assays (2.5μg/ml) appeared to form aggregates when resolved on 9% acrylamide gels (*fig. 5.7a*). Another possibility is that naturally occurring multimers of protein S present in plasma (Heeb et al., 2006) can also bind to apoptotic neutrophils.

Although our data point towards a pro-phagocytic role for protein S over the C4BP-protein S complex, there may be additional benefits of complex binding independent of phagocytic function. Apoptotic neutrophils can activated and become opsonised with complement proteins due to the loss of membrane complement regulatory molecules DAF (CD55), MCP (CD46), and CR1 (CD35) (Jones and Morgan, 1995). Loss of protection against excessive complement attack would leave apoptotic neutrophils vulnerable to complement-mediated lysis if they did not acquire other

means of protection. Despite the suggested inhibitory effect on apoptotic cell clearance by untreated MDMϕ, protein S localisation of C4BP, a fluid-phase regulator of complement activation, to the surface of apoptotic neutrophils (Furmaniak-Kazmierczak et al., 1993) during later stages of apoptosis may provide local regulation of the complement system to suppress complement-mediated lysis and inflammation in the vicinity of apoptotic cells (Webb et al., 2003, Kask et al., 2004, Trouw et al., 2005, Trouw et al., 2007), a process that is essential for maintanence of normal physiologic function of host tissues and organs.

5.4.4 *Protein S deficiency in vivo: consequences for coagulation and apoptotic cell clearance pathways*

The functional importance of protein S as an anti-coagulant is demonstrated by the high risk of venous thrombosis in patients with hereditary protein S deficiency (Comp and Esmon, 1984, Schwarz et al., 1984, Broekmans et al., 1985). Although the prevalence in the general population is unknown, an investigation using 3788 Scottish blood donors found protein S deficiency to have a relatively low prevalance of between 0.03% and 0.13% (Dykes et al., 2001). Inherited protein S deficiency may result either from reduced plasma levels of protein S (quantitative; 95% of cases) or a defect in protein S function with normal protein level (qualitative; 5% of cases) (Rezende et al., 2004). Several reports have indicated an acquired aspect to protein S deficiency, and protein S levels may be amenable to biological influences such as age and hormonal levels (Comp et al., 1986, Boerger et al., 1987). Interestingly, several studies have shown men to have higher levels of free and total protein S than females (Boerger et al., 1987, Henkens et al., 1995), a factor that must be taken into consideration with regards to phagocytic variability in my system, as serum donors used were not sex-specific. Given the importance of free and complexed protein S in regulation of inflammation, apoptosis and phagocytosis pathways, the *in vivo* implications of reduced protein S levels could be immense in terms of inflammatory and/or autoimmune consequences. Indeed, protein S deficiency has been suggested to provoke exaggerated inflammatory responses (Kasuno et al., 1997). Moreover, anti-protein S autoantibodies and acquired protein S

deficiency are often observed in patients with SLE, an autoimmune disease characterised by ineffecnt clearance of apoptotic cells (Song et al., 2000). The *in vivo* consequence of protein S-deficiency on the apoptotic cell clearance capacity of macrophages remains to be determined, and could be investigated *in vitro* using serum obtained from protein S-deficient human donors. From our protein S immunodepletion data, we would predict that Dex-MDMϕ phagocytic capacity may be reduced in the presence of protein S-deficient human serum and could be recovered upon restoration of protein S levels. Protein S may therefore represent a novel therapeutic agent for the treatment of autoimmune diseases such as SLE due to the combined anti-coagulant and pro-resolving effects. However, administration would need to be tightly regulated to prevent hyper-anticoagulation. Interestingly, protein S infusion in a murine model of ischaemic stroke resulted in decreased motor neurologic deficit, infarction and oedema volumes, possibly as a consequence of the pro-resolving apoptotic cell clearance effects of protein S (Liu et al., 2003).

In this chapter, I performed chromatography techniques to isolate a protein fraction that contains the pro-phagocytic activity of serum. Our data in Chapter 3 indicated that a common pro-phagocytic component present in sera from different mammalian species can enhance Dex-MDMϕ phagocytosis of apoptotic neutrophils (Chapter 3, *fig. 3.5*). We propose that protein S, present in human, bovine, murine, porcine, monkey, rabbit and rat sera with much identity between protein S species (Lundwall et al., 1986, Dahlback, 1986, Dahlback, 2007), represents the common serum factor required. To our knowledge, protein S has not been identified in goat serum and may account for the lack of enhancing activity when added to phagocytosis assays. The pattern of protein S interaction with C4BP or membranous structures, and the phagocytic potential of this molecule appears to be extremely complex and subject to modulation by several environmental factors. The degree by which protein S enhances Dex-MDMϕ phagocytosis is mainly determined by the C4BP-protein S complex from which we propose protein S must dissociate from to confer this augmentation.

CHAPTER 6: PROTEIN S AUGMENTS DEX-MDMφ PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS THROUGH STIMULATION OF MERTK

6.1   Introduction

The ability of glucocorticoids to augment human MDMφ phagocytic capacity for apoptotic neutrophils was found to be highly dependent upon the stage of maturation that monocytes were exposed to glucocorticoids, with a greater potentiation observed in first 24 hours of culture (Liu et al., 1999, Giles et al., 2001). The glucocorticoid effect could be reversed following treatment with RU38486 glucocorticoid antagonist (Liu et al., 1999), indicating that GR-mediated alterations in the monocyte gene expression profile during maturation "programme" a highly phagocytic MDMφ phenotype. The acquisition of phagocytic ability during MDMφ differentiation may be due to altered expression of cell surface molecules or intracellular signalling components that facilitate the uptake of apoptotic neutrophils. Indeed, Dex-enhanced MDMφ phagocytic potential is associated with increased levels of active Rac (Giles et al., 2001). Previous work in our lab provided some insight into the effect of changes in gene expression at the level of the GR on Dex-MDMφ phagocytic phenotype. By using synthetic glucocorticoid analogues that predominately mediated transactivation (ZK57740, ZK77945) or transrepression (RU24782, RU24858) by the GR (Heck et al., 1997, Vayssiere et al., 1997), Giles *et al* found that Dex-enhanced MDMφ phagocytosis required both mechanisms of transcriptional regulation (Katherine Giles, unpublished data). It is possible that the two mechanisms of action are not mutually exclusive, and optimal induction of MDMφ phagocytic capacity probably requires combinatorial transactivation and transrepression actions to induce alterations in the transcriptional profile during monocyte maturation. Interestingly, Dex-enhanced MDMφ phagocytosis of apoptotic neutrophils could be reversed by treatment with the protein synthesis inhibitor cycloheximide, supporting the requirement for a GR-induced transactivation process during monocyte differentiation, possibly the induction of a pro-phagocytic receptor (Liu et al., 1999). Previous investigations into the effects of glucocorticoids on

monocyte/macrophage surface receptor expression, either through specific mAb binding and flow cytometry or by oligonucleotide arrays, revealed glucocorticoid-induced alterations in the levels of cell surface expression of CD163 scavenger receptor, mannose receptor, CD36 and αvβ3 and β1 integrins (Giles et al., 2001), FPR1 and Mertk (Ehrchen et al., 2007). However, the relevance of these alterations in glucocorticoid-enhancement of phagocytic capacity has remained elusive.

The function performed by macrophages depends on their activation state, and can be highly influenced by the cytokine microenvironment. The presence of bacterial LPS and IFNγ during monocyte maturation, mediators associated with "classical" macrophage activation (M-1 cells) and induction of pro-inflammatory responses (Goerdt et al., 1999, Mills et al., 2000), may regulate phagocytic potential through effects on macrophage morphology and phenotype, possibly by altering the predominance for utilisation of a specific apoptotic cell clearance pathway. Treatment of monocytes with IFNγ *in vitro* induces the formation of MNGCs (Most et al., 1990, Fais et al., 1994) that phagocytose apoptotic neutrophils poorly (Heasman et al., 2004). Interestingly, treatment of human monocytes with Dex overrides the effect of IFNγ on morphology, inducing a homogeneous population of MDMϕ devoid of MNGCs, with similar alterations in intracellular signalling molecules and adhesion status observed for Dex-only MDMϕ cultures (Heasman et al., 2004). Co-treatment of human monocytes with Dex and IFNγ (Heasman et al., 2004), LPS or TNFα (Michlewska et al., 2009) abolished the potentiating effect of Dex on MDMϕ phagocytosis, suggesting that the cytokine microenvironment can significantly modulate glucocorticoid effects on macrophage function.

In chapter 5 of this study, I demonstrated the requirement of protein S for enhanced removal of apoptotic neutrophils by Dex-MDMϕ. Protein S has been reported to enhance phagocytosis of apoptotic lymphoma cells by untreated human MDMϕ through stimulation of Mertk (Uehara and Shacter, 2008). In this chapter, I wanted to investigate whether the Mertk receptor pathway was utilised by Dex-MDMϕ for protein S-dependent clearance of apoptotic neutrophils, and to determine whether

pro-inflammatory mediators such as LPS and IFNγ could modulate this receptor mechanism.

6.2   Materials and methods

6.2.1   *Immunoprecipitation and western blotting for Mertk on human MDMϕ*

12×10^6 MNCs were added per well of 6-well tissue culture plates and allowed to adhere for 1 hour at 37°C in 5% CO_2. Non-adherent lymphocytes were removed by washing 3 times with IMDM and adherent monocytes (roughly 1.2×10^6) were cultured for 5 days in IMDM containing 10% fresh autologous serum $\pm$ 1μM Dex. Cells were washed with 1 x TBS containing 0.1mM NaVO3 before addition of 300μl RIPA lysis buffer (1mM Na3VO4, 50mM NaCl, 50mM Tris pH7.4, 0.5% Deoxycholate, 0.1% SDS, 1% NP-40, 1mM EGTA pH8, 1mM NaF. 10mM NaV and 10μl of protease inhibitor cocktail (Sigma) were added to 1ml of lysis buffer immediately prior to lysis) for 30min, 4°C with continual shaking and cells detached using a cell scraper. The contents of the wells were transferred to appropriately labelled eppendorfs and centrifuged at 13,000 x *g*, 4°C for 15min. 10μl of lysate was removed to estimate protein concentration by the Pierce BCA kit procedure and the protein concentration in different cell lysates were made equivalent using RIPA lysis buffer. 100μl lysate (approximately 150μg protein) was incubated with 1:100 mouse IgG1 control or 5μg/ml anti-Mer at room temperature on ice for 30min, and immunoprecipitation of Mertk was achieved by incubation for 1 hour with agarose-coupled goat anti-mouse IgG at 4°C on a rotator. Immunoprecipitates were then washed twice (13,000 x *g*, 4°C for 1min) in 1 x TBS, pH7.5, 0.1% Triton X-100 to remove non-specifically bound proteins, then washed once (13,000 x *g*, 4°C for 1 minute) in 25mM Tris, pH8, 0.05% SDS to remove salt. Agarose beads were boiled for 5min in 2x non-reducing sample buffer and 10μl was loaded per well of a 9% acrylamide gel to resolve proteins by SDS PAGE (see Chapter 2, 2.13 SDS polyacrylamide gel electrophoresis). Immunoblotting for Mertk was performed as described in Chapter 2, 2.14 Western blotting, using 1:1000 dilutions of anti-Mer mAb and 1:2500 of goat anti-mouse HRP.

6.2.2 *LPS and IFNγ treatment of human MDMφ*

Human monocytes were cultured in 48-well plates for 5 days in IMDM containing 10% fresh autologous serum in the absence or presence of 1μM Dex plus 10ng/ml IFNγ. To examine the effect of short-term LPS treatment, 5-day Dex-MDMφ were either untreated (No LPS) or treated for 1, 2 or 3 hours with 10ng/ml LPS prepared in IMDM containing 10% autologous serum.

6.2.3 *Induction of neutrophil apoptosis*

CMFDA-labelled neutrophils were cultured at 4 x 10^6/ml in <u>serum-free medium</u> (IMDM) at 37°C for 20-24 hours to render neutrophils apoptotic. Neutrophil populations were then centrifuged at 200 x g for 5min and resuspended in fresh IMDM prior to assessment of phagocytosis.

6.3 Results

6.3.1 *Mertk expression is upregulated on the surface of Dex-MDMϕ*

Protein S bound to the surface of apoptotic lymphoma cells has been suggested to promote their clearance through Mertk on untreated human MDMϕ (Uehara and Shacter, 2008). We hypothesised that engagement of this receptor pathway may be responsible for high capacity clearance of apoptotic neutrophils by Dex-MDMϕ. Initially, we performed indirect immunofluorescence as described in Chapter 2, 2.15 Flow cytometry, using a anti-Mer mAb to confirm surface expression of Mertk on MDMϕ. Mertk expression on Dex-MDMϕ was increased 2.2-fold compared to untreated MDMϕ (*fig. 6.1a*) (McColl et al., 2009). Downregulation of CD44 expression on human MDMϕ (1.8-fold) following Dex treatment confirms previous findings (Giles et al., 2001). The percentage of MDMϕ expressing Mertk was also increased, possibly as a result of induction of greater homogeneity in macrophage Mertk expression, whereas the percentage of cells expressing CD44 was unaltered i.e. the surface expression of CD44 decreased, but all cells remained positive for CD44 (*fig. 6.1b*). Interestingly, the percentage of Mertk-positive Dex-MDMϕ (~80%) corresponded well with the percentage of Dex-MDMϕ capable of protein S-dependent phagocytosis of apoptotic neutrophils (~70-80%). We have confirmed Mertk expression (~210-kDa) on untreated and Dex-MDMϕ by western blotting, performed as described in 6.2.1 Immunoprecipitation and western blotting for Mertk on human MDMϕ (*fig. 6.1c*).
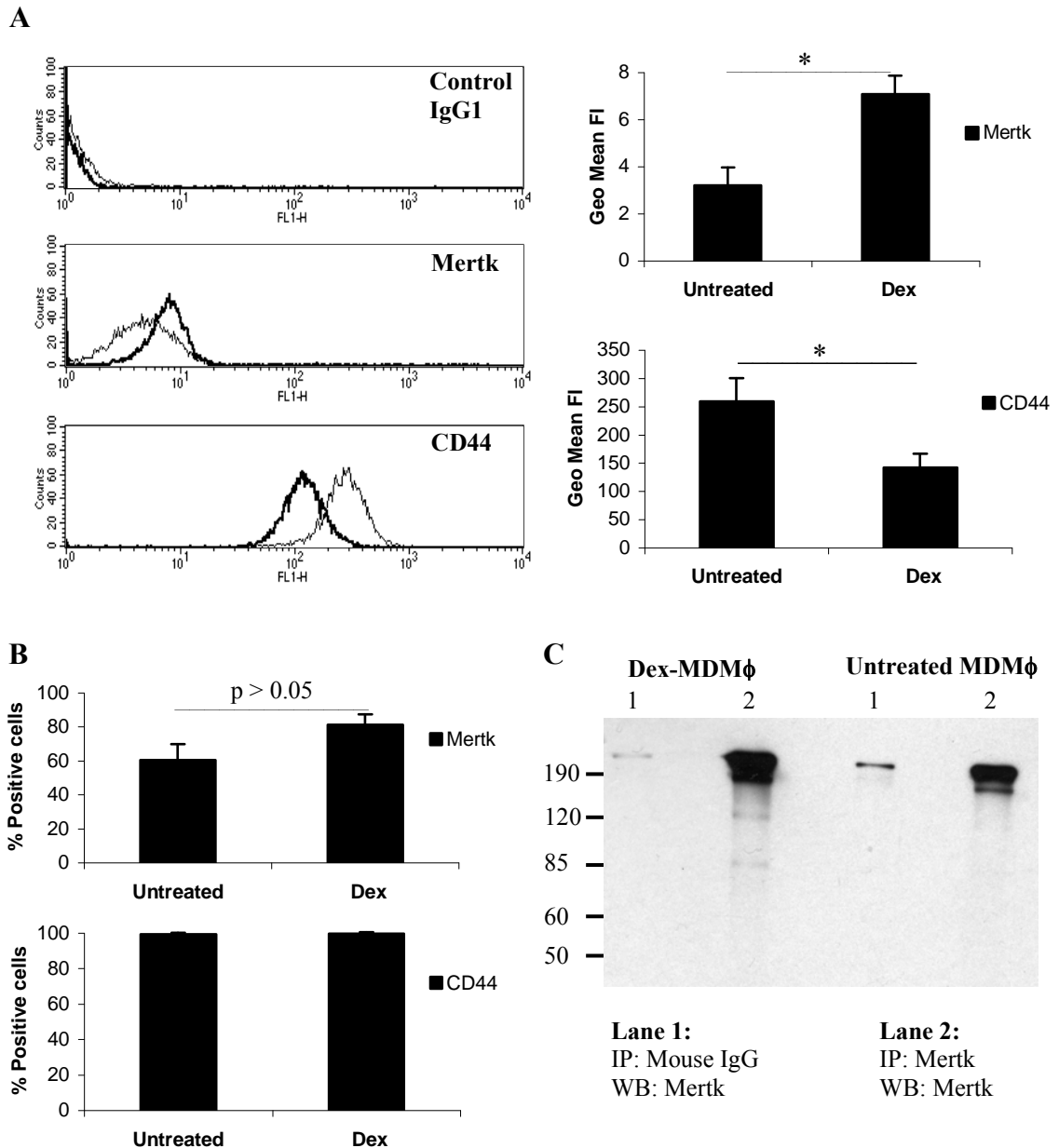
Figure 6.1: Effect of Dex treatment on human MDMɸ expression of Mertk and CD44.

A and **B**, Analysis of surface receptor expression by untreated (thin lines) and Dex-MDMɸ (dark lines) by flow cytometry. Data are presented as Mean ± SEM, n=7 where n = number of different macrophage donors used (* p<0.05; paired t test). **A**, Treatment with Dex resulted in increased expression of Mertk (2.2-fold), but decreased expression of CD44 (1.8-fold). **B**, Treatment with Dex increased the percentage of MDMɸ positive for Mertk expression, whereas the percentage of MDMɸ positive for CD44 was unaltered. **C**, MDMɸ lysates were resolved by SDS PAGE on 9% gels under non-reducing conditions and immunoblotted for Mertk with an anti-Mer mAb (1:1000). Lane 1, IgG1 control; lane 2, Mertk.

6.3.2 *Blocking Mertk on Dex-MDMϕ significantly inhibits serum- and protein S-dependent phagocytosis of apoptotic neutrophils*

We next wanted to evaluate whether this receptor pathway was important for protein S-dependent clearance of apoptotic neutrophils by Dex-MDMϕ. For this, we pre-treated Dex-MDMϕ with 10μg/ml anti-human Mer mAb for 10min prior to phagocytosis to "block" Mertk function. Although anti-Mer had no effect on phagocytosis by Dex-MDMϕ in the absence of protein S, anti-Mer significantly reduced Dex-MDMϕ phagocytosis of serum-free cultured apoptotic neutrophils in the presence of 2.5μg/ml of protein S (*fig. 6.2a*; *** $p < 0.001$, n=3) (McColl et al., 2009). Blockade of Mertk also significantly inhibited Dex-MDMϕ phagocytosis in the presence of 10% fresh autologous serum (*fig. 6.2b*; 67% reduction, ** $p < 0.01$, n=3) (McColl et al., 2009). However, full inhibition of serum-dependent phagocytosis by Dex-MDMϕ was never achieved with anti-Mer, suggesting that another receptor pathway may be responsible for the residual phagocytosis observed. Importantly, we found that anti-Mer had no effect on number of Dex-MDMϕ recovered and therefore represents a specific inhibitory effect on phagocytosis, implying that the Mertk pathway is critical for glucocorticoid augmentation of MDMϕ phagocytosis of apoptotic neutrophils.
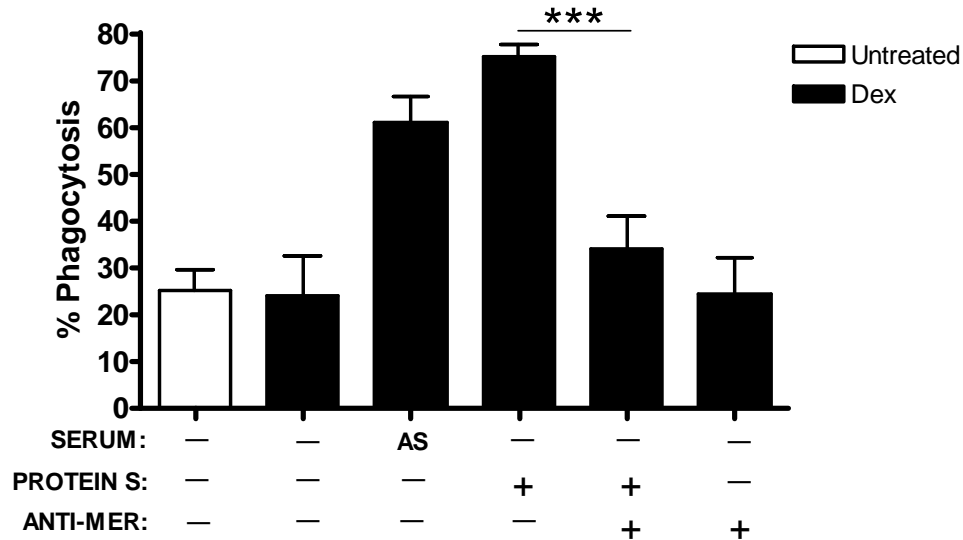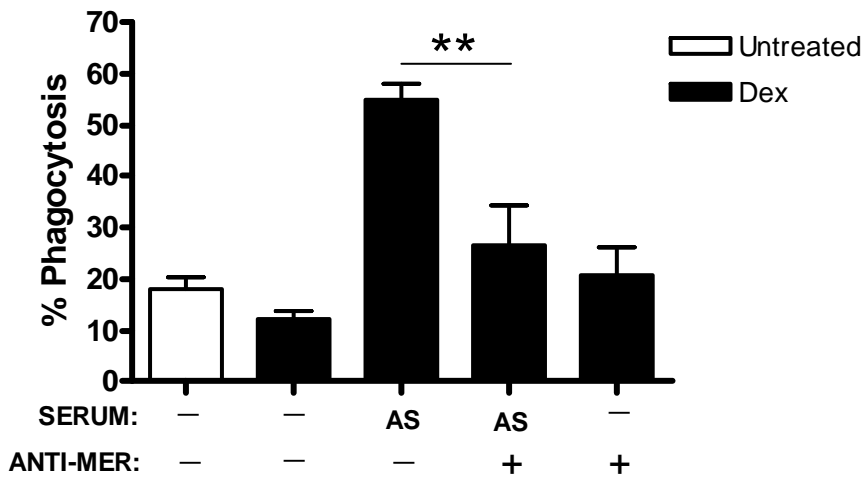
Figure 6.2: Effect of anti-Mer on serum- and protein S-enhanced Dex-MDMφ phagocytosis of apoptotic neutrophils.

Pre-incubation of Dex-MDMφ (black bars) with 10μg/ml anti-Mer mAb for 10min inhibited the enhancing effect of 2.5μg/ml protein S (**A**) and 10% fresh autologous serum (**B**) on Dex-MDMφ phagocytosis of serum-free cultured heterologous apoptotic neutrophils. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (** $p<0.01$ and *** $p<0.001$ when compared to uptake by Dex-MDMφ in the presence of autologous serum and 2.5μg/ml protein S, respectively, one-way ANOVA with a Bonferroni post test). Phagocytosis by untreated MDMφ (white bars) is shown for comparison.

6.3.3 *Mertk expression on the surface of Dex-MDMφ is downregulated following short-term treatment with LPS: effect on Dex-MDMφ phagocytic capacity*

Surface expression of Mertk is dynamically regulated during cell culture, with metalloproteinase-mediated cleavage of the extracellular domain of Mertk resulting in the production a soluble form of the receptor that is constitutively released from cultured cells (Sather et al., 2007). Mertk cleavage is amenable to environmental signals, and can be enhanced by treatment with bacterial LPS (Sather et al., 2007). Since untreated and Dex-MDMφ display differential capacities for serum-dependent phagocytosis of apoptotic neutrophils (Chapter 3, *fig. 3.2*), we hypothesised that upregulation of macrophage Mertk surface expression following treatment with Dex may be responsible for efficient phagocytosis by Dex-MDMφ and that LPS would inhibit Dex-induced expression of Mertk.

LPS was found to rapidly downregulate surface expression of Mertk on Dex-MDMφ, with a significant decrease in the geometric mean fluorescence intensity observed after 1 hour, with further reductions in the level of Mertk expression after 3 hours (*figs. 6.3a and b*). Furthermore, LPS treatment significantly reduced the percentage of Dex-MDMφ positive for Mertk expression (*fig. 6.3c*). We hypothesised that LPS-mediated downregulation of Mertk on Dex-MDMφ would result in a mirrored downregulation of phagocytic capacity to untreated control levels. However, we were surprised to find that downregulation of Mertk expression resulted in only a partial inhibition of protein S-dependent phagocytosis of apoptotic neutrophils by Dex-MDMφ, with LPS treatment for 1, 2 and 3 hours resulting in only 35%, 29.8% and 37.1% reduction, respectively (*fig. 6.4*; p<0.05, n=3). Interestingly, LPS-induced downregulation of Mertk had a more profound impact on serum-enhanced Dex-MDMφ phagocytosis, with LPS treatment for 1, 2 and 3 hours resulting in 48.7%, 47% and 62.2% reduction, respectively (*fig. 6.4*; * p<0.05, ** p<0.01, n=3).
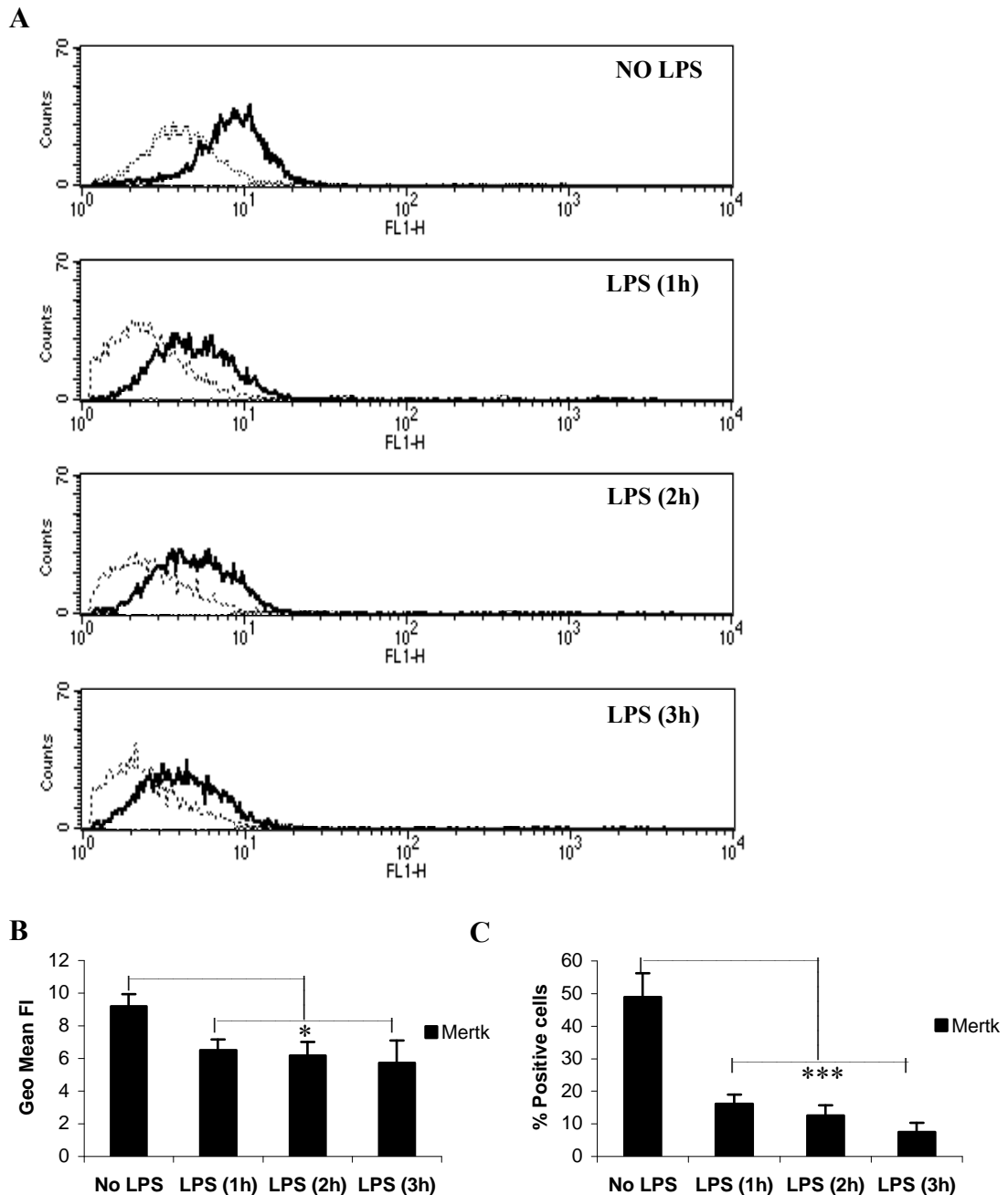
Figure 6.3: Effect of short-term LPS treatment on Dex-MDMφ expression of Mertk.
A, Analysis of Dex-MDMφ surface expression of Mertk (thick line) by flow cytometry. Binding of mouse IgG1 (thin line) is shown as a control. **A** and **B**, LPS treatment of 5-day Dex-MDMφ for 1, 2, or 3 hours resulted in downregulation of Mertk expression by 1.4-, 1.5-, and 1.6-fold, respectively. **C**, LPS treatment also decreased the percentage of human MDMφ positive for Mertk expression. Data are presented as Mean ± SEM, n=3 where n = the number of different macrophage donors. (* p<0.05, *** p<0.001, one-way ANOVA with a Bonferroni post test).
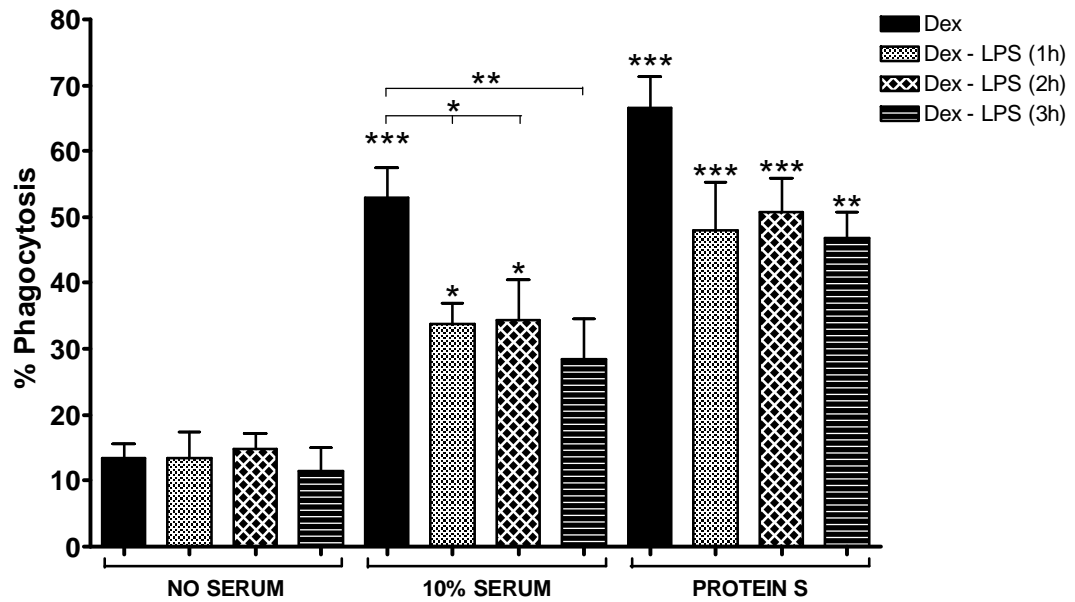
Figure 6.4: Effect of short-term LPS treatment on serum- and protein S-enhanced phagocytosis of apoptotic neutrophils by Dex-MDMφ.

MDMφ were cultured in the presence of 1μM Dex for 5 days, and Dex-MDMφ phagocytosis of serum-free cultured heterologous apoptotic neutrophils was assessed in the absence or presence of 10% fresh autologous serum or 2.5μg/ml human protein S. Short-term treatment of Dex-MDMφ with LPS (1, 2, or 3 hours) significantly reduced the pro-phagocytic effect of serum, but not protein S. Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared to uptake by Dex-MDMφ in the absence of serum, * $p < 0.05$ and ** $p < 0.01$ when compared to uptake by Dex-MDMφ in the presence of serum, one-way ANOVA with a Bonferroni post test).

6.3.4 *Mertk expression on the surface of Dex-MDMϕ is downregulated upon co-culture with IFNγ: effect on Dex-MDMϕ phagocytic capacity*

Previous studies in our lab showed that the potentiating effect of Dex on human MDMϕ phagocytosis could be reversed with the pro-inflammatory cytokine, IFNγ (Heasman et al., 2004). As found for short-term LPS treatment (*fig. 6.3*), increased Mertk expression induced by Dex treatment could be reversed by co-culture with IFNγ (*figs. 6.5a and b*). Furthermore, whilst the percentage of human MDMϕ positive for Mertk expression increased almost two-fold upon Dex treatment, this was returned to untreated control levels upon co-culture with IFNγ (*fig. 6.5c*). Phagocytosis of <u>serum-free cultured</u> apoptotic neutrophils by 5-day untreated-MDMϕ, Dex-MDMϕ and Dex/IFNγ-MDMϕ was assessed in the absence or presence of 10% fresh autologous serum or 2.5μg/ml human protein S. In confirmation of our previous data, we found that uptake of apoptotic neutrophils by Dex-MDMϕ was enhanced in the presence of human serum or 2.5μg/ml protein S (*fig. 6.6*). However, IFNγ had no significant effect on serum-dependent or protein S-dependent (*fig. 6.6*; 36% reduction; $p > 0.05$, $n = 3$) Dex-MDMϕ phagocytosis of apoptotic neutrophils.

Together, these results show that pro-inflammatory mediators such as LPS and IFNγ negatively regulate Mertk expression on Dex-MDMϕ, but fail to block acquisition of a pro-phagocytic phenotype by Dex, suggesting that additional molecular mechanisms independent of Mertk expression may be required.
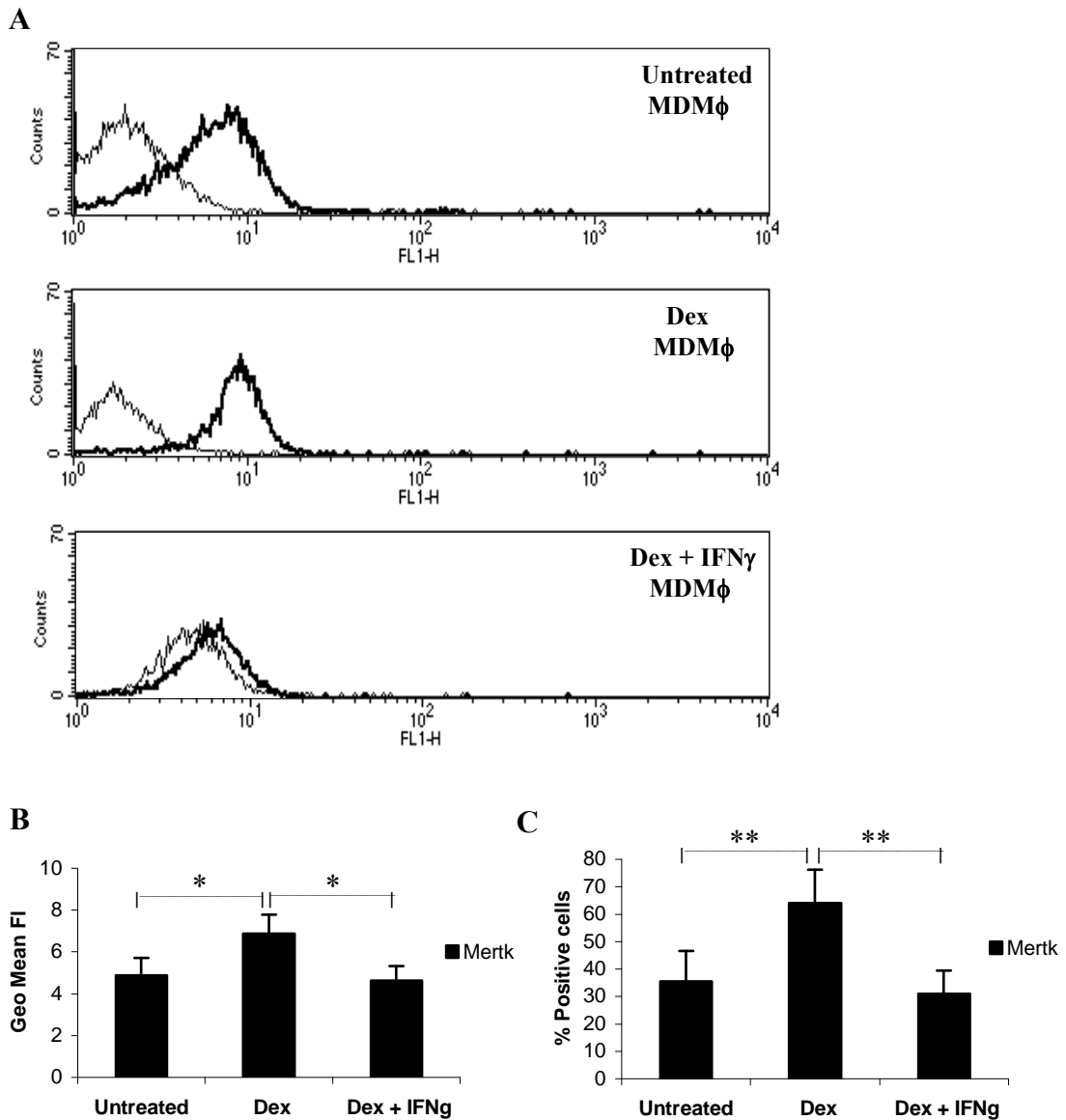
Figure 6.5: <u>Effect of IFNγ on Dex-MDMφ expression of Mertk.</u>
A, Analysis of human MDMφ surface expression of Mertk (thick line) by flow cytometry. Binding of mouse IgG1 (thin line) is shown as a control. **A** and **B**, Co-treatment of human MDMφ with 1μM Dex and 10ng/ml IFNγ for 5 days inhibited Dex-induced upregulation of Mertk expression, with geometric mean fluorescence intensity of binding (**B**) and the percentage of human MDMφ positive for Mertk expression (**C**) equivalent to untreated MDMφ. Data are presented as Mean ± SEM, n=3 where n = the number of different macrophage donors. (* $p<0.05$, ** $p<0.01$, one-way ANOVA with a Bonferroni post test).
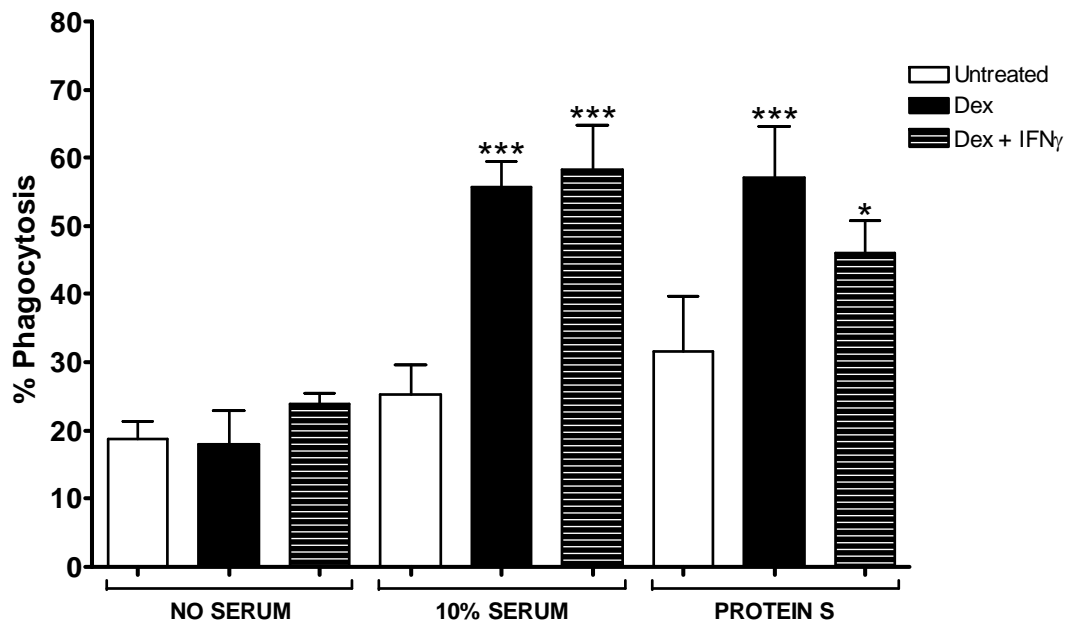
Figure 6.6: Effect of IFNγ on serum- and protein S-enhanced phagocytosis of apoptotic neutrophils by Dex-MDMφ.

MDMφ were cultured in the absence or presence of 1μM Dex ± 10ng/ml IFNγ for 5 days, and MDMφ phagocytosis of serum-free cultured heterologous apoptotic neutrophils was assessed in the absence or presence of 10% fresh autologous serum or 2.5μg/ml human protein S. Co-treatment of human MDMφ with Dex and IFNγ for 5 days did not alter the potentiating effect of Dex on MDMφ phagocytosis (p>0.05). Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (* p<0.05 and *** p<0.001 when compared to uptake by Dex/IFNγ-MDMφ (striped bars) in the absence of serum or 2.5μg/ml protein S, *** p<0.001 when compared to uptake by Dex-MDMφ (black bars) in the absence of serum or 2.5μg/ml protein S, one-way ANOVA with a Bonferroni post test). Phagocytosis by untreated MDMφ (white bars) is shown for comparison.

6.3.5 *Mertk expression is upregulated on the surface of untreated MDMφ following treatment with a TACE-inhibitor peptide: effect on untreated MDMφ phagocytic capacity*

Since the Mertk pathway was found to be critically required for protein S-dependent phagocytosis of apoptotic neutrophils by Dex-MDMφ, we next investigated whether increased surface expression of Mertk on untreated-MDMφ could enhance the phagocytic potential of these cells. For this experiment, 5-day untreated MDMφ were either untreated or treated with 50μg/ml TNFα-converting enzyme (TACE) inhibitor peptide (KD–1X–73.5) for 1 hour to inhibit Mertk shedding. We observed a 1.7-fold increase in Mertk surface expression following TACE inhibitor treatment when compared to untreated controls (*fig. 6.7a*). Interestingly, CD44 expression also increased 2-fold (*fig. 6.7a*), suggesting that expression of Mertk and CD44 on the surface of human MDMφ can be dynamically regulated by TACE metalloproteinase. Although the increase in Mertk expression by untreated-MDMφ following treatment with TACE-inhibitor peptide (1.7-fold) was similar to that induced by long-term Dex treatment of monocytes (2.2-fold), this was not paralleled by an increase in protein S- or serum-dependent phagocytosis of apoptotic neutrophils (*fig. 6.7b*; p>0.05, n=3), suggesting that additional mechanisms independent of Mertk expression are required for Dex-induced acquisition of a highly phagocytic MDMφ phenotype.
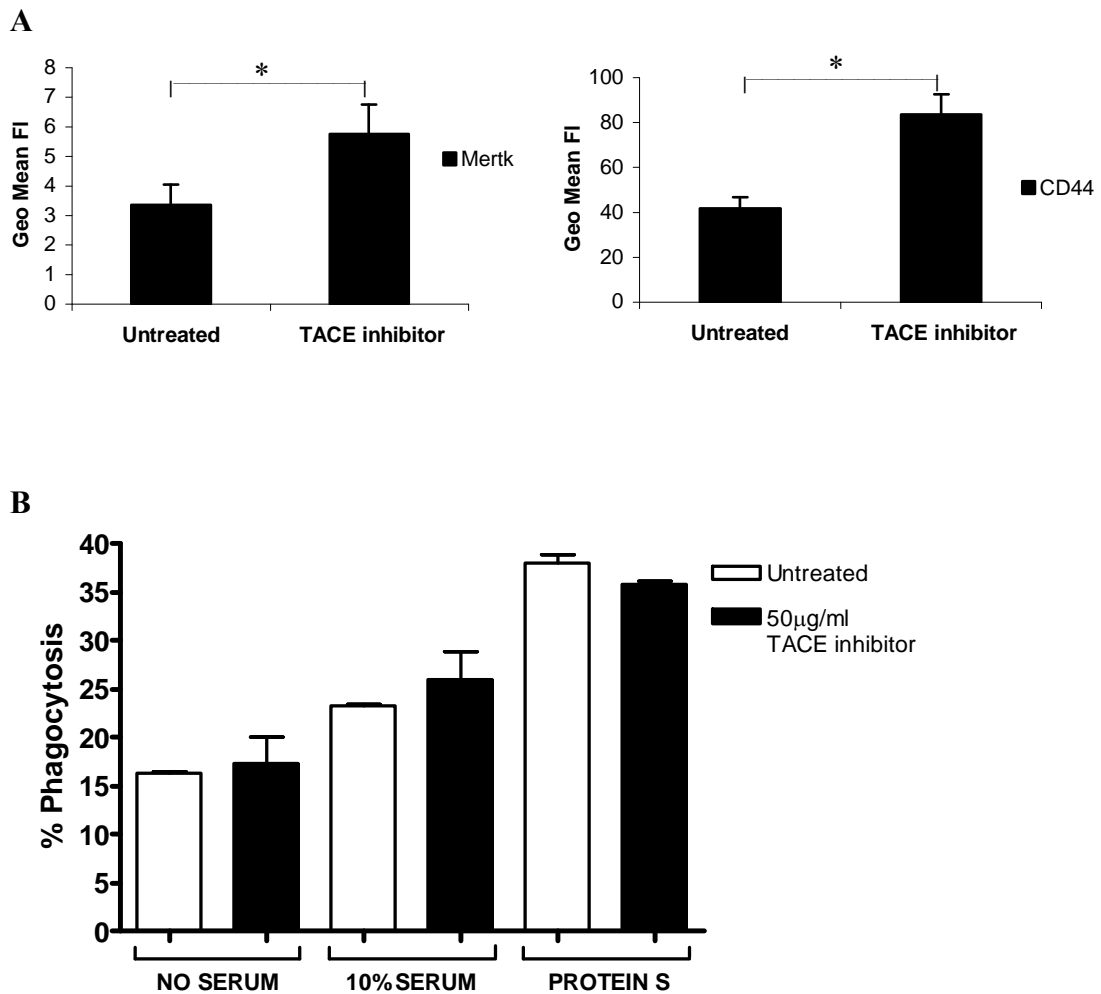
Figure 6.7: Effect of inhibition of TACE/ADAM17 metalloproteinase on Mertk expression and phagocytic ability of untreated MDMφ.

A, Analysis of human untreated MDMφ surface expression of Mertk by flow cytometry. Treatment of 5-day untreated MDMφ with 50μg/ml TACE inhibitor peptide for 1 hour increased expression of Mertk and CD44. Data are presented as Mean ± SEM, n=3 where n = the number of different macrophage donors. (* $p<0.05$; paired t test). **B**. Enhanced Mertk expression was not accompanied by increased MDMφ phagocytic potential (black bars) for heterologous apoptotic neutrophils in the presence of 10% fresh autologous serum or 2.5μg/ml human protein S. Data are presented as Mean ± SEM, n=3 where n = the number of different macrophage donors. ($p>0.05$, when compared to phagocytosis by untreated MDMφ not exposed to TACE inhibitor peptide (white bars), one-way ANOVA with a Bonferroni post test).

6.3.6 *Human MDMφ express αvβ5 integrin on their surface and this is downregulated on Dex-MDMφ*

We next wanted to determine whether the differential capacities of untreated and Dex-MDMφ for protein S-dependent phagocytosis of apoptotic neutrophils was due to altered association of Mertk with other receptors on the cell membrane following Dex treatment. In light of results presented by Wu and co-workers, where efficient phagocytosis of human lymphocytes appeared to require molecular cooperation of Mertk with αvβ5 integrin (Wu et al., 2005), we were interested in examining whether this mechanism was required for protein S-enhanced phagocytosis by Dex-MDMφ. Interestingly, flow cytometric analysis revealved that surface expression of αvβ5 on human MDMφ was downregulated (1.4-fold) following long-term treatment of human monocytes with Dex (*fig. 6.8a*). Furthermore, Dex treatment was associated with a decrease (2-fold) in the percentage of αvβ5-positive MDMφ from ~47.8% to ~24.2%.

6.3.7 *Blocking αvβ5 on Dex-MDMφ has no significant effect on serum- and protein S-dependent phagocytosis, but may affect the adhesive ability of these cells*

To evaluate the potential contribution of the αvβ5 integrin receptor pathway to protein S-dependent clearance of apoptotic neutrophils by Dex-MDMφ, 5-day Dex-MDMφ were pre-treated with 10μg/ml anti-human αvβ5 antibody for 10min prior to phagocytosis to "block" αvβ5 function. Anti-αvβ5 had no effect on the basal levels of phagocytosis by Dex-MDMφ (*fig. 6.9*). Although blockade of αvβ5 had no effect on the protein S-dependent phagocytosis of apoptotic neutrophils by Dex-MDMφ, there was a significant inhibitory effect on serum-dependent phagocytosis by Dex-MDMφ (*fig. 6.9*; 39% inhibition, ** $p < 0.01$. n=3). Interestingly, we found that anti-αvβ5 had a notable effect on the number of Dex-MDMφ recovered under basal conditions (10% autologous serum in the absence of neutrophils; 29% less), and also when neutrophils were added in the presence of 10% autologous serum (24.2% less) but not 2.5μg/ml human protein S (3.4% less), suggesting that αvβ5 may be an important adhesion receptor for Dex-MDMφ in the presence of serum.
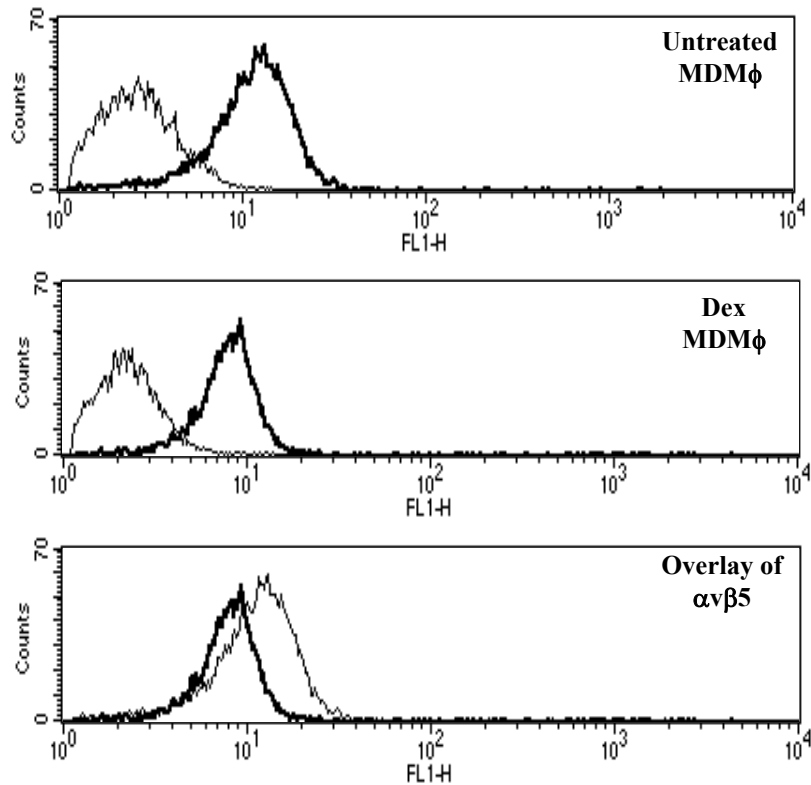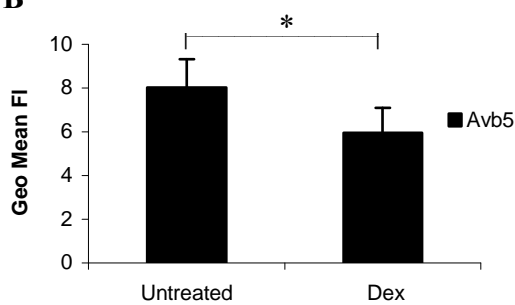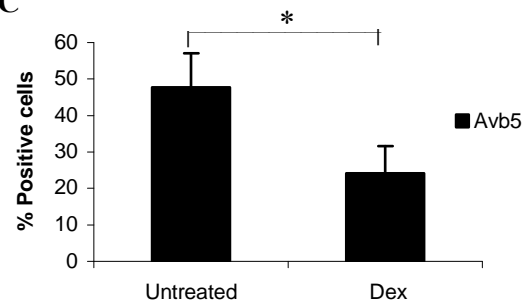
Figure 6.8: Effect of Dex treatment on human MDMφ expression of αvβ5.
A, Analysis of human MDMφ surface expression of αvβ5 (thick line) by flow cytometry. Binding of mouse IgG1 (thin line) is shown as a control. **A** and **B**, Human MDMφ expression of αvβ5 integrin was reduced (1.4-fold) by 5-day treatment of monocytes with Dex. **C**, Dex treatment also decreased the percentage of human MDMφ positive for αvβ5 expression. Data are presented as Mean ± SEM, n=3 where n = the number of different macrophage donors (* $p<0.05$; paired t test).
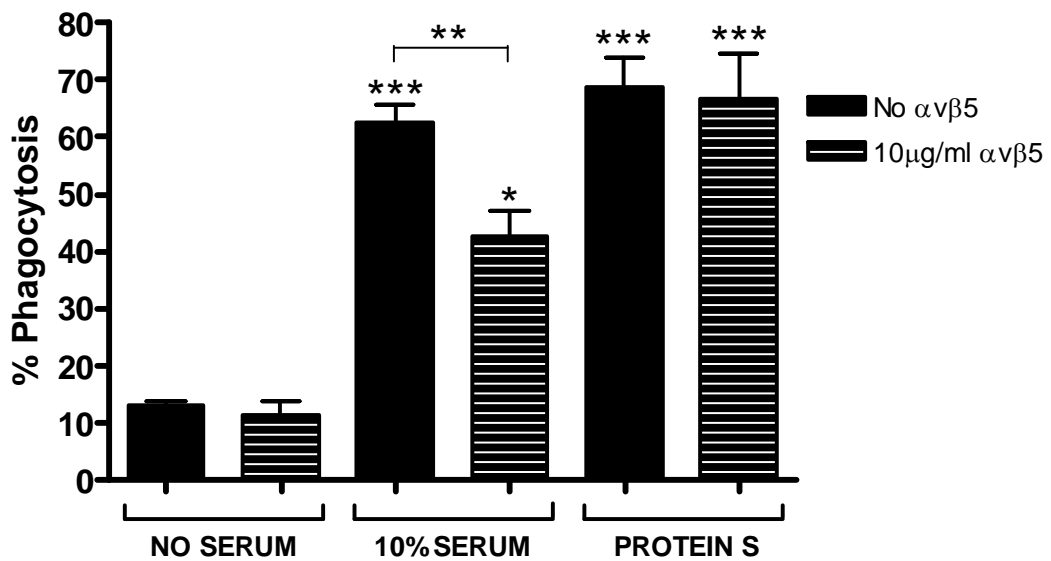
Figure 6.9: Effect of anti-αvβ5 on serum- and protein S-enhanced Dex-MDMφ phagocytosis of apoptotic neutrophils.

5-day Dex-MDMφ were untreated (black bars) or treated with 10μg/ml anti-αvβ5 mAb for 10min prior to phagocytosis assays. Dex-MDMφ phagocytosis of serum-free cultured heterologous apoptotic neutrophils was assessed in the absence or presence of 10% fresh autologous serum or 2.5μg/ml human protein S. Blocking αvβ5 receptor significantly reduced the pro-phagocytic effect of serum but not protein S on Dex-MDMφ, an effect that may be associated with reduced number of Dex-MDMφ recovered in serum-containing cultures (24.2% less) compared to protein S-containing cultures (3.4% less). Mean phagocytosis ± SEM, n=3 where n = the number of different macrophage donors. (* $p<0.05$, *** $p<0.001$ when compared to uptake by Dex-MDMφ in the absence of serum, ** $p<0.01$ when compared to uptake by Dex-MDMφ in the presence of serum, one-way ANOVA with a Bonferroni post test).

6.4 Discussion

6.4.1 *Mertk is critically required for protein S-dependent clearance of apoptotic neutrophils by Dex-MDMφ*

Mertk, a member of the Tyro-3/Axl/Mer (TAM) family of receptor tyrosine kinases, is composed of an extracellular region comprising two immunoglobulin-like and two fibronectin type III motifs, a transmembrane region, and an intracellular tyrosine kinase domain (Graham et al., 1994, Graham et al., 1995). Mertk expression has been detected in peripheral blood monocytes, monocyte-derived macrophages and dendritic cells, but not by granulocytes or lymphocytes, and is highly expressed in testis, ovary, prostate, lung, kidney, and eye (Graham et al., 1994, Graham et al., 1995, Behrens et al., 2003, Seitz et al., 2007). Moreover, Lu *et al* performed *in situ* hybridisation to show that Mertk is expressed in macrophage zones of the spleen, lymph node and thymus (Lu and Lemke, 2001). Thus, Mertk is expressed predominately by macrophages that are required for efficient clearance of apoptotic cells *in vivo* and in organs where high cell turnover is evident. Mertk expression at the protein level changes with both developmental and activation status (Seitz et al., 2007), being essentially absent from peripheral blood monocytes and increases as these cells differentiate into tissue macrophages (Behrens et al., 2003). Long-term treatment of human monocytes with Dex resulted in increased surface expression of Mertk, consistent with previous observations that Mertk is a glucocorticoid-regulatable protein (Ehrchen et al., 2007). Pre-treatment of Dex-MDMφ with an anti-Mer mAb significantly reduced the pro-phagocytic effect of human serum and protein S on apoptotic neutrophil clearance. However, the Mertk-independent phagocytosis observed for Dex-MDMφ suggests that additional serum-dependent pathways are involved. Interestingly, a calcium-independent serum opsonisation event was responsible for a partial augmentation of Dex-MDMφ phagocytosis, representing 38% of the serum-enhancing effect (Chapter 3, *fig. 3.8*), a percentage that is similar to the Mertk-independent pro-phagocytic effect of serum (33% augmentation). However, it must be noted that anti-Mer had a slight stimulatory effect on the basal levels of Dex-MDMφ phagocytosis which may account for these effects.

6.4.2 *Mertk is required for phagocytosis of apoptotic neutrophils by Dex-MDMϕ, but not untreated-MDMϕ: a glucocorticoid-induced "switch" in apoptotic cell recognition mechanisms*

The results presented in this thesis support the requirement of the Mertk pathway for protein S-dependent phagocytosis of "early", membrane-intact apoptotic neutrophils by Dex-MDMϕ. However, we were unable to show a requirement for Mertk-dependent phagocytosis by untreated MDMϕ. Since both untreated and Dex-MDMϕ populations examined in this study expressed Mertk, the reason why Dex-MDMϕ are enabled to utilise a protein S-dependent clearance pathway is not clear. The predominance for utilisation of a specific molecular pathway for apoptotic cell clearance is not constant between different populations of phagocytes and can be influenced by environmental stimuli. Untreated MDMϕ represent a highly heterogeneous population of cells in terms of morphology and receptor expression (Giles et al., 2001), and may have been "imprinted" with different mechanisms for apoptotic cell clearance during differentiation. This is in contrast to the morphological and functional homogeneity exhibited by Dex-MDMϕ, and homogeneity in Mertk expression may explain why more MDMϕ are capable of using this recognition pathway. Another possibility is that the observed upregulation of Mertk expression on the surface of Dex-MDMϕ was sufficient to confer phagocytic potential. We found that exposure of Dex-MDMϕ to bacterial LPS (short-term) and IFNγ (long-term) resulted in downregulation of Mertk expression to levels expressed by untreated MDMϕ. However, this alteration was not accompanied by loss of protein S-dependent phagocytosis by Dex-MDMϕ. This is an important observation, as cytokine inhibition of a Dex-MDMϕ pro-phagocytic phenotype would have implications for the efficacy of glucocorticoid treatment *in vivo* when stimuli such as LPS or IFNγ might also be present. Interestingly, inhibition of serum-dependent phagocytosis following pre-treatment of Dex-MDMϕ with LPS for 3 hours (62.2% reduction) was remarkably similar to effects of blockade of Mertk with an anti-Mer mAb (67% reduction; ** $p<0.01$, n=3) (*fig. 6.2b*). The lack of IFNγ-mediated inhibition of serum-dependent phagocytosis by Dex-MDMϕ is in contrast to previous reports where co-treatment with Dex and IFNγ for 5 days abolished the

effect of Dex on MDMϕ phagocytic capacity (Untreated: ~13%; Dex: ~66%; Dex/IFNγ: ~23%) (Heasman et al., 2004). Similar human MDMϕ culture conditions and IFNγ concentration, albeit a different source of IFNγ, were used in this study. Based upon the inhibitory effect of LPS on Dex-MDMϕ phagocytic potential reported in this study and by Michlewska *et al* (Michlewska et al., 2009), inhibition of Dex-MDMϕ phagocytosis as reported by Heasman *et al* may not have been a specific effect mediated by IFNγ but instead a consequence of LPS-contamination of the IFNγ. Addition of polymyxin B, a substance that binds and neutralises LPS (Ferrari et al., 2004), during culture would have permitted analysis of IFNγ on the pro-phagocytic effect of Dex in the absence of LPS contamination, although this experiment would need to be tightly controlled to exclude false negative (cytotoxicity) or false positive results (stimulation by polymyxin).

Proteolytic cleavage of cell surface proteins, or ectodomain "shedding", plays an essential role in regulating the pattern of surface protein expression and/or function. TACE/ADAM17 metalloproteinase has been implicated in the shedding of a number of distinct cell surface proteins, including cytokines (TNFα), cytokine receptors (TNF receptors I and II), and adhesion molecules (L-selectin) (Black et al., 1997, Moss et al., 1997, Peschon et al., 1998, Reddy et al., 2000, Li et al., 2006). By using a TNFα protease inhibitor (TAPI-0), Sather *et al* recently demonstrated that Mertk expression on the surface of the mouse macrophage cell line, J774, can be regulated by TACE metalloproteinase (Sather et al., 2007). Inhibition of TACE-mediated cleavage of Mertk on the surface of untreated human MDMϕ revealed that enhanced expression of Mertk is not sufficient to confer a pro-phagocytic MDMϕ phenotype. Surface expression is not always an indicator of receptor function, as demonstrated by loss of β2 integrin function during neutrophil apoptosis independently of expression (Dransfield et al., 1995). Alternatively, efficient phagocytosis may require a conformational change in the Mertk receptor on Dex-MDMϕ to increase affinity for protein S, in a manner analogous to that described for the integrin-dependent interaction of activated leukocytes with endothelial cells (Hogg et al., 2002).

Mertk may also interact with other receptors on the cell membrane following Dex treatment. Induction of co-operative action of receptors may allow regulation of phagocytosis of apoptotic cells in response to different environmental cues encountered during the inflammatory response. Co-precipitation studies with fusion proteins of Tyro-3/Axl/Mertk, composed of the extracellular ligand-binding domain of the receptors fused to the Fc region of human IgG1, suggested that TAM receptors are expressed as disulphide-linked homodimers capable of binding Gas6, but not protein S (Nagata et al., 1996). Human Gas6, a protein S homologue (44% amino acid sequence identity) (Manfioletti et al., 1993), has been suggested to preferentially bind and stimulate Axl, yet can bind to Tyro-3 and Mertk albeit with a lower affinity (Ohashi et al., 1995, Godowski et al., 1995, Nagata et al., 1996, Chen et al., 1997, Sather et al., 2007, Seitz et al., 2007). Gas6 may opsonise apoptotic cells for phagocytosis mediated by Axl or Mertk (Ishimoto et al., 2000, Scott et al., 2001, Wu et al., 2005), and Gas6 expression in the retina may be important for Mertk-mediated phagocytosis of rod outer segments by RPE cells (D'Cruz et al., 2000). Interestingly, murine macrophages express both Gas6 and protein S and this may endow macrophages with all three receptors pathways (Seitz et al., 2007). However, the human plasma concentration of Gas6 is 1000-fold lower than protein S (Balogh et al., 2005), making it unlikely that Gas6 in human serum could also confer Mertk-dependent phagocytosis by Dex-MDMϕ in our system. Ligand-activated Mertk forms dimers in the membrane, resulting in Mertk autophosphorylation and activation (Schlessinger, 2000), and heterodimerisation with other TAM family receptors following glucocorticoid treatment may be required for interaction with protein S or to provide synergistic signalling for efficient Mertk-dependent phagocytosis (Seitz et al., 2007). Interestingly, platelets also express all three receptors and appear to require heterodimerisation for receptor tyrosine phosphorylation (Angelillo-Scherrer et al., 2005). Since murine macrophages express all three TAM receptors on their surface (Seitz et al., 2007), it would be interesting to determine the effect of Dex on Axl and Tyro-3 surface expression and membrane localisation on human Dex-MDMϕ with respect to Mertk, and whether anti-Axl or anti-Tyro-3 antibodies can similarly inhibit protein S-dependent phagocytosis of apoptotic neutrophils by Dex-MDMϕ.

Alternatively, protein S-dependent phagocytosis by Dex-MDMϕ may require collaboration of Mertk with other membrane receptors involved in the phagocytic process. Cooperation of receptor pathways for opsonin-dependent recognition of apoptotic neutrophils has been demonstrated for untreated MDMϕ, where a αvβ3–CD36 molecular complex is required for recognition of TSP-1-opsonised apoptotic neutrophils (Savill et al., 1992). Molecular crosstalk between Mertk and scavenger receptor A (SR-A) or αvβ5 receptor pathways may provide synergistic signalling required for efficient phagocytosis of apoptotic cells (Wu et al., 2005, Todt et al., 2008). Expression of αvβ5 integrin has been suggested to be important for binding outer segments by RPE cells (Anderson et al., 1995, Finnemann et al., 1997), and subsequent triggering of Mertk-dependent internalisation (Finnemann, 2003). Although our results indicate that Mertk-dependent phagocytosis of apoptotic neutrophils by Dex-MDMϕ does not involve an αvβ5-dependent clearance mechanism, we found that Dex-MDMϕ were prone to detachment when phagocytosis assays were performed in the presence of anti-αvβ5. Ligation of αvβ5 integrin, an important modulator of cell adhesion, induces the formation of the DOCK180/Crk/p130cas signalling complex (Albert et al., 2000). Interestingly, co-immunoprecipitation studies indicate that Mertk under conditions of limited receptor expression, such as that found on untreated MDMϕ, induces p130cas in an αvβ5-dependent manner (Wu et al., 2005). We propose that downregulation of αvβ5 integrin on the surface of Dex-MDMϕ may contribute to the loss of p130cas expression and reduced formation of adhesive contacts in these cells (Giles et al., 2001) and may be required for acquisition of a Mertk-dependent phagocytic phenotype, whereas functional cooperation between αvβ5 and Mertk on untreated MDMϕ may contribute to the more adhesive nature of these cells. One possible approach to testing this hypothesis would be to "knock-down" αvβ5 expression in human untreated MDMϕ using siRNA and examine the effects on morphology and adhesion status. Interestingly, phagocytosis of apoptotic neutrophils by Dex-MDMϕ was markedly inhibited in the presence of 500μg/ml fucoidin, indicating scavenger receptor involvement (Giles et al., 2001). Furthermore, SR-A mediates divalent cation-independent adhesion to serum-coated cell culture plastic (Fraser et al., 1993),

and it would be interesting to investigate whether this receptor pathway mediates the calcium-independent effect of serum on Dex-MDMφ, possibly acting as a Mertk-independent tethering receptor.

Receptor tyrosine kinases play an essential role in transducing signals from the extracellular environment to influence a variety of cellular processes including migration, survival, proliferation, and differentiation (Ullrich and Schlessinger, 1990, Fantl et al., 1993, Schlessinger, 2000). Glucocorticoids may influence engagement of downstream signalling pathways critical for Mertk-dependent phagocytosis independently of Mertk expression, including regulation of cytoskeletal organisation (Guttridge et al., 2002). Dex-MDMφ exhibit reduced phosphorylation and localisation of paxillin and pyk2 to podosome-like adhesion structures, together with increased Rac activity (Giles et al., 2001). Interestingly, the Rac guanine nucleotide exchange factor Vav1 is activated downstream of Mertk (Mahajan and Earp, 2003). The phagocytic defect in Mertk[kd] mice may be due to the loss of residues in the activation-loop domain of the Mer kinase region, including the autophosphorylation sites Tyr749, Tyr753, and Tyr754 (Ling et al., 1996). One possibility is that phosphorylation of Mertk at Tyr867 (Tibrewal et al., 2008), a residue required for binding of Grb2 and subsequent recruitment of PI3-kinase (PI3K) to Mertk (Georgescu et al., 1999), in the absence of assembly of adhesion structures promotes MDMφ phagocytic activity. Pre-treatment of Dex-MDMφ for 1 hour with 100μM of a PI3K inhibitor, LY294002, significantly inhibited Dex-MDMφ phagocytosis of apoptotic neutrophils (from ~41% to ~8% phagocytosis) (Katherine Giles, unpublished data). PI3K is required for membrane recycling during extension of cellular processes (Cox et al., 1999), suggesting that the high membrane activity displayed by Dex-MDMφ may contribute to their phagocytic potential. Furthermore, ligation of Mertk receptor induces rapid activation of ERK1 and ERK2, Akt and p38 MAPK (Guttridge et al., 2002). Thus, altered association of Mertk with the actin cytoskeleton may produce a more phagocytic phenotype.

6.4.3 *Consequences of Mertk deficiency for apoptotic cell clearance in vitro and in vivo*

The physiological role of Mertk in apoptotic cell clearance has been addressed *in vivo* through targeted disruption of Mertk using homologous recombination to delete 53 residues in the activation-loop domain of the Mertk cytoplasmic kinase domain, generating Merk kinase-dead mice (Mertk^{kd}) (Lu et al., 1999). Mertk^{kd} mice contain abnormally high numbers of apoptotic cells in many of their tissues due to an *in vivo* defect in the apoptotic cell clearance capacity of macrophages (Lu et al., 1999, Scott et al., 2001). Mertk^{kd} macrophages demonstrate a marked impairment in their ability to phagocytose apoptotic thymocytes *in vitro* (83-94% less efficient than wild-type mϕ), yet FcR-mediated phagocytosis and uptake of other particles including bacteria and latex beads was unaffected, suggesting an essential role for Mertk in facilitating macrophage phagocytosis of apoptotic cells (Scott et al., 2001). In contrast to dramatically reduced clearance of apoptotic thymocytes observed in Mertk^{kd} mice (Seitz et al., 2007), with a seven-fold increase in remnant apoptotic thymocytes in the thymi of Mertk^{kd} mice compared to wild-type control mice following injection of Dex to induce thymocyte apoptosis (Scott et al., 2001), apoptotic thymocyte removal was found to be normal in Axl/Tyro3-deficient mice (Seitz et al., 2007), indicating that the Mertk receptor pathway is critically required for efficient macrophage clearance of a high load of apoptotic cells in the thymus. Interestingly, the opposite is true for dendritic cell phagocytosis, which does not require Mertk and instead has been suggested to be mediated by Tyro-3 and Axl (Behrens et al., 2003, Seitz et al., 2007), indicating that Tyro-3 and Axl may be involved in the normal homeostatic response to apoptotic cells. Mertk-deficient mice develop an SLE-like autoimmune disease characterised by high levels of autoantibodies to nuclear chromatin and DNA, probably resulting from impaired removal of apoptotic cells before lysis (Lu et al., 1999, Scott et al., 2001, Lu and Lemke, 2001, Cohen et al., 2002). Interestingly, the functional mutation in Mertk was found to render Mertk^{kd} mice blind (Scott et al., 2001). The Royal College of Surgeons (RCS) rat model of retinal degeneration has a naturally occurring splice variation that renders Mertk non-functional, resulting in retinal dystrophy and eventually blindness due to impaired clearance of rod outer segments (ROS) by associated retinal pigmented epithelial (RPE) cells (D'Cruz et al.,

2000). A Mertk-specific defect in apoptotic cell clearance has also been reported for RPE cells of the human eye (Gal et al., 2000).

Scott *et al* performed binding assays to determine whether the phagocytic defect of Mertk[kd] macrophages was due to an inability to bind apoptotic thymocytes in the absence of this receptor (Scott et al., 2001). Analysis by scanning electron microscopy showed that wild type and Mertk[kd] macrophages were equally capable of binding apoptotic thymocytes. However, while wild-type macrophages proceeded to ingest bound thymocytes within the 1 hour assay, apoptotic thymocytes remained bound to the surface of Mertk[kd] macrophages with negligable ingestion. Hence, Mertk-deficient macrophages display normal recognition and binding, but a deficiency in internalisation of apoptotic thymocytes. Analysis of Dex-MDMϕ phagocytosis of apoptotic neutrophils by confocal microscopy (Chapter 3, *fig. 3.7*) indicated that Dex-MDMϕ were capable of binding apoptotic neutrophils in the absence of serum, but internalisation required the presence of human serum during phagocytic interaction, probably as a source of protein S for stimulation of Mertk. Thus, binding and internalisation of apoptotic neutrophils by Dex-MDMϕ may be separate events requiring distinct receptor mechanisms. We have shown anti-Mer to inhibit serum- and protein S-dependent uptake of apoptotic neutrophils by Dex-MDMϕ, however it would be interesting to determine whether Dex-MDMϕ binding of apoptotic neutrophils is Mertk-dependent. The contribution of Mertk to apoptotic neutrophil binding could be assessed by pre-treating Dex-MDMϕ with anti-Mer before performing binding experiments at 4°C in the absence or presence of human serum or protein S. Additional experiments are required to determine whether a tethering receptor is employed by Dex-MDMϕ for serum-independent binding of apoptotic neutrophils, and if this interaction is critical for efficient uptake of apoptotic neutrophils. Several receptor mechanisms have been implicated in clearance of apoptotic cells *in vitro*, yet relatively few of these clearance mechanisms impair apoptotic cell clearance *in vivo* when tested in genetically deficient mice. Intriguingly, expression of Mertk, MFG-E8 and C1q, proteins critically required for apoptotic cell clearance *in vivo* (Botto, 1998, Scott et al., 2001, Taylor et al., 2000,

Hanayama et al., 2004), were upregulated in human monocytes upon exposure to glucocorticoids (Ehrchen et al., 2007). We have demonstrated that Dex-MDMφ phagocytosis of apoptotic neutrophils is Mertk-dependent, but C1q-independent and MFG-E8 independent (via inhibition of αvβ5).

6.4.4 *Mertk signalling is linked to anti-inflammatory effects in macrophages*

Glucocorticoids dramatically alter the expression profile of monocytes during maturation to induce a more anti-inflammatory macrophage phenotype (Ehrchen et al., 2007) that displays enhanced capacity for nonphlogistic phagocytosis of apoptotic neutrophils (Liu et al., 1999, Heasman et al., 2004). For example, uptake of apoptotic neutrophils by methylprednisolone-treated MDMφ failed to trigger the release of IL-8 and MCP-1, chemokines that can promote influx of more leukocytes and therefore prolong inflammation (Liu et al., 1999). Therefore, glucocorticoids promote "safe" clearance of cells dying by apoptosis and may directly contribute to the resolution of inflammation. Importantly, Dex-MDMφ are not completely dissociated from pro-inflammatory signalling, retaining the ability to respond to LPS and IFNγ via modification of surface receptor expression. Members of the TAM family of receptor tyrosine kinases perform an essential immunoregulatory role by regulating macrophage responses to environmental stimuli, for example by limiting toll-like receptor (TLR)-mediated immune responses (Camenisch et al., 1999, Lu and Lemke, 2001, Lemke and Lu, 2003). The repression of the pro-inflammatory macrophage responses is directly coupled to the recognition of apoptotic cells (Cvetanovic and Ucker, 2004). Interestingly, Gas6-mediated ligation of Mertk in the presence of apoptotic cells enhanced apoptotic cell clearance by microglial cells, associated with Vav phosphorylation and activation of Rac, while actively inducing anti-inflammatory signalling that was sufficient to suppress LPS-induced expression of iNOS and IL-1β (Grommes et al., 2008). Furthermore, apoptotic cells protect mice against LPS-induced shock (Ren et al., 2008). Mertk expression on the surface of human MDMφ was subject to positive and negative regulation by anti- (Dex) and pro-inflammatory (LPS or IFNγ) molecules, respectively. Altered expression of

Mertk would be critically required for the ability of macrophages differentiated in the presence of Dex respond to bacterial infection *in vivo* by releasing pro-inflammatory mediators upon stimulation of TLRs.

Mertk may therefore play a dual role in the pro-resolving effect of glucocorticoids. Induced expression of Mertk and protein S by glucocorticoids may promote acquisition of a negative-feedback pathway to both switch off pro-inflammatory cytokine production (Rothlin et al., 2007) while enhancing phagocytic capacity for apoptotic cells. The efficacy of glucocorticoids in treatment of autoimmune diseases like SLE that are characterised by impairment of apoptotic cell clearance may be due, in part, to engagement of these pro-resolution mechanisms. Manipulation of the Mertk pathway may represent a novel approach to engage aspects of glucocorticoid action that favour resolution of inflammation without promoting deleterious side effects.

CHAPTER 7: SUMMARY AND FUTURE DIRECTIONS

The aim of this study was to determine the molecular mechanisms required for efficient clearance of apoptotic neutrophils by human MDMϕ, a process that may be conducive for the resolution of inflammation. Glucocorticoids are potent anti-inflammatory agents that exhibit pro-resolving actions through modulation of inflammatory neutrophil survival and function, and augmentation of macrophage phagocytic capacity for apoptotic neutrophils *in vitro* (Liles et al., 1995, Cox, 1995, Meagher et al., 1996, Liu et al., 1999, Giles et al., 2001). These findings have serious implications in the clinical setting where unresolving chronic inflammatory diseases characterised by inefficient or dysregulated resolution processes cause considerable morbidity and untimely death. Indeed, it has recently been demonstrated that manipulation of apoptosis *in vivo* may result in enhanced resolution of inflammation (Rossi et al., 2006, Sawatzky et al., 2006), thereby highlighting the potential to manipulate processes involved in the resolution of inflammation for therapeutic gain (Serhan et al., 2007, Rossi et al., 2007). When the work in this thesis was started, little was known of the phagocytic receptor mechanisms(s) required for enhanced uptake of apoptotic neutrophils by human MDMϕ following treatment with the dexamethasone. We hypothesised that glucocorticoids specifically induce a distinct mechanism for apoptotic cell clearance. Apoptotic cell clearance is a complex process thought to involve numerous interactions between the apoptotic prey and macrophages, and soluble bridging molecules can bind to the apoptotic surface to modulate the phagocytic interaction. The diversity of receptor pathways involved in the phagocytic interaction may represent redundancy in receptor mechanisms, or may equip different phagocyte populations with specific receptor profiles required for specialisation of phagocytic function, targeted recognition of different apoptotic prey, or to allow adaptability in macrophage phagocytic capacity based upon the tissue load of apoptotic cells. We focused on understanding the interaction between Dex-MDMϕ and apoptotic neutrophils at the receptor level, and we have found that exposure of human monocytes to Dex immediately following isolation for the duration of their maturation into macrophages induced a highly phagocytic phenotype associated with a "switch" from a serum-independent clearance

mechanism employed by untreated MDMϕ to a highly efficient serum-dependent mechanism. In summary, long-term treatment with Dex:

- Increased phagocytosis of 20 hour <u>serum</u>-cultured neutrophils 2-fold, consistent with results from previous studies (Liu et al., 1999, Giles et al., 2001)
- Failed to enhance uptake of 20 hour <u>serum-free</u> cultured neutrophils
- Increased phagocytic capacity critically required the presence of serum during the phagocytic interaction
    - Serum promotes internalisation of "early" apoptotic neutrophils by Dex-MDMϕ, but not untreated MDMϕ
    - Human albumin, IgG, complement C1q and C3b, fibronectin, pentraxin-3, annexin-1, a platelet-derived factor and alpha-2 macroglobulin were found to be devoid of pro-phagocytic activity
    - High molecular weight fractions, containing protein S and C4BP, conferred the pro-phagocytic activity of human serum
    - Protein S binds to the surface of apoptotic neutrophils in a calcium-dependent but PS-independent manner to enhance clearance by Dex-MDMϕ
    - Immunodepletion of protein S abolished the pro-phagocytic activity of 0.2M anion exchange fractions and phagocytosis was restored upon addition of purified protein S to depleted fractions
    - Reduction and alkylation abolished the pro-phagocytic activity of high molecular weight gel filtration fractions

Although our data indicated that a high molecular weight complex of C4BP-protein S was required for augmented phagocytosis of apoptotic neutrophils by Dex-MDMϕ, we found that purified protein S was sufficient to confer augmentation. The failure of low molecular weight fractions to enhance Dex-MDM phagocytosis may be due to inefficient isolation of the free form of protein S during anion exchange. Incubation of C4BP-protein S complexes with guanidium chloride to dissociate C4BP with the β chain from protein S followed by gel filtration and dialysis, as performed by Kask *et al* (Kask et al., 2004), may be a valuable future experiment for testing the phagocytic contribution of C4BP and protein S in their free and complexed forms.

By performing inhibitor studies of Dex-MDMϕ phagocytosis using mAbs, we found that the Mertk receptor pathway was critically required for protein S-dependent clearance of apoptotic neutrophils by Dex-MDMϕ, but not untreated MDMϕ. The environmental stimuli monocytes are exposed to during differentiation are therefore likely to enable the promotion of specific phagocytic mechanisms. We propose that long-term exposure of monocytes to Dex induces a "switch" to a Mertk-dependent pathway for efficient apoptotic cell clearance. We hypothesised that Dex-mediated transactivation of Mertk expression was sufficient for increasing the phagocytic potential of human MDMϕ. However, LPS- and IFNγ-mediated downregulation of Mertk on Dex-MDMϕ did not abolish the phagocytic potential of these cells. Furthermore, we demonstrated that serum-enhanced Dex-MDMϕ phagocytosis of apoptotic neutrophils:

- Involved a partial calcium-independent opsonisation event
- Was only partially inhibited by blocking Mertk
- By promoting internalisation of bound apoptotic neutrophils, with binding possibly mediated by a serum-independent mechanism

These data suggest that while Mertk mediates efficient internalisation of protein S-opsonised apoptotic neutrophils, other pathways may contribute to the phagocytic potential of Dex-MDMϕ. Indeed, it may be naïve to expect such a complex process to be influenced by a single molecule. The enhancing effect of serum on Dex-MDMϕ phagocytosis of apoptotic neutrophils is likely complex and multifaceted, and alterations in intracellular cytoskeletal organisation in macrophages may also be important (Giles et al., 2001).

The studies in this thesis have been performed entirely *in vitro* using cells isolated from human peripheral blood. Although this approach allows the intricate dissection of molecular pathways, it cannot replicate the *in vivo* tissue microenvironment that has the potential to further influence the mechanisms involved in clearance of apoptotic neutrophils. The cytokine and matrix composition will determine the

differentiation status of MDMϕ populations during progression of an inflammatory response. Although untreated human MDMϕ differentiated in the presence of M-CSF may utilise a protein S-dependent apoptotic cell clearance mechanism (Uehara and Shacter, 2008), our data indicates that untreated human MDMϕ cultured in the presence of autologous serum do not utilise a protein S-dependent recognition pathway. The production and release of potential opsonins (complement components, pentraxins, annexins, protein S, etc.) is also regulated during inflammation, and production of both protein S and C4BP in the liver appears to be controlled by inflammatory mediators including IL-6 and TNFα (Hooper et al., 1995, de Wolf et al., 2006). Levels of protein S are reduced in patients with ischaemic stroke (Akyol et al., 2006), possibly via the effects of TNFα on endothelial cells (Hooper et al., 1994). In contrast, glucocorticoids have been reported to elevate levels of protein S (Oner et al., 2005). Based upon data presented in this thesis, we propose that a major effect of glucocorticoids on MDMϕ differentiation is the induction of the capacity to recognise a distinct set of molecular cues that are presented on the apoptotic neutrophil surface. The surface molecular signature of an apoptotic cell may therefore be interpreted differently by different MDMϕ populations, allowing functional diversity in MDMϕ responses, for example whether apoptotic cells are encountered during normal homeostasis, in the presence of inflammatory signals, or during the resolution of inflammation. What receptor mechanism(s) are engaged likely depends upon the phagocytic and apoptotic target cell types (Fadok et al., 1992a), with the cytokine milieu and presence of activatory signals (e.g. bacterial LPS) also having an influence over the activation state of the phagocyte, surface receptor expression and the availability of opsonins.

An alternative approach to investigating the role of Mertk in protein S-dependent phagocytosis by Dex-MDMϕ would be to use specific genetic mouse "knock-outs" of Mertk, or to "knock-down" Mertk expression in human MDMϕ using siRNA, to explore how the absence of Mertk affects monocyte to macrophage differentiation and aqcuisition of phagocytic capability when exposed to Dex. Murine Mertk shows remarkable homology to the human form (88% amino acid sequence identity)

(Graham et al., 1995), and murine Mertk may be similarly involved in serum-enhanced clearance of apoptotic neutrophils following Dex treatment of <u>murine</u> MDMφ (Liu et al., 1999, Cortes-Hernandez et al., 2002). It would be interesting to compare the phagocytic efficiency of Mertk-deficient macrophages *in vivo* under homeostatic conditions or in the presence of inflammatory signals. It is possible that a Mertk-independent receptor mechanism may mediate apoptotic neutrophil clearance during normal homeostasis. However, higher rates of neutrophil apoptosis evident during persistent or chronic inflammation may require a more efficient apoptotic cell clearance mechanism by MDMφ, such as the Mertk pathway facilitated by glucocorticoid treatment. Indeed, upregulation of Mertk mRNA in alveolar macrophages in response to an increased apoptotic cell load may reflect adaptation of macrophages to increase their apoptotic cell clearance capacity (Kazeros et al., 2008). Thus, we would predict that the pro-resolving effects of glucocorticoid treatment would be impaired in Mertk-deficient mice compared to wild type control mice, which could be tested experimentally by assessing the effect of Dex treatment on the secretory responses and apoptotic neutrophil clearance by Mertk-deficient macrophages when compared to wild type macrophages. However, the inability of anti-Mer to entirely abolish phagocytosis by human Dex-MDMφ in the presence of 10% autologous serum suggests that Mertk-deficient macrophages may utilise additional Mertk-independent receptor pathways. Furthermore, this approach may be complicated by the redundancy in apoptotic cell clearance mechanisms, and it is impossible to predict whether Tyro-3 and Axl, additional members of the TAM receptor family, may compensate for the loss of Mertk *in vivo*, particularly during development (Lu et al., 1999). The development of a macrophage-specific Mertk knock-out using Cre-Lox recombination may represent a more targeted approach to determining the contribution of Mertk to macrophage clearance capacity *in vivo*. However, it would be beneficial to assess the contribution of Axl and Tyro-3 to Dex-MDMφ phagocytic capacity *in vitro* prior to *in vivo* experiments.

An alternative to specific genetic deletion of Mertk to define the role of this receptor in glucocorticoid-enhanced phagocytosis would be to specifically over-express Mertk in cell lines previously deficient as phagocytes i.e. 'knock in' or transfection.

This approach has been reported by Ren *et al* to confer a phagocytic phenotype following transfection of CD36 into human Bowes melanoma cells and monkey COS-7 cell lines (Ren et al., 1995). However, there are potential limitations to this approach as additional uncharacterised receptor mechanisms may be required for "binding" of apoptotic neutrophils by Dex-MDMϕ independent of Mertk (Scott et al., 2001). Furthermore, the effect of Dex on MDMϕ phagocytic capacity may also require modification of MDMϕ morphology and adhesive ability through altered expression/phosphorylation of cytoskeletal proteins (Giles et al., 2001) that would be absent from transfected macrophages. Indeed, we found that increased Mertk expression on the surface of untreated MDMϕ was not sufficient to confer a pro-phagocytic MDMϕ phenotype. Thus, the glucocorticoid effect is probably too complex for such an approach to be successful. Addition of soluble Mertk during *in vitro* phagocytosis assays was found to inhibit Mertk-dependent clearance of apoptotic Jurkat T cells by the mouse macrophage cell line, J774 (Sather et al., 2007), and we predict that similar inhibitory effects on protein S-dependent phagocytosis by Dex-MDMϕ would be achieved using soluble Mertk. We hypothesise that uptake of apoptotic neutrophils could also be inhibited by adhering Dex-MDMϕ to protein S-coated surfaces, but not to surfaces coated with control proteins, before addition of apoptotic neutrophils in the presence of soluble protein S, due to redistribution of Mertk from the apical surface where it is accessible to protein S-coated neutrophils, to the lateral surface where it mediates adherence. This experiment may also be a useful indicator of whether other receptors can compensate for the loss of Mertk.

We have characterised the molecular mechanism of glucocorticoid action on macrophage phagocytic capacity using human neutrophils as apoptotic targets throughout our study. Dex has been shown to augment macrophage phagocytosis of alternative phagocytic targets, including human eosinophils and lymphocytes (Liu et al., 1999). Moreover, Van der Goes *et al* suggested that glucocorticoids enhance the general phagocytic capacity of human MDMϕ for several particles including latex beads and bacteria (van der Goes et al., 2000). Whether the protein S-Mertk receptor

mechanism is a pattern recognition mechanism induced by glucocorticoids to enhance MDMφ phagocytic capacity in general remains to be examined, although our data suggests that the mechanism utilised by MDMφ for apoptotic cell recognition depends upon the subpopulation of MDMφ and is independent of the type of apoptotic cell. Ligation of CD44 with a monoclonal antibody promotes uptake of apoptotic neutrophils by human MDMφ, however the precise mechanism remains to be fully characterised. Previous suggestions include redistribution of ligated CD44 to faciliate the apoptotic neutrophil-MDMφ phagocytic interaction, or CD44-mediated signal transduction to modify MDMφ phagocytic potential (Vivers et al., 2004). An interesting future experiment would be to investigate whether the protein S-Mertk pathway is required for enhanced phagocytosis mediated by CD44 and other pro-phagocytic stimuli such as IL-10.

The resolution of inflammation is a dynamically regulated process that may be subverted in many pathological conditions. Pharmacological manipulation of the processes involved in physiological clearance of neutrophils from inflamed sites may therefore represent a therapeutic approach to treatment of inflammatory diseases. Glucocorticoids are potent modulators of macrophage phagocytic capacity, however glucocorticoid action is pleiotropic and relatively non-specific, and there are side effects associated with long-term treatment of chronic disease (Rosen and Miner, 2005). If their desired anti-inflammatory actions could somehow be uncoupled from their side effects, the clinical benefits would be immense. It has become widely accepted that such uncoupling might be achieved through separation of activatory and inhibitory effects of glucocorticoids on gene expression. The development of dissociated steroids may appear to be a good strategy in theory, yet it does not account for the likelihood that the *in vivo* situation might be more complex. Glucocorticoids modulate inflammatory processes during various stages of inflammation, via distinct cell- and tissue-specific effects that require different mechanisms of action, be it induction of anti-inflammatory mediators or suppression of NFκB-mediated gene transcription. It is likely that the optimal effect of glucocorticoids on macrophage phagocytic ability requires dynamic integration of

both immunoenhancing and immunosuppressive actions during monocyte maturation. Production of relatively non-specific steroid analogues devoid of transactivational ability may lack an important component of glucocorticoid-mediated anti-inflammatory action, so it is debatable whether such compounds would yield the predicted clinical benefits.

A way of treating inflammatory diseases is to tackle the major underlying mechanisms involved in disease pathogenesis; cytokine dysregulation and accumulation of potentially injurious apoptotic neutrophils may play a contributory role. Grommes *et al* have shown ligation of Mertk in the presence of apoptotic cells attenuates pro-inflammatory signalling in macrophages (Grommes et al., 2008). Our results suggesting that a protein S-dependent pathway for apoptotic cell clearance could be inducible in already differentiated human MDMϕ, and that Dex-MDMϕ could modify receptor expression independent of phagocytic function in response to pro-inflammatory stimuli is consistent with the ability of macrophages to functionally adapt to changing environmental signals (Stout and Suttles, 2004). It would be interesting to assess whether short-term Dex treatment could induce acquisition of a protein S-mediated phagocytic pathway in monocytes that have been differentiated in the presence of pro-inflammatory mediators such as LPS, TNFα or IFNγ that may be present during non-resolving inflammation and produce MDMϕ that are normally poorly phagocytic for apoptotic cells (Heasman et al., 2004, Michlewska et al., 2009). A more comprehensive understanding of whether macrophages show plasticity in phagocytic function, and indeed in other important pro-resolving qualities, may allow therapeutic targeting of these cells in disease. We therefore propose that dissection of the signalling pathways triggered following protein S stimulation of Mertk on Dex-MDMϕ may provide a valuable insight into the intracellular molecular mechanisms that charaterise a pro-phagocytic macrophage phenotype. The high level of active Rac suggests that guanine nucleotide exchange factor (GEF) activity may be elevated in Dex-MDMϕ (Giles et al., 2001). GEFs such as Grb2 or Vav1 may couple Mertk to activation of Rac1 (Bustelo, 2001, Turner and Billadeau, 2002, Mahajan and Earp, 2003, Grommes et al., 2008). Future experiments are required to determine whether tyrosine phosphorylation and

therefore GEF activity in MDMɸ is altered following exposure to Dex. It would be interesting to evaluate whether there is chronic suppression of MDMɸ Mertk expression due to the presence of abundant pro-inflammatory mediators during chronic inflammation that may impede the pro-resolving function of macrophages. Since Mertk expression is relatively restricted to cells of the monocyte/macrophage lineage (Graham et al., 1994, Graham et al., 1995), manipulation of the Mertk pathway may allow the development of novel strategies that are more selective in their action for promoting inflammatory resolution *in vivo* of chronic inflammatory diseases characterised by neutrophilic inflammation, such as rheumatoid arthritis, inflammatory bowel disease, COPD and SLE.

In conclusion, this study has provided a novel insight into the molecular mechanisms required for a pro-phagocytic macrophage phenotype. The data presented in this thesis suggests that long-term glucocorticoid treatment promotes a "switch" to a Mertk-dependent apoptotic cell recognition mechanism that is pivotal to immune regulation and for processes involved in the resolution of inflammation.

CHAPTER 8: BIBLIOGRAPHY

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REVIEW

Clearance of dying cells and autoimmunity

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Abstract
Phagocytic clearance of apoptotic cells is an important physiologic homeostatic mechanism that is associated with non-
inflammatory or anti-inflammatory sequalae. Disruption of the process of apoptotic cell clearance may contribute to
development of a number of inflammatory and autoimmune diseases. In this review, we summarize the molecular pathways
that have been suggested to account for phagocytic clearance of apoptotic cells. We discuss potential mechanisms for
regulation of phagocytosis and the implications for development of autoimmunity.

Keywords: *Phagocytosis, apoptosis, macrophage, inflammation, autoimmunity*

Introduction

Apoptosis, in contrast to necrosis (cell disintegration),
does not provoke pro-inflammatory responses and
plays a fundamental role in almost all physiological
processes [1,2]. Apoptotic cells are characterised by
rapid shrinkage of the cytoplasm, nuclear coalescence,
membrane budding and formation of one or more
apoptotic bodies. Importantly, membrane integrity
during apoptosis is maintained preventing liberation
of intracellular histotoxic contents thereby limiting the
potential for propagation and exaggeration of inflam-
matory processes. To avoid cell disintegration (sec-
ondary necrosis), apoptotic cells must be efficiently
and rapidly removed by macrophages [3], dendritic
cells (DC) [4] or in certain circumstances other cell
types such as endothelial cells [5], vascular smooth
muscle cells [6], and fibroblasts [7]. Moreover,
clearance of apoptotic cells results in diminished
pro-inflammatory mediator release and an augmented
secretion of anti-inflammatory cytokines TGF-β, IL-
10, [8], whereas uptake of necrotic cells including
secondarily necrotic cells derived from nonengulfed
apoptotic cells, stimulates release of pro-inflammatory
mediators (TNFα, IL-1β and NO) [9]. It is now
widely accepted that failed clearance of apoptotic cells
and consequent secondary necrosis may lead to the
development of diseases that have an autoimmune
component such as systemic lupus erythematosus
(SLE) [10], type II diabetes [11], cystic fibrosis
[12] and chronic obstructive pulmonary disease
(COPD) [13].

Recognition of apoptotic cells by phagocytes

Removal of apoptotic cells usually involves three
central elements: (1) attraction of phagocytes *via*
soluble "find me" signals, (2) recognition and
phagocytosis *via* displayed "eat me" and absence of
"don't eat me" signals, and (3) altered production of
pro- and anti-inflammatory cytokines. In higher
organisms secretion of specialised chemotactic signals
by apoptotic prey appears to be an important factor in
recruiting phagocytic populations and preventing
secondary necrosis. Although "find me" signals are
so far poorly characterised, recent studies demon-
strated that apoptotic bodies secrete the phospholipid
lysophosphatidylcholine (LPC) attracting phagocytic
cells to apoptotic cells in a caspase-3-dependent

fashion [14]. Other examples of recruitment signals include S19 (ribosomal protein dimer) [15], split human tyrosyl-tRNA synthetase [16] and thrombospondin-1 (TSP-1) [17]. Recognition and engulfment of apoptotic particles involves multiple ligand–receptor interactions including "eat me" signals (molecules enabling recognition of apoptotic cells by phagocytes), bridging molecules and phagocytic receptors. One of the most extensively studied "eat me" signal is loss of phospholipid asymmetry and translocation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane during apoptosis [18]. Inhibition of the flippase that normally confines PS to the inner leaflet together with activation of a bidirectional non-specific phospholipid scramblase may lead to loss of lipid asymmetry [19]. In addition the ATP binding cassette transporter ABC1 has also been reported to have a role in phospholipid redistribution [20]. Masking of external PS on the apoptotic cell surface with annexin V usually blocks phagocytosis [21]. However, some viable cells, such as CD45RB low T lymphocytes [22], neutrophils in Barth syndrome [23] or activated B cells [24] also expose PS on the outer leaflet of the cell membrane and these cells fail to be ingested by either amateur or professional phagocytes.

Attempts to identify a phagocyte receptor that recognises PS using phage display and monoclonal antibody 217 (mAb 217) that inhibited binding of PS liposomes and engulfment of apoptotic cells by macrophages lead to cloning of this putative phosphatidylserine receptor (PSR) [25]. Although interaction of PS and this putative PSR promoted engulfment of apoptotic targets by phagocytes [26] Bose et al. showed that the ablation of putative PSR function resulted in developmental abnormalities during embryogenesis but failed to affect macrophage capacity to remove apoptotic cells both *in vitro* and *in vivo*. Furthermore, no differences in expression of the antigen recognised by mAb217 was found between wild-type and knockout mice whilst blotting with commercially generated antibody against PSR showed that this protein did disappear from knockout mice [27]. In addition, Cui et al. demonstrated nuclear localisation of GFT-tagged putative PSR which was consistent with the presence of five nuclear localisation signals [28]. One conclusion is that a phage display approach has the potential to identify weak cross-reacting epitopes present on the nuclear protein belonging to iron-oxidase family [29] and that the real PS receptor remains to be identified [30].

- Recently it has been shown that PS interacts with a number of bridging molecules including annexin I (Anx I or lipocortin) [31], milk-fat-globule-EGF-factor 8 (MFG-E8), growth-arrest-specific 6 (Gas 6) and β2-glycoprotein-I (β2-GPI) [32]. These interactions may facilitate PS interaction with other phagocyte receptors, e.g. $\alpha_v\beta_3$-integrin, the receptor-tyrosine kinase Mer, and the β2-GPI receptor by acting as a bridge between the apoptotic cell and the phagocyte [33]. Interestingly, a recent manuscript has demonstrated that the macrophage class B scavenger receptor CD36 interacts with membrane associated oxidised PS (oxPS) on the surface of apoptotic cells [34].

Apart from translocation of PS (or oxPS), apoptotic cells are characterised by surface exposure of oxidised low-density lipoproteins (oxLDL) [35] recognised by diverse scavenger receptors: SR-A (class A macrophage scavenger receptor) [36], lectin-like oxLDL-receptor-1 (LOX-1) [37], CD-68 [38] and CD36 [39]. Another class of rather poorly characterised "eat me" signals are sites binding TSP-1 [40], complement proteins C1q or C3b/bi [41] or collectins like mannose binding lectin (MBL) or lung surfactant proteins-A and -D (SP-A and SP-D) [42]. CD36 and integrin $\alpha_v\beta_3$ bind apoptotic cells to the phagocyte *via* TSP-1 [43,44], integrins $\alpha_m\beta_2$ and $\alpha_x\beta_2$ (complement receptors: CR3, CR4) recognise complement protein C3b/bi [45], whereas scavenger complex calreticulin (CRT)–CD91 interacts with lung surfactant proteins-A and -D (SP-A and SP-D), MBL and collectin-like complement protein C1q [46]. Exposure of CRT on the cell surface increases during apoptosis and this is consistent with a potential role in phagocytic clearance. Removal of apoptotic CRT-deficient cells by macrophages is impaired and this effect is reversed by addition of soluble CRT. Moreover CRT interacts with CD91 also known as low-density lipoprotein receptor-related protein (LRP1) or α2 macroglobulin receptor which is a highly effective internalisation receptor on phagocytes [47]. Since CRT may be exposed on the apoptotic cell or the phagocyte surface, CRT activation of LRP may occur either in *trans* or *cis* action [48]. The differential anti-inflammatory or pro-inflammatory outcomes of clearance of apoptotic and late apoptotic/secondarily necrotic cells may be a consequence of engagement of specific receptors and adaptor molecules. For example, CD36 triggers strong anti-inflammatory responses from both monocytes and macrophages including inhibition of TNFα, IL-1β, IL-12 production and up-regulation of IL-10 or TGF-β [49,50]. In contrast, complement components C1q, C3 and C4 seem to bind only to primary or secondary necrotic cells, opsonising those cells not cleared by other mechanisms [51]. C1q cooperates with serum DNase 1 in the degradation of chromatin from necrotic cells, enabling uptake of chromatin fragments by monocyte-derived phagocytes [52] and C-reactive protein (CRP) fails to bind to early apoptotic neutrophils but binds to late apoptotic cells, membrane-permeable cells [53]. Some receptors including

TSP–CD36–$\alpha_v\beta3$ complex, CD14 and putative PSR may also be involved in uptake of both apoptotic and necrotic cells [54,55].

The presence of "eat me" signals on viable cells raises the possibility that inhibitory signals, termed "don't eat me" signals, prevent phagocytosis of those cells [56]. One paradigm for this might be the expression of inhibitory receptors by NK cells, e.g. killer cell Ig-like receptor (KIR) which binds self major histocompatibility complex (MHC) class 1 molecules and subsequently suppress elimination of target cells by NK cells. This effect may be due to recruitment of src-homology 2—containing protein tyrosine phosphatase-1 (SHP-1) and SHP-2 to two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [57]. A similar mechanism may also be operative in macrophage activation and viable cells may express surface molecules that bind to macrophage inhibitory receptors such as signal regulatory protein α (SIRPα) display markers of "self" in form of ubiquitously expressed surface molecules that bind to macrophage inhibitory receptors such as signal regulatory protein α (SIRPα) [58].

-Brown et al. demonstrated that homophilic interaction of CD31 promotes detachment of viable cells from phagocytes, whereas apoptotic cells are unable to detach and become internalised by macrophages. In addition CD31 expressed by apoptotic cells failed to associate with the cytoplasmic signalling molecules SHP-1 and SHP-2 indicating disruption in CD31-mediated signalling in apoptotic cells [59]. CD47 may also suppress removal of "self" viable cells when binding to extracellular domain of SIRPα on macrophages [60]. CD47 acts as a "don't eat me" signal on normal red blood cells (RBC) [61] and is also highly express on the surface of some cancer cells (i.e. ovarian cancer cells) and virally-infected cells leading to impaired immune defence [62].

Engulfment pathways

Studies in the nematode *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster* and mammalian cells revealed conservation of the engulfment machinery [63,64] involving two signalling pathways influencing the balance of membrane Rho family GTPases activity [65]. RhoA and Rac-1 regulate phagocytosis of apoptotic cells in opposite manner—RhoA inhibits this process while Rac-1 is obligatorily required for engulfment [66]. Assembly of a trimolecular complex trimolecular complex of ELMO, CrkII and DOCK180 in mammals (known respectively as CED-12, CED-2 and CED-5 in the worm) [67] activates the guanine nucleotide guanine nucleotide exchange activity of DOCK180 for Rac (CED-10), which in turn promotes cytoskeletal rearrangements crucial for the uptake of target particles [68]. The upstream components required for Rac activation are different. One pathway identified in both mammalian cells and worms involves RhoG/MIG-2 and the guanine nucleotide exchange factor TRIO/UNC-73 [69], although the surface receptor remains unknown. The second pathway involves the cell surface receptor proteins ABC1/CED-7 and CD-91/CED1 and the adaptor protein GULP/CED-6. ABC1/CED-7 downstream ligands have not been identified yet [70].

Pharmacological modulation of phagocytosis

Our group and others have shown that macrophage phagocytic capacity is modulated by pharmacological or immunological intervention. For example, anti-inflammatory glucocorticoids augment the ability of macrophages to phagocytose apoptotic cells in a concentration—and time-dependent manner *via* the glucocorticoid receptor [71]. Glucocorticoid treatment was suggested to reprogram macrophage differentiation towards a highly phagocytic phenotype with high levels of active Rac, increased formation of lamellipodia and cellular extensions as assessed by time lapse video microscopy and decreased cellular adhesion [72,73]. Furthermore, one important mediator of glucocorticoid action, the anti-inflammatory protein Anx-1, has been suggested to be essential for phagocytosis. Macrophages from Anx-1 null mice have reduced phagocytic capacities *in vitro* and *in vivo* associated with decreased expression of CD11b receptor protein [74].

Extracellular matrix (ECM) proteins such as fibronectin and collagen may also influence macrophage phagocytic abilities. Their interaction with macrophages through $\beta1$ integrin receptor was shown to upregulate phagocytosis [75]. However, exposure to cigarette smoke causes post-translational carbonyl modification of ECM proteins as well as lipid peroxidation resulting in increased macrophage adherence but decreased macrophage uptake of apoptotic neutrophils [76].

CD44 has been demonstrated to act as an adhesion receptor for ECM molecules and mediates a number of cellular functions such as adhesion and proliferation of lymphocytes, cytocidal activity of NK cells and tumor metastasis [77]. Cross linking of human monocyte-derived macrophage CD44 with bivalent antibodies rapidly and specifically augmented uptake of apoptotic neutrophils *in vitro* [78] suggesting the possibility that ligation of CD44 *in vivo* may selectively promote clearance of apoptotic neutrophils and subsequently resolution of inflammation. Studies on CD44 deficient mice administrated with bleomycin to induce inflammation in alveolar interstitium, confirmed this suggestion. A 13-fold increase in the number of apoptotic cells was detected in lung tissue of CD44 deficient animals in comparison to control, indicating impaired clearance of apoptotic cells resulting in development of lung injury in these mice [79].

The close relationship between cell adhesion and phagocytosis is further revealed in studies with agents that elevate intracellular cAMP levels such as prostaglandins (PG). Pretreatment of macrophages with PGE_2 or PGD_2 or cAMP stable analogues such as dibutyryl-cAMP and 8-bromo-cAMP significantly reduced the proportion of macrophages that phagocytosed apoptotic cells and caused alterations in macrophage adhesion, loss in membrane activity and cell locomotion [80]. It has been reported that variety of inflammatory mediators, including PG, that stimulate activity of adenylate cyclase and PKA are likely to inhibit clearance of apoptotic cells [81]. Furthermore, PKA is able to phosphorylate Rho, a key negative regulator of cytoskeletal organisation, indicating that cAMP may play a pivotal role in regulation of the phagocytic process [82]. All Rho family GTPases require prenylation (covalent attachment of lipid adducts) and subsequent membrane insertion for activity. Since the cholesterol lowering drugs (statins) inhibit activity of HMG-CoA reductase they also reduce production of prenylation substrates and it has been recently reported that lovastatin may enhance phagocytosis of apoptotic cells *via* suppression of prenylation and membrane localization of RhoA, altering the RhoA–Rac-1 membrane balance towards Rac-1—a positive regulator of phagocytosis [83].

The lipoxins represent further arachidonic acid metabolites that play an important role in resolution of inflammation. Their strong inhibitory effects on neutrophil chemotaxis and adhesion is well documented, but more recent data show that LXA_4 and its stable synthetic analogues augmented clearance of apoptotic neutrophils in a concentration-dependent manner [84]. It has been also demonstrated that Anx-1 and LXA_4 converge onto the same specific membrane lipoxin A_4 receptor [85]. In addition the stable cAMP analogue 8-bromo-cAMP attenuated and the PKA inhibitor, Rp-cAMP, mimicked LXA_4 effects indicating that lipoxin may inhibit PKA activity. The lack of additive effects of Rp-cAMP and LXA_4 suggested they act through the same pathway. Interestingly, LXA_4 was shown to inhibit PKA activity induced by PGE_2, consistent with an important role for PKA in regulation of phagocytosis. One potential substrate for PKA is scavenger receptor CD36 [86] and loss of CD36 phosphorylation in platelets results in increased cytoadhesion [87]. Monoclonal antibodies against CD36 blocked macrophage phagocytosis of apoptotic neutrophils induced by LXA_4 raising the possibility that LXA_4-mediated dephosphorylation of CD36 may promote phagocyte-apoptotic cell recognition [88].

Clearance deficiency and autoimmunity

Impairment of apoptotic cell clearance may result in exacerbation of inflammation and tissue injury,

together with loss of production of cytokines (i.e. IL-10) that maintain an anti-inflammatory environment [89]. On the contrary, enhancement of phagocyte clearance capacity by IL-10 [90] may further promote an environment of tissue repair and resolution of inflammation. There is now a wealth of evidence that support the hypothesis that defects in clearance of apoptotic cells have a role in pathogenesis of human autoimmune diseases. For an instance in SLE patients phagocytic activity is impaired [91], including clearance of autologous apoptotic material [92,93].

Apoptotic cells undergoing secondary necrosis within tissue represent a source of pro-inflammatory stimuli and potentially immunogenic autoantigens. As shown at Figure 1 presentation of autoantigens by DC and macrophages may drive production of autoantibodies [94]. Nucleosomal proteins released from DNA as a result of internucleosomal cleavage during apoptosis represent one of the most abundant class of autoantigens found in patients with autoimmune disorders [95]. Antibodies against nucleosomes are a serological hallmark of SLE but also other autoimmune diseases. Additionally, delayed uptake of apoptotic cells may generate caspase-derived neoantigenic peptides that are capable of triggering autoimmune responses, inducing loss of tolerance by B cells and formation of immune complexes (IC) [96]. Autoantibodies opsonising apoptotic corpses may further alter phagocytic clearance pathways. It has been demonstrated that anti-phospholipid antibodies may bind to externalised PS on apoptotic cell surfaces and promote Fc receptor-dependent phagocytosis which may have pro-inflammatory sequelae [97]. It could be postulated that whilst low number of apoptotic cells are disposed of in a non-inflammatory manner, problems may arise when tissue phagocytes are faced with an overwhelming load of apoptotic cells following infection or serious tissue injury. We have recently demonstrated that IC bind to "enabled" FcγRIIA on apoptotic human neutrophils [98]. Surprisingly, opsonisation of apoptotic neutrophils with IgG-containing IC allowed their rapid clearance by macrophages without production of pro-inflammatory cytokines, thus providing a mechanism for preventing release of intracellular antigens (particularly nuclear constituents) even in the face of a heavy apoptotic cell load. Paradoxically, therefore, IC may have beneficial effects, acting to limit development of autoimmunity in some circumstances.

Impairment of apoptotic cell removal by macrophages in peripheral or lymphoid tissues may result in late apoptotic cells become accessible to tissue or follicular DC. The capacity of DC to induce tolerance or immune activation is strongly related to the levels of "maturation" and expression of co-stimulatory molecules. Thus, cross-presentation of self or foreign antigens to $CD8^+$ cytotoxic T lymphocytes (CTL) and stimulation of production of antibodies by B cells may depend on local micro-environmental conditions that

A. Efficient clearance of apoptotic cells

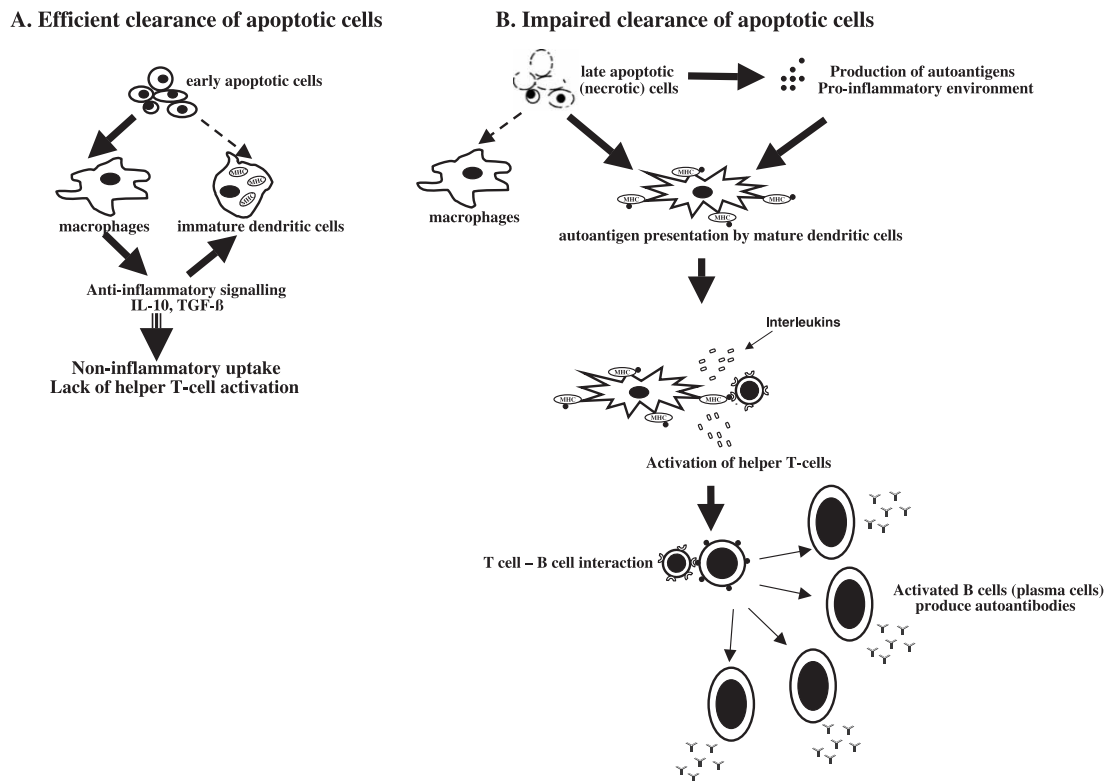**B. Impaired clearance of apoptotic cells**

Figure 1. Diagram illustrating cascade of events resulting from efficient (A) or impaired (B) clearance of apoptotic cells. Efficient clearance of apoptotic cells prevents cell necrosis and stimulates production of anti-inflammatory mediators by phagocytes. In anti-inflammatory environment maturation of antigen presenting cells (i.e. DC) and immune response is inhibited. On the contrary, when clearance of apoptotic cells is affected, accumulated apoptotic cells undergo secondary necrosis resulting in production of autoantigens and pro-inflammatory mediators. In the presence of pro-inflammatory mediators maturation of DC is induced. Mature DC, after gaining access to autoantigens, present them to T helper cells followed by subsequent B cell production of autoantibodies and development of autoimmunity.

regulate levels of DC maturation [99]. Pro-inflammatory signals such as LPS, TNFα, IL-1β or IFN-α as well as viral or bacterial proteins, high antigen load or uptake of necrotic cells appear to be very powerful inductors of DC maturation and activators of immune response [100]. On the other hand, anti-inflammatory cytokines such as TGF-β or IL-10 or more importantly uptake of apoptotic cells strongly suppress maturation of DC and promotes peripheral tolerance [100]. It has been shown that engulfment of apoptotic material by DC may inhibit even such strong pro-maturation signal as LPS, indirectly reducing DC capacity to stimulate T cells [101]. Furthermore, uptake of apoptotic cells by DC fails to stimulate effective antigen presentation to lymphocytes. In contrast, uptake of necrotic cells induces maturation of DC and a break in peripheral tolerance [102,103]. Therefore, the current model for the development of autoimmunity involves two important factors: (i) production of autoantigens, and (ii) a strongly pro-inflammatory environment, both of which are closely linked to deficiency in clearance of apoptotic cells.

However, although this association is supported by some studies using mice lacking apoptotic cell recognition molecules, e.g. C1q, in some cases defective clearance of apoptotic cells may have no consequences in terms of development of autoantibodies and autoimmunity, for example, CD-14 deficient animals show a defect in the clearance of apoptotic cells in the absence of an overt autoimmune phenotype [104]. There is convincing evidence linking complement protein C1q deficiency with development of SLE. It has been reported that lack of complement pathway proteins results in accumulation of apoptotic material and elevated levels of self-antigens and IC, and consequently in direct induction of tissue inflammation, maturation of DC and production of autoantibodies. Complement fragments also deliver regulatory signals to T and B lymphocytes maintaining peripheral tolerance to self-antigens and absence of stimulation results in decrease thresholds of activation for T and B cells [100,105].

Another example of condition with an autoimmune component and clearance deficiency is cystic fibrosis characterised by massive recruitment of inflammatory cells and release of intracellular sources into the lung. Patients are also characterised by increased number of apoptotic cells in airways and it is highly probable that impaired clearance may result from elastase-mediated inhibition of phagocytosis causing chronic inflammation and progressive lung tissue damage [106].

Type-1 diabetes is another disease condition in which autoimmunity and clearance of dying cells play a crucial role. This autoimmune disease usually emerges in genetically predisposed children, most likely in response to an environmental insult. Humoral and cell-mediated immunities trigger chronic apoptotic cell death amongst the insulin-secreting β-cells of the pancreatic islets of Langerhans [107]. It has since been postulated that the apoptotic β-cells themselves constitute a trigger for autoimmunity and that impaired clearance of apoptotic cells is central to development of the disease, perhaps on account of harmful autoantigens released during the apoptotic process and resultant infiltration of T-cells [108]. Results from animal studies suggest that the underlying cause of impaired clearance is likely to be due to a deficiency in the macrophages themselves [109] but the mechanism has yet to be fully elucidated.

Closing remarks

Apoptosis and subsequent clearance by professional or non-professional phagocytes is a complex and dynamic process crucial for normal tissue homeostasis and modulation of immune responses and development of autoimmunity. Uptake of late apoptotic or necrotic cells engages different receptors and signalling pathways than early apoptotic cells with profound implications for regulation of immunity. As our knowledge of the phagocytic clearance mechanisms and the potential for modulation of macrophage phagocytosis increases, we may uncover new ways to treat patients with autoimmune conditions arising from defective removal of apoptotic cells.

Acknowledgements

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Effects of Glucocorticoids on Apoptosis and Clearance of Apoptotic Cells

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The glucocorticoid (GC) drugs are one of the most commonly prescribed and effective anti-inflammatory agents used for the treatment of many inflammatory disorders through their ability to attenuate phlogistic responses. The glucocorticoid receptor (GCR) primarily mediates GC actions via activation or repression of gene expression. GCs directly induce the expression of proteins displaying anti-inflammatory activities. However, the likely predominant effect of GCs is the repression of multiple inflammatory genes that invariably are overexpressed during nonresolving chronic inflammation. Although most GC actions are mediated through regulation of transcription, rapid nongenomic actions have also been reported. In addition, GCs modulate inflammatory cell survival, inducing apoptosis in immature thymocytes and eosinophils, while delaying constitutive neutrophil apoptosis. Importantly, GCs promote noninflammatory phagocytosis of apoptotic cell targets, a process important for the successful resolution of inflammation. Here, the effects and mechanisms of action of GC on inflammatory cell apoptosis and phagocytosis will be discussed.

KEYWORDS: glucocorticoids, apoptosis, inflammation, macrophage phagocytosis

INTRODUCTION

Inflammation is an important physiological host defence mechanism against infection and injury. Granulocytes, such as neutrophils and eosinophils, are crucial in innate immune defence against bacterial and parasitic infections, respectively[1]. However, the persistent recruitment and/or enhanced survival of granulocytes at inflamed sites may result from dysregulated expression of proinflammatory genes, such as cytokines (IL-1, TNFα, GM-CSF, etc.), chemokines (IL-8, IL-5, MIP-1α), and adhesion molecules (ICAM-1 and E-selectin)[2]. Uncontrolled leukocyte responsiveness will lead to release of inflammatory mediators, such as eicosanoids (prostaglandins, leukotrienes, and thromboxanes), cytokines (IL-8, etc.), reactive oxygen/nitrogen species (O_2^-, NO), and granular enzymes (e.g., elastase)[3], resulting in damage to the surrounding tissue and propagation of the inflammatory response. This likely contributes to persistent dysregulated inflammation, resulting in the pathogenesis of disorders such as chronic obstructive pulmonary disease (COPD) and bronchial asthma[4].

Apoptosis is a programmed form of cell death[5] that regulates the number and fate of leukocytes at inflamed sites and, in contrast to necrosis, is associated with maintenance of cell membrane integrity and damage limitation[6]. Granulocytes are terminally differentiated cells and undergo constitutive apoptosis during *in vitro* culture[6,7], which is amenable to lineage-specific modulation by intrinsic and extrinsic factors including cAMP[8], IL-5[7], GM-CSF[9], prostaglandins[10], and TNFα[11]. Synthetic glucocorticoids (GCs), such as dexamethasone, initially demonstrated to induce apoptosis of immature thymocytes[12], also accelerate apoptosis in eosinophils while, surprisingly, prolong neutrophil survival[13,14]. This observation has led to speculation that GCs may be exerting part of their beneficial effect in eosinophil-dominant diseases (e.g., asthma) by inducing apoptosis of lymphocytes and eosinophils while maintaining beneficial neutrophil-dependent antimicrobial responses. Consequently, it is possible that direct pharmacological triggering of cell-specific apoptosis may be a novel therapeutic strategy in the treatment of inflammatory disorders[15,16]. However, for this to be considered as a successful tactic, efficient removal of the apoptotic leukocytes is also required. Failure to do so may lead to apoptotic cells undergoing secondary necrosis with deleterious consequences in terms of tissue damage and the outcome of the inflammatory response. An additional anti-inflammatory effect of GCs is the profound potentiation of phagocytic clearance of intact apoptotic leukocytes described *in vitro*[17,18] and possibly *in vivo*[19]. These recently described effects of GCs, together with limiting inflammatory cell recruitment and activation, may prevent further injury through the release of noxious intracellular contents and may be important for promoting the resolution of inflammation. However, there are potential limitations to the application of GCs in disease, particularly as a consequence of the undesirable side effects associated with long-term treatment. Delineating the precise mechanisms of GC action would provide a significant insight into the anti-inflammatory role of GCs and allow the development of novel strategies that are more selective in their action.

THE GLUCOCORTICOID RECEPTOR

GCs mediate most of their effects by binding to glucocorticoid receptors (GCRs). The GCR is a member of the nuclear receptor superfamily, which includes receptors for mineralocorticoids and sex hormones[20]. Alternative splicing of the *gcr* gene generates two or more GCR isoforms with distinct functions. Although GCRα is the predominant isoform and is responsible for GC binding, GCRβ is a c-terminally truncated variant that cannot bind GC or regulate transcription, but can form heterodimers with GCRα to modulate GCRα function[21,22]. Inactive GCR resides in the cytoplasm as part of a multiprotein complex, being bound to chaperone molecules including heat shock proteins (HSP90) and immunophilin[23]. HSP90 forms interactions with GCR in the c-terminal domain essential for maintaining the correct configuration of GCR and also masks a nuclear localisation signal to prevent translocation of the unoccupied GCR to the nucleus[24]. Upon binding of GC to GCR, these chaperone proteins dissociate from the GCR, unmasking the nuclear localisation signal required for the activated GC-GCR complex to translocate to the nucleus[24]. Here, GCR can directly or indirectly modulate the transcription of multiple target genes.

MECHANISMS OF GC ANTI-INFLAMMATORY ACTION

Transactivation: Induction of Anti-Inflammatory Gene Expression

One mechanism whereby GCs can mediate their action is via direct binding of GCRs to DNA to increase the transcription of anti-inflammatory genes, a process known as transactivation (Table 1). Ligand-activated GCRs translocate to the nucleus and bind specifically to palindromic glucocorticoid-responsive elements (GREs) found in the promoter region of GC-responsive genes[25,26]. The central domain of the GCR contains two zinc fingers essential for GCR dimerisation and binding to GRE sequences[26,27],

with a point mutation in the D-loop of the central domain abolishing transactivation[28]. Thus, transcription of GC-responsive genes requires GCRs to be in the homodimeric form. GCR "switching on" of gene transcription also requires the recruitment of specific transcriptional coactivator proteins, which are important for localised chromatin remodelling and stabilisation of the basal transcriptional machinery[29].

This mechanism directly modulates the expression of secretory leukocyte proteinase inhibitor (SLPI)[30], IL-1 receptor antagonist[29], and C1q [31]. Annexin I (or Lipocortin 1) is an additional GC-inducible protein thought to mediate many of the anti-inflammatory actions of GCs. This is confirmed by studies in deficient mice, which have altered expression of annexins, COX-2, and cPLA2; exaggerated responses to carrageenin- or zymosan-induced inflammation; and partial resistance to the anti-inflammatory effects of GCs[32]. Administration of exogenous annexin I confers anti-inflammatory activity in some models of inflammatory disease[33]. Conversely, infusion of annexin I antibodies neutralises the effect of annexin I and abrogates the anti-inflammatory activities of GCs[33]. It has been suggested that autoantibodies to annexin I may contribute to GC resistance and the pathogenesis found in inflammatory diseases where GCs may be used as a treatment, such as rheumatoid arthritis and systemic lupus erythematosus (SLE)[34]. Additionally, annexin I–derived peptides have been shown to mimic some of the anti-inflammatory effects of endogenous annexin I, including a role in phagocytic clearance of apoptotic cells[35]. GCs also enhance the expression of other anti-inflammatory proteins that may be important in switching off signalling pathways engaged during persistant inflammation. Mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) dephosphorylates and inactivates MAPKs, such as p38 MAPK and c-Jun terminal kinase (JNK), which contribute to enhanced expression of proinflammatory mediators[36]. The suppressive effect of GCs on MAPK signalling is impaired in MKP-1 knockout mice, which show enhanced expression of proinflammatory genes, including COX2, TNFα and IL-1[36]. Thus, the anti-inflammatory action of GCs via transactivation may be important in aiding the resolution of inflammation and in boosting innate immune defence through the induction of protective proteins. Nevertheless, it is doubtful that all the effects mediated by pleiotropic GCs could be explained by enhanced production of a small number of proteins with anti-inflammatory properties.

Transrepression: Inhibition of Proinflammatory Gene Expression

Although a small number of genes can be regulated directly, many more genes are regulated indirectly by the GCR via suppression of gene expression, a process known as transrepression (Table 1). Originally, this repression was thought to result from homodimeric GCRs binding to putative negative GRE sites in the promoter regions of proinflammatory genes to switch off their transcription[37]. However, the majority of genes repressed by GCs do not contain negative GRE sequences; therefore, alternative mechanisms to DNA binding must be engaged.

Ligand-activated GCRs can "switch off" inflammatory gene expression by a DNA-independent mechanism through direct protein-protein interactions with activated NFκB and AP-1 transcription factors[38,39]. Since NFκB and AP-1 induce the transcription of multiple inflammatory and immune genes characteristic of various inflammatory disorders[40,41], this mechanism of GC action is extremely important and probably accounts for most of the inhibitory effects of GCs on inflammation. Although cytoplasmic interaction of ligand-activated GCR with transcriptional regulators has been reported, more recent data indicate that GCR may interfere with NFκB and AP-1 at a later stage after they have bound to DNA to influence transcription[39,42]. Reichardt and colleagues introduced a point mutation A458T into the GCR gene by a gene-targeting technique involving Cre/loxP recombination[28,42,43]. Specifically, the mutation was "knocked in" to the D-loop of the central domain of the GCR, preventing DNA binding, GCR dimerisation (GCR^{dim}), and transactivation. However, transrepression of NFκB- and AP-1–mediated gene expression remained intact, indicating that this process is mediated by monomeric GCR. Application of the GCR^{dim} mouse model would allow investigation into the process of transrepression in the absence

of transactivation and allow elucidation of GC mechanism of action in various processes during inflammation *in vivo*. Interestingly, higher concentrations of GCs are required for induction of anti-inflammatory gene expression, while gene repression by the GCR can occur at much lower, more clinically relevant concentrations of GC[24]. Indeed, it has been questioned whether DNA binding is required at all for physiological GCR function[44]. Thus, although GCs can induce anti-inflammatory gene expression, the repression of activated proinflammatory transcription factors appears to be the dominant mechanism of GC action. Moreover, these modes of GC effect may even occur concurrently, where the induction of IκBα and glucocorticoid-inducible leucine zipper (GILZ) may contribute to later onset inhibition of NFκB and AP-1, respectively[20].

Table 1.

Mechanisms of Glucocorticoid Anti-Inflammatory Action

Mechanism of GC Action	Genes Affected
Transactivation: Induction of anti-inflammatory gene expression	SLPI, IL-1 receptor antagonist, C1q, annexin I, MKP-1, GILZ, IκBα
Transrepression: Suppression of proinflammatory gene expression	Cytokines (IL-1, TNFα, GM-CSF), chemokines (IL-8, MIP-1α), adhesion molecules (ICAM-1, E-selectin)

Chromatin Remodelling, HATs, and HDACs

It is now apparent that an additional DNA binding-independent mechanism of GC action may involve reversible alterations in chromatin structure and histone acetylation. Gene expression and repression are associated with alterations in chromatin structure through modification of core histones[45] (Fig. 1). In a resting cell, the DNA is tightly coiled around core histone proteins and is inaccessible to transcriptional regulators, a configuration referred to as "closed" and is associated with gene silencing[46]. Initiation of transcription by NFκB and AP-1 requires slackening of chromatin and unwinding of DNA for transcriptional cofactors and RNA polymerase II to gain access to genes. Upon binding to DNA, NFκB and AP-1 recruit and activate coactivator proteins, such as cAMP response element binding (CREB) binding protein (CBP) and p300/CBP-associated factor, which have intrinsic histone acetyltransferase (HAT) activity[46]. Acetylation of key lysine residues in histones by coactivator proteins initiates chromatin remodelling required for gene transcription[45]. Reversal of histone acetylation results in tightening of chromatin around DNA and hinders binding of NFκB and AP-1. Deacetylation is controlled by corepressor proteins, such as histone deacetylases (HDACs) and nuclear receptor corepressor (NcoR), and is associated with gene repression[29]. This process may be important for "switching off" genes once the stimulus is removed and inflammation is no longer required, and failure to do so could contribute to the development of persistent or chronic inflammation.

GCR Interacts with HATs and HDACs to Regulate Their Function

GCR can directly interact with NFκB and AP-1 to suppress the expression of proinflammatory genes, a process that may involve reversal of histone acetylation[39] (Fig. 1). The exact mechanism involved here is uncertain, although a competitive role between GCR, NFκB, and AP-1 for binding to coactivators remains controversial[47]. GCRs may carry out their repressive function by directly suppressing the HAT

activity of coactivators[2]. Alternatively, activated GCRs may recruit corepressors, such as HDAC2, to the transcriptional complex to reverse histone acetylation[48].
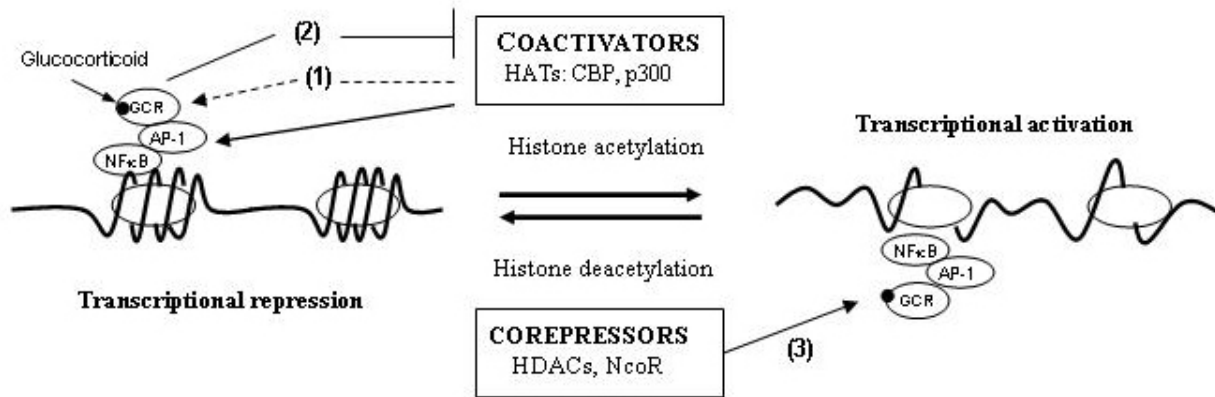

FIGURE 1. Effect of GCR on chromatin remodelling, HATs, and HDACs. Gene expression and repression are regulated by modification of core histones. In the resting state, DNA is tightly coiled around histones and is inaccessible to transcriptional cofactors and RNA polymerase II. Upon binding to DNA, NFκB and AP-1 recruit and activate coactivator proteins, such as CBP and p300/CBP-associated factor, which have intrinsic HAT activity. Histone acetylation results in slackening of chromatin and unwinding of DNA, allowing the transcriptional machinery to gain access to genes and initiate transcription. Deacetylation is controlled by corepressor proteins, such as HDACs and NcoR, and is associated with gene repression. Activated GCRs may interact with HATs and HDACs to regulate their function. Suggested mechanisms of GR transrepression include (1) GCR competes with NFκB and AP-1 for binding to coactivators, (2) direct suppression of HAT activity of coactivators by GCR, (3) recruitment of corepressors by GCR to reverse histone acetylation.

Nongenomic Effects

The genomic effects of GCs have a lag period of around 30 min to a few hours/days[20], result from GC binding to cytoplasmic GCR, and are blocked by inhibitors of transcription and translation, such as cycloheximide[49]. Some data indicate that GCs may exert more rapid actions (seconds to minutes) that are most likely transcriptional independent[50]. Furthermore, it has been suggested that some immediate GC effects may be GCR independent, for instance, inhibition of superoxide production by macrophages[51] or alteration of plasma membrane properties[50]. Other investigations have implicated GCR-dependent nongenomic effects of GCs via post-transcriptional regulation, both at the level of mRNA and protein[52]. This may provide an immediate inhibitory effect of GCs on the expression, synthesis, and release of proinflammatory mediators. For this, a putative membrane-associated form of the GCR (mGCR) may bind GC and transduce signals via MAPKs, PI-3K, and PKC, which mediate the rapid effects[49,53]. Investigations by Croxtall and colleagues have implicated GCR-dependent effects of dexamethasone via altered phosphorylation status of annexin I, leading to inhibition of cPLA2 activation and arachidonic acid release[54]. Whether any of these nongenomic effects described occur at physiological concentrations of GC and have a contributory role to the anti-inflammatory effect of these steroids remains controversial.

GC RESISTANCE

GCs are pleiotropic steroids with the ability to attenuate many phlogistic responses, making them the most effective clinical treatment for various inflammatory disorders, e.g., asthma. However, a small proportion of asthmatic patients do not respond well to GC treatment and may require high doses of GC[55]. Several mechanisms have been postulated to contribute to GC resistance. Increased expression of GCRβ has been suggested to interfere with GCRα function[21,22]. Interestingly, the level of GCRβ is increased by proinflammatory cytokines[56] and in peripheral blood mononuclear cells from patients with GC-resistant rheumatoid arthritis[57]. The level of intracellular GC is much lower than the extracellular concentration due to expulsion of GC from the cell by members of the ABC family of transporter molecules[52]. Overexpression of these proteins may contribute to the development of GC resistance. COPD is an inflammatory disorder where GCs are not usually a beneficial treatment and patients are relatively resistant to these steroids even when given high doses[24]. This ineffectiveness has been linked to factors such as smoking and oxidative stress[58].

GC SIDE EFFECTS

Despite being the mainstay therapy for treatment of inflammatory disease, long-term GC use is associated with many undesirable side effects, the molecular mechanisms of which are extremely complex and are not entirely understood. Endocrine and metabolic side effects leading to diabetes and osteoporosis have been linked to transactivation of genes by the ligand-activated GCR[59]. For example, GCs may increase glucose synthesis through transactivation of enzymes involved in gluconeogenesis[59]. GCR^{dim} mutants, which are transactivation deficient, retain many of their beneficial anti-inflammatory effects through transrepression of NFκB- and AP-1–mediated gene expression[28,42,43]. Hence, identification of "dissociated steroids" that can mediate transrepression of proinflammatory genes without induction of transactivation would represent an attractive therapeutic strategy. Such compounds may provide a safer approach to treatment of inflammatory diseases through this ability to convey the beneficial actions of GCs with reduced adverse effects[60]. However, some side effects, such as skin atrophy and suppression of the hypothalamic-pituitary-adrenal axis, may be mediated by the transrepressive function of the GCR[59] or may even involve a combination of both GCR mechanisms that would complicate the use of dissociated steroids in therapy. It is imperative, therefore, that the specific molecular mechanisms involved in GC-induced side effects are delineated to allow the development of more selective therapies.

GCS AS A THERAPY FOR INFLAMMATORY DISEASE

Inflammation is Normally Self-Resolving

Under normal circumstances, inflammation is beneficial to the host and is self-resolving, requiring controlled clearance of granulocytes from inflamed sites[6]. Apoptosis of granulocytes, in contrast to necrosis, is associated with maintenance of membrane integrity[6] and down-regulation of potentially injurious secretory responses[61]. For granulocytes, functional attenuation is also achieved by surface changes during the apoptotic process, including down-regulation of FcγRIII (CD16)[62] and L-selectin expression[63], and uncoupling β2 integrins[63]. Cells undergoing apoptosis display specific surface alterations important for signalling rapid recognition and internalisation by phagocytes[64,65,66]. Many receptor mechanisms have been acknowledged in the direct recognition and engulfment of apoptotic cells by phagocytes[67,68,69], including the vitronectin receptor[70], scavenger receptors[71], CD31[72], putative phosphatidylserine receptor (PSR)[73], C1q receptor calreticulin[74], and the tyrosine kinase receptor, MER[75]. Blocking individual receptors on phagocytes only partially inhibits phagocytosis of apoptotic cells, suggesting redundancy in the engulfment pathways and underlining the importance of

phagocytic clearance of apoptotic cells. During resolving inflammation, macrophages (or other cells with phagocytic capacity) are required to remove the potentially histotoxic apoptotic cells rapidly and efficiently[76]. Phagocytic removal of apoptotic cells normally occurs early on in the death process when the dying cell is still intact[6] and, in contrast to phagocytosis of necrotic cells, fails to induce the release of proinflammatory mediators, such as eicosanoids, GM-CSF, or IL-8, and MCP-1 chemoattractants from the phagocytic cell[77,78,79]. Engulfment of apoptotic cells also actively induces anti-inflammatory and immunosuppressive effects in the phagocyte by, for example, inducing the production of IL-10, TGFβ, PGE2, and platelet-activating factor[80,81]. Thus, rapid recognition, ingestion, and degradation of dying cells by phagocytes are nonphlogistic and equally as vital as the process of apoptosis itself. Both apoptosis and subsequent phagocytic clearance are prerequisites for efficient clearance of granulocytes and other inflammatory cells from tissues and, ultimately, for the resolution of inflammation.

Disease: Failure of Natural Resolution Process?

Neutrophils and eosinophils have been implicated in the pathogenesis of many inflammatory and allergic disorders, such as COPD and bronchial asthma. Accumulation of leukocytes reflects a mismatch between their infiltration into the inflamed site in response to cytokines, chemokines, and adhesion molecules, which are overexpressed during chronic inflammation and the mechanisms required for their clearance. Defective apoptosis or failure to remove intact apoptotic cells efficiently before lysis may play a contributory role in the disease pathogenesis through release of histotoxic granulocyte contents that have the capacity to damage the surrounding tissue and by stimulating proinflammatory macrophage secretory responses. Indeed, impaired phagocytosis of apoptotic cell material may contribute to the severity of disease in human SLE[82,83]. Mouse models of C1q deficiency develop spontaneous/persistant inflammation that resembles an SLE-like disease and may be due to defective clearance of apoptotic cells[84,85]. In both cases, a systemic autoimmunity is developed against autoantigens that may be displayed on or released by the noningested apoptotic cells. This underlines the importance for "safe" and efficient removal of these potentially harmful cells and the therapeutic value of agents that promote this. Our laboratory and others have identified GCs as important regulators of both apoptosis and phagocytic removal of apoptotic cells in diseases where these processes may be inefficient or dysregulated. Indeed, it has recently been demonstrated that manipulation of apoptosis *in vivo* may result in enhanced resolution of inflammation[86,87], thereby highlighting the potential to manipulate processes involved in the resolution of inflammation for therapeutic gain[15,16]. Consequently, the effects of glucocorticoids on the resolution process, in our opinion, are highly clinically relevant.

GLUCOCORTICOID REGULATION OF APOPTOSIS IN INFLAMMATORY CELLS

Internal Controls of Apoptosis

Apoptosis plays a crucial role in regulation of the inflammatory response. Neutrophils have a short life span with a circulating half-life of 6–10 h, which is delayed by redundant mediators present at inflamed sites (24–48 h)[88]. Eosinophil apoptosis occurs at a much slower rate than in neutrophils[7]. Despite their derivation from a common precursor cell, apoptosis in these granulocytes is controlled quite differently. Many of the inflammatory mediators present at an inflamed site can exert either anti- or proapoptotic influences. For example, G-CSF, GM-CSF, C5a, and LPS profoundly inhibit neutrophil apoptosis[14,89,90], as does increased cAMP[8] or a hypoxic environment[91]. Eosinophil survival is promoted in response to GM-CSF, IL-3, and IL-5[92], and PGD$_2$[10].

    T cells are an essential component of the adaptive immune system, and apoptosis is important for their development and maintenance. Apoptosis is fundamental in regulating T-cell development in the thymus during selection of developing thymocytes bearing a functional TCR[93]. Regulation of apoptosis

in T cells differs from that in granulocytes. Thymocytes undergo apoptosis upon elevation of intracellular calcium concentrations in response to calcium ionophores[94], whereas increased levels of intracellular calcium delays eosinophil apoptosis[10]. Additionally, cycloheximide accelerates the constitutive rate of apoptosis in both neutrophils[95] and eosinophils[13], but not T cells[4]. Apoptosis is also essential for maintenance of mature T cells in the periphery. Upon encounter with cognate antigen, mature T cells undergo significant clonal expansion and removal of these expanded cells once the stimulus has been cleared is by activation-induced cell death[93], which involves activation of the extrinsic pathway of apoptosis by ligation of death receptors such as FasL[96]. Hence, apoptosis is an indispensable process to avoid autoimmunity.

From the knowledge gained on the internal controls of apoptosis, it may be possible to induce apoptotic death of specific inflammatory cells involved in the pathogenesis of diverse inflammatory diseases. In support of this suggestion, *in vitro* studies suggest that apoptosis in the closely related neutrophil and eosinophil granulocytes appears to be controlled by different mechanisms.

GC Modulation of Apoptosis

Physiological concentrations of GCs can regulate the intrinsic rate of apoptosis in many leukocytes and their efficacy depends upon a number of modulatory factors, including the GC type and concentration. GC regulation of granulocyte survival is dose dependent and occurs through the GCR (blocked by mifepristone)[13,14]. The GCs dexamethasone, methylprednisolone, and hydrocortisone all prolonged neutrophil viability from 12 to 48 h, however non-GC progesterone failed to inhibit development of apoptosis[14,97], indicating this is not due to nonspecific effects of high dose GCs. Endogenous cortisone also mediates these effects on granulocyte survival, albeit to a lesser extent[13]. Differential effects of GCs on granulocyte apoptosis have also been shown in a rat peritoneal model[98], where various GCs induce eosinophil apoptosis, but delay neutrophil apoptosis with most significant effects observed at GC concentrations of 10^{-6} and 10^{-8} M, respectively. Thus, GCs used at optimal concentrations for eosinophil apoptosis prolong neutrophil survival, indicating that the beneficial effects of GC treatment for asthma may involve suppressing eosinophilic airway inflammation while maintaining antimicrobial responses. This is a more specific therapeutic approach to treatment of inflammatory diseases compared to general nonspecific immunosuppressive agents, such as cyclosporine. However, the anti-inflammatory action of GCs is not reproducible for COPD, a neutrophil-dependent disease. Here, GC-mediated neutrophil survival may contribute to tissue damage with increased potential for release of intracellular contents. NFκB plays a vital role in the regulation of granulocyte apoptosis by inducing the expression of prosurvival genes[99] and targeted inhibition may be an approach to induce neutrophil apoptosis in COPD. Indeed, the NFκB inhibitor gliotoxin induces apoptosis in granulocytes alone or synergistically with TNFα[99].

GCs can also induce apoptosis in thymocytes[12] and, hence, are used in the treatment of lymphoid diseases. Interestingly, thymocyte sensitivity to GCs seems to be dependent upon their developmental stage where GCs induce apoptosis in CD4$^+$CD8$^+$ double positive thymocytes, but not upon maturation to single positive thymocytes[93]. In contrast, mature T cells are relatively resistant to GC-induced apoptosis, and some investigations have even reported GCs to prevent activation-induced cell death in these cells[100]. The effect of GCs on monocyte viability is more controversial. Although GCs have been suggested to induce apoptosis in monocytes[101], a recent study by Ehrchen et al. showed that monocytes were protected from staurosporine-induced apoptosis, mediated by reactive oxygen species (ROS), by increasing the expression of antioxidant molecules upon GC treatment[102].

GC influence on cell survival is initiated by GC binding to the cytoplasmic GCR and involves alterations in gene expression. However, there has been much controversy over the mechanism used by the GCR, whether by transactivation of death genes, transrepression of survival genes or both.

MECHANISMS OF GC EFFECTS ON APOPTOSIS

Evidence for Transrepression

Helmberg and colleagues[103] have suggested that interference with proinflammatory signalling through the transrepressional activity of GCR is an important mechanism of GC-induced apoptosis. This was derived from studies using T-cell leukaemia cell lines expressing mutant GCRs that were DNA-binding and transactivation defective, but retained the ability to repress NFκB and AP-1 transcriptional capability. These mutants were sufficient to induce apoptosis in these cells in response to GCs, implicating a role for GCs in apoptosis via the transrepression of prosurvival genes. Transrepression by GCR is suggested to be the main mechanism of suppressing the expression of cytokines and other inflammatory mediators that modulate granulocyte survival, and this may contribute to GC-induced apoptosis. In a recent study, Novac and colleagues provide data that support the role of GCR repression of gene expression in GC-induced apoptosis[104]. Here, GCs transiently suppress the level of the death receptor FasL and subsequently inhibit activation-induced T-cell apoptosis. Regulation of FasL expression was suggested to be a result of GCR binding directly to DNA, specifically to negative GREs that overlap an NFκB site. Hence, repression of FasL by GCR binding *in cis* may be due to competition for a common binding site and their rationalisation of this mechanism is that it would slow down T-cell apoptosis so that macrophages are not overwhelmed with a massive apoptotic cell load.

Evidence for Transactivation

Ramdas and Harmon[105] used a transactivation-defective mutant GCR that retained transrepressional activity, but did not mediate GC-induced apoptosis in human leukaemic T cells, suggesting that DNA binding by the GCR is important. Similarly, Reichardt and coworkers used a GCR^{dim} mutant to show the requirement for transactivation in GC-induced thymocyte apoptosis[28]. New protein synthesis has been implicated in the regulation of apoptosis and, indeed, GC-induced thymocyte apoptosis and neutrophil survival can be abolished with cycloheximide[106]. This indicates that neutrophil survival requires ongoing gene expression and continuous synthesis of a prosurvival factor(s), a process that requires continued presence of GC[106], whereas GC-induced thymocyte apoptosis may be due to transactivation of death genes. Alternatively, GC induction of IκB expression may block proinflammatory signalling and contribute to induction of apoptosis in responsive cells[105].

Effects of GCs on the Mitochondrial Pathway–Regulated Apoptosis

Regulation of apoptosis may occur at the level of the mitochondria, an intracellular organelle with a central role in the apoptotic process. During the early stages of apoptosis, loss of mitochondrial membrane potential and increase in outer mitochondrial membrane permeability allow the release of factors such as Cytochrome C[8], which initiates downstream effects culminating in caspase activation and ultimately apoptosis[107]. Human eosinophils stimulated with dexamethasone show morphological changes characteristic of apoptosis, including DNA fragmentation, caspase-3 activation, and loss of mitochondrial membrane permeability[107]. Inhibition of mitochondrial permeabilisation, but not caspase-3 activity, prevented both mitochondrial disruption and apoptosis in response to dexamethasone. This suggests that GC-induced eosinophil apoptosis occurs through activation of the mitochondrial pathway, possibly resulting from the release of caspase-independent apoptosis-inducing factor (AIF)[107]. Loss of mitochondrial membrane potential and induction of eosinophil apoptosis may result from oxidant-induced mitochondrial injury, a process enhanced by GCs through production of ROS and sustained activation of proapoptotic JNK[3]. Ruiz and coworkers used the glucose-glucose oxidase system to achieve a constant production of hydrogen peroxide as a source of ROS to stimulate oxidative

stress in neutrophils and, hence, apoptosis[108]. Dexamethasone delayed ROS-induced apoptosis in a concentration-dependent manner, with most significant inhibition at $10^{-6} M$ dexamethasone at 12 and 24 h time points[108].

Although some studies described above do not implicate a role for caspases, GC-induced apoptosis in thymocytes and lymphoma cells can be blocked by pharmacological inhibition of caspases (z-VAD-fmk)[109], showing an additional level of apoptosis regulation, at least in these cell types, by GCs.

Involvement of Bcl-2 Family Members in GC-Induced Apoptosis

Bcl-2 proteins play a crucial role in apoptosis by regulating mitochondrial membrane stability[88]. Neutrophils express proapoptotic (Bak and Bax) and antiapoptotic (A1 and Mcl-1) members of the bcl-2 family[88]. GCs may delay neutrophil apoptosis resulting from mitochondrial dysfunction by increasing the abundance of antiapoptotic A1 mRNA, while decreasing the level of proapoptotic Bak mRNA in a GCR-dependent manner[88]. GC-induced thymocyte apoptosis may also result from mitochondrial dysfunction[110] as a consequence of alterations in the levels of pro- and antiapoptotic bcl-2 factors. Indeed, thymocyte apoptosis in response to dexamethasone is accelerated by bcl-2 deficiency[111], whereas bcl-2 overexpression inhibits GC-induced lymphoma cell apoptosis[112]. Furthermore, alterations in the phosphorylation status of bcl-2 family members in response to GCs may lead to mitochondrial injury and subsequent eosinophil apoptosis[3]. Thus, the intrinsic mitochondrial pathway may contribute significantly to determining the fate of cells by integrating signals from pro- and antiapoptotic members of the bcl-2 family of proteins and this may be regulated by GCs. This apoptotic pathway is also inhibited in human neutrophils by a cell permeable analogue of cAMP, dibutyryl cAMP (db-cAMP), resulting in neutrophil survival[8], whereas proinflammatory mediators G-CSF and GM-CSF increase the expression of proapoptotic bcl-2 proteins and, hence, may promote apoptosis[88].

MODULATION OF PHAGOCYTIC REMOVAL OF APOPTOTIC CELLS BY MACROPHAGES

In situations where cells are stimulated to undergo apoptosis at a high rate (for example, when there are overwhelming proapoptotic signals), the tissue load of apoptotic cells may be in danger of exceeding the removal capacity by phagocytes and noningested apoptotic cells may then undergo secondary necrosis with detrimental consequences[113]. Thus, the phagocytic clearance of cells dying by apoptosis plays a pivotal role in determining the inflammatory outcome and may be a prerequisite for effective resolution of inflammation. Hence, in order for deliberate induction of apoptotic cell death to be considered as a therapeutic strategy, parallel strategies for their efficient removal will also be required.

Pharmacological Modulation

Our laboratory is interested in the way inflammation resolves and we have made attempts to modulate macrophage phagocytic potential via pharmacological means. Increased levels of cAMP in human monocyte-derived macrophages (MDMφ), using db-cAMP, specifically inhibits phagocytosis of apoptotic neutrophils[114], whereas ligation of macrophage surface CD44 rapidly and specifically augments phagocytic uptake of apoptotic neutrophils, but not apoptotic lymphocytes[115]. Adhesion to the matrix protein fibronectin also rapidly increases macrophage capacity for internalisation of apoptotic neutrophils[116]. Particular focus is being made now on interpreting the influence of GCs on the mechanisms by which acute inflammation normally resolves. We have demonstrated that GCs augment phagocytic potential for nonphlogistic clearance of apoptotic leukocytes, a process that is essential for the resolution of inflammation. Pretreatment of 5-day human MDMφ for the first 24 h of culture with GCs

methylprednisolone, dexamethasone, and hydrocortisone induced a prophagocytic phenotype displaying augmented phagocytosis of neutrophils undergoing apoptosis[17]. GCs also enhance macrophage capacity for uptake of other apoptotic targets, including Jurkat T cells and eosinophils, and also promote uptake of apoptotic neutrophils by alternative phagocytes, including human glomerular mesangial cells. GC action on phagocytosis is specific, as non-GCs aldosterone, estradiol, and progesterone did not exert this effect and GCs did not promote ingestion of Ig-opsonised erythrocytes. These observed effects are GCR dependent, being inhibited by the GCR antagonist mifepristone, and are reversed by cycloheximide, indicating the requirement for new protein synthesis.

Reprogramming of Monocyte Differentiation by GCs

Freshly isolated monocytes lack the capacity to ingest apoptotic targets, an ability that is gradually acquired during *in vitro* culture as adherent monocytes differentiate into macrophages[117]. GCs potentiate the phagocytic capacity of MDMɸ cultured for 5 days *in vitro*, with a greater potentiation observed the earlier monocytes were exposed to GCs during maturation[17]. Furthermore, we have demonstrated that long-term exposure (5 days) of monocytes to GCs induced differentiation of monocytes into MDMɸ displaying enhanced phagocytosis of apoptotic cells (up to threefold) and, hence, represents a proresolution phenotype[18]. Macrophages that had been treated with GC for this period were a more homogenous population of smaller, more rounded, and less well-spread cells with a phenotype characterised by reduced phosphorylation and, hence, recruitment of paxillin and pyk2 to sites of adhesion, with consequential loss of actin- and paxillin-containing podosomes, and loss of p130cas expression, an important adaptor molecule in integrin adhesion signalling through the DOCK180/Crk/p130cas pathway. Despite their altered adhesion, time-lapse video microscopy revealed that GC-treated MDMɸ remained membrane active with extension and retraction of cellular process, probably as a result of increased levels of active Rac. GC-treated MDMɸ also displayed homogeneity in surface receptor expression, as shown by their laser scatter properties from flow cytometry, which identified a more uniform expression of HLA-DR, CD14, and CD44, consistent with reprogramming during monocyte differentiation. Although GC-treated MDMɸ exhibit elevated surface expression of the haemoglobin scavenger receptor, CD163, no single surface receptor was identified that would define a prophagocytic macrophage phenotype. Combinatorial treatment with GC and the "classical activator" IFNγ abolished GC potentiation of macrophage phagocytic capacity for apoptotic cells[118]. The first 24 h of culture with GC was critical for acquisition of a prophagocytic phenotype as this was overridden by subsequent treatment with IFNγ, with less pronounced inhibition the later IFNγ was added. Interestingly, IFNγ did not have an observed effect on GC-mediated morphology or surface receptor expression indicating that GC-treated MDMɸ adhesion status can be dissociated from phagocytic capacity. This has implications for the application of GC therapy in Th1-mediated diseases characterised by high levels of IFNγ.

Induction of an Anti-Inflammatory Macrophage Phenotype by GCs

GCs are associated with induction of an anti-inflammatory macrophage phenotype. Importantly, GC potentiated phagocytosis of apoptotic cells did not stimulate the release of proinflammatory mediators, including MCP-1 and IL-8 chemokines[17]. Therefore, GCs promote "safe" clearance of cells dying by apoptosis and may directly contribute to the resolution of inflammation. The GC-inducible protein annexin I undergoes caspase-dependent recruitment from the cytosol to the outer plasma membrane during apoptosis to colocalise with phosphatidylserine and may be required for efficient clearance of dying cells[33]. Alternatively, GC-induced expression of annexin I in macrophages and subsequent release may enhance apoptotic cell uptake[35]. Indeed, macrophages from annexin I knockout mice show

defective phagocytosis of apoptotic cells acting through the FPRL1[35]. This has been furthered by a recent study by Scannell et al., who report that annexin I is a prophagocytic factor released by apoptotic cells and actively promotes the FPRL1-dependent clearance of apoptotic cells by macrophages[119]. Thus, transactivation of annexin I expression by the GCR may contribute to enhanced recognition and internalisation of apoptotic cells in response to GC treatment.

C1q is an important subcomponent of complement C1, which activates the classical pathway upon binding to immune complexes or CRP and its expression in monocytes/macrophages can be induced by GCs or inhibited by IFNγ and LPS[31,120]. C1q binds to apoptotic cells[121] and may aid in their removal by phagocytes. Interestingly, Botto and colleagues demonstrated C1q deficiency in mice to cause an SLE-like disease with high titres of autoantibodies and accumulation of apoptotic cells in glomeruli[85] and defective clearance of apoptotic cells is also observed in human SLE patients[82,83]. Thus, C1q may protect against development of SLE by targeting apoptotic cells for clearance. This has been supported by *in vitro* studies where C1q-deficient human macrophages show impaired phagocytosis of apoptotic cells, and this is restored with purified human C1q[84]. Hence, induction of innate proteins by GCs may contribute to their anti-inflammatory effect by enhancing removal of dying cells.

A recent study by Ehrchen and coworkers used a microarray system to analyse the expression profile in human monocytes treated with GCs[102]. Their data signify the multitude of GC effects, where the expression of over 100 genes were GC regulated with a more pronounced induction of genes that are important in monocyte/macrophage functions such as phagocytosis, apoptosis, and adhesion. Important to the process of phagocytosis, there was induced expression of CD163, FPR1, and MER tyrosine kinase receptors, and MFG-E8 and C1q serum proteins, however, the relevance of this in GC-potentiated phagocytic capacity is unclear. These results indicate that GC effects on monocytes are not simply immunosuppressive, but also immunomodulatory. GCs promote induction of an anti-inflammatory phenotype important for the resolution of inflammation, challenging the concept that transrepression is the dominant mechanism of GC action on monocytes.

CONCLUSION

Apoptosis is a fundamental process in cell and tissue homeostasis that, in contrast to necrosis, is associated with maintenance of cell membrane integrity and noninflammatory clearance by phagocytes. Failure or inefficient apoptosis and/or phagocytosis may result in necrosis with detrimental proinflammatory consequences. GCs modulate inflammatory cell survival and promote nonphlogistic phagocytosis of apoptotic cell targets *in vitro*, a situation that could be deemed proresolution with consequent implications in the clinical setting where unresolving chronic inflammatory diseases cause considerable morbidity and untimely death. Thus, considerable effort to elucidate the precise molecular and cellular mechanisms of action of GCs, as well as improve existing GCs, is being made.

ACKNOWLEDGEMENTS

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Glucocorticoids Induce Protein S-Dependent Phagocytosis of Apoptotic Neutrophils by Human Macrophages[1]

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During resolution of an inflammatory response, recruited neutrophil granulocytes undergo apoptosis and are removed by tissue phagocytes before induction of secondary necrosis without provoking proinflammatory cytokine production and release. Promotion of physiological neutrophil clearance mechanisms may represent a viable therapeutic strategy for the treatment of inflammatory or autoimmune diseases in which removal of apoptotic cells is impaired. The mechanism underlying enhancement of macrophage capacity for phagocytosis of apoptotic cells by the powerful anti-inflammatory drugs of the glucocorticoid family has remained elusive. In this study, we report that human monocyte-derived macrophages cultured in the presence of dexamethasone exhibit augmented capacity for phagocytosis of membrane-intact, early apoptotic cells only in the presence of a serum factor. Our results eliminate a role for a number of potential opsonins, including complement, pentraxin-3, and fibronectin. Using ion-exchange and gel filtration chromatography, we identified a high molecular mass serum fraction containing C4-binding protein and protein S responsible for the augmentation of phagocytosis of apoptotic neutrophils. Because the apoptotic neutrophils used in this study specifically bind protein S, we suggest that glucocorticoid treatment of macrophages induces a switch to a protein S-dependent apoptotic cell recognition mechanism. Consistent with this suggestion, pretreatment of macrophages with Abs to Mer tyrosine kinase, a member of the Tyro3/Axl/Mer family of receptor tyrosine kinases, prevented glucocorticoid augmentation of phagocytosis. Induction of a protein S/Mer tyrosine kinase-dependent apoptotic cell clearance pathway may contribute to the potent anti-inflammatory effects of glucocorticoids, representing a potential target for promoting resolution of inflammatory responses. *The Journal of Immunology,* 2009, 183: 2167–2175.

S uccessful restoration of a tissue to its original state after an inflammatory insult requires that large numbers of extravasated neutrophil granulocytes are cleared from the inflamed site. During this resolution phase of inflammation, recruited neutrophils undergo apoptosis and are subsequently removed by phagocytosis (1), a rapid and efficient process that does not stimulate proinflammatory macrophage responses (2). Conversely, inefficient or defective clearance of membrane-intact apoptotic neutrophils may result in release of their histotoxic intracellular contents as a consequence of secondary necrosis, potentially causing local tissue damage and contributing to pathogenesis of inflammatory disease (3). An attractive approach for therapeutic intervention in inflammatory diseases would therefore be to manipulate the processes involved in physiological clearance of neutrophils from inflamed sites. Although promotion of neutrophil apoptosis may be achievable pharmacologically (4), under some circumstances in vivo it will be important to ensure that the capacity for apoptotic cell clearance within tissues is matched to avoid potential deleterious consequences of the presence of non-phagocytosed apoptotic cells (5).

We have previously reported that the powerful anti-inflammatory drugs of the glucocorticoid family (methylprednisolone, hydrocortisone, or dexamethasone (Dex))[3] specifically enhance noninflammatory phagocytosis of apoptotic cells by human and murine macrophages (6, 7). Glucocorticoids have been shown to modulate the expression of over 100 genes, including those known to be associated with apoptotic cell phagocytosis, such as CD163, FPR1, and Mer tyrosine kinase (Mertk) receptors and MFG-E8 and C1q serum proteins (8). Furthermore, we have shown that human monocytes differentiated for 5 days in the presence of glucocorticoids exhibit a more homogeneous phenotype with reduced phosphorylation of molecules involved in integrin signaling and cytoskeletal rearrangement (7). However, the precise mechanism(s) by which glucocorticoids augment phagocytosis of apoptotic cells has remained elusive.

In this study, we have examined the mechanism underlying augmentation of human monocyte-derived macrophage (MDMϕ) capacity for phagocytosis of early membrane-intact apoptotic human neutrophils following exposure to glucocorticoids. Apoptotic neutrophils display a distinct surface molecular phenotype important for attenuation of functional responses (9) with additional surface changes that target dying cells for removal by phagocytes (10). A number of soluble factors present in serum, including complement C1q and C3b, properdin, collectins, long pentraxin-3, MFG-E8, galectin-3, and α_2-macroglobulin, have been reported to bind to apoptotic human cells (11–18) and consequently modulate their

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[3] Abbreviations used in this paper: Dex, dexamethasone; C4BP, C4-binding protein; CMFDA, 5-chloromethylfluorescein diacetate; MDMϕ, monocyte-derived macrophage; Mertk, Mer tyrosine kinase.

recognition and uptake by macrophages via a number of different surface receptors, including scavenger receptors, complement receptors, receptors for phosphatidylserine, and Mertk (19–22). However, it is important to note that some of these opsonization events occur relatively late during the apoptotic process and accompany loss of membrane integrity (23).

In this study, we demonstrate that glucocorticoid augmentation of MDMϕ phagocytosis is associated with a switch from a serum-independent to a serum-dependent apoptotic cell recognition mechanism, which can be recapitulated with purified protein S, a 75-kDa vitamin K-dependent anticoagulation factor that is present in plasma at a relatively high concentration of ~25 μg/ml (24), and involves macrophage Mertk, a member of the Tyro3/Axl/Mer family of immunoregulatory receptor tyrosine kinases (25). Our data strongly suggest that glucocorticoids critically regulate a switch in apoptotic cell clearance mechanisms used by macrophages, potentially contributing to their potent anti-inflammatory effects and thus representing a target for promoting inflammatory resolution.

Materials and Methods

Sera, serum proteins, and other reagents

All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. Culture medium (IMDM), buffers (HBSS and PBS without divalent cations), and trypsin-EDTA were from PAA Laboratories. Percoll was from GE Healthcare. Dextran T500 was from Pharmacosmos. Dex was obtained from Organon. Roscovitine was from Merck. Serum, from coagulated whole blood, was obtained by cardiac puncture from wild-type, annexin I-deficient (26), and C1q-deficient (27) mice on a C57BL/6 background. C1q-depleted human serum was obtained from Merck. A soluble recombinant form of human complement receptor 1 was used for inhibition of C3 activation in serum (28). A dose of 250 μg/ml completely blocks complement activity, as assessed by hemolytic assays. Proteins purified from human serum/plasma were obtained from the following sources: protein S (Enzyme Research Laboratories), C1q (Merck), and α_2-macroglobulin (Sigma-Aldrich).

Antibodies

Primary Abs were from the following sources: polyclonal rabbit anti-protein S Ab (1:5000; DakoCytomation), anti-human Mer mAb (clone 125508, murine IgG2b, 1:50; R&D Systems), and CD44 mAb (clone 5A4, IgG1, 1:50; provided by G. Dougherty, University of California, San Francisco, CA). Control mouse Igs (IgG1, IgG2b; 1:50) were from Serotec. HRP-conjugated goat anti-rabbit Igs (1:2500) and FITC-conjugated F(ab')$_2$ goat anti-mouse Igs (1:50) were from DakoCytomation. PE-conjugated anti-CD16 mAb (clone 3G8, IgG1) was obtained from BD Biosciences. Agarose-coupled goat anti-rabbit Ig was obtained from Sigma-Aldrich.

Cell isolation

Mononuclear and polymorphonuclear leukocytes were isolated from freshly drawn, citrated human blood by dextran sedimentation and centrifugation over a discontinuous Percoll gradient (final concentrations of 55, 70, and 81% Percoll), as previously described (29). Mononuclear cells were aspirated from the 55/70% interface, and neutrophils from the 70/81% interface. Autologous serum was prepared by recalcification of platelet-rich plasma (final CaCl$_2$ concentration: 22 mM), as previously described (24).

In vitro culture of human MDMϕ

Mononuclear cells were resuspended at 4 × 10^6/ml in IMDM and adhered to 48-well tissue culture plates for 1 h at 37°C in 5% CO$_2$. Nonadherent lymphocytes were removed by washing with IMDM, and adherent monocytes were cultured for 5 days in IMDM containing 10% autologous serum ± 1 μM Dex. These cells are >90% CD14$^+$ at 5 days with functional and phenotypic characteristics of macrophages (Dex-MDMϕ) (7).

In vitro culture of neutrophils to induce apoptosis

Neutrophils were cultured at 4 × 10^6/ml in IMDM either in the absence of serum or in the presence of 10% autologous serum at 37°C in 5% CO$_2$ atmosphere for 20–24 h, during which time a proportion of the cells underwent apoptosis (1). Apoptosis and secondary necrosis were determined by annexin V-FITC binding (Roche Applied Sciences) and

propidium iodide staining (Sigma-Aldrich), respectively. Alternatively, neutrophils were resuspended in IMDM at 2 × 10^7/ml and labeled with the fluorescent cell tracker dye 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen), 2 μg/ml for 15 min at 37°C in 5% CO$_2$. Neutrophils were then washed and cultured for 20–24 h, as described above.

Apoptotic cell phagocytosis assay

Phagocytosis of apoptotic cells was assessed essentially as described (30), using a method that has been carefully characterized and shown to discriminate between bound and internalized apoptotic cells, comparing favorably with microscopy analysis. CMFDA-labeled apoptotic neutrophils were centrifuged at 200 × g and resuspended at 2.5 × 10^6/ml in IMDM, and 0.5 ml was overlaid onto MDMϕ that had been cultured in 48-well plates (~200,000 MDMϕ/well, a ratio of ~10 neutrophils per macrophage) and then coincubated for 30 min at 37°C in 5% CO$_2$. Medium was then gently aspirated from the wells, and all cells were detached with 500 μl of trypsin-EDTA before determination of phagocytosis (percentage of FL-1-positive MDMϕ, identified by their distinct forward and side scatter properties) by flow cytometry using a FACScan (BD Biosciences). For assays conducted in the presence of serum or purified proteins, neutrophils were resuspended in IMDM containing either 1% autologous serum, 10 μg/ml either purified protein, or 1 mg/ml protein fractions from ion-exchange or gel filtration chromatography, unless otherwise stated in the figure legends. For experiments requiring preincubation with either Abs or other protein fractions from ion-exchange or gel filtration chromatography, MDMϕ or neutrophils were incubated with saturating concentrations of Abs (final concentration of ~10 μg/ml as determined by flow cytometric analysis) or 10 μg/ml either purified protein or 1 mg/ml protein fractions from ion-exchange or gel filtration chromatography, unless otherwise stated in the figure legends. Cells were washed and resuspended in IMDM before use in phagocytosis assays.

Serum fractionation

Human serum was dialyzed against 50 mM HEPES buffer (pH 7.0) containing 0.14 M NaCl overnight before anion-exchange chromatography using Q Sepharose (Sigma-Aldrich). Proteins were eluted using 50 mM HEPES containing 0.2 M NaCl, and fractions containing the highest amount of protein were combined before dialysis against 50 mM Tris (pH 7.4) containing 0.14 M NaCl (TBS) and concentrated before gel filtration chromatography using Sephacryl S-300 (GE Healthcare). The protein concentration in eluted fractions was estimated by measurement of absorbance at 280 nm (A280) using a spectrophotometer or using a bicinchoninic acid protein assay kit, as specified by the manufacturer (Pierce). Gel filtration fractions containing phagocytic activity were analyzed by SDS-PAGE, immunoblotting, and mass spectrometry to determine the proteins present (two separate analyses; J. Creanor, University of Edinburgh, Edinburgh, U.K.).

Immunodepletion and Western blotting

The 0.2 M NaCl eluate from anion-exchange chromatography was incubated with protein S Ab (5 μg/ml eluate) for 1 h on ice. Immunodepletion was achieved by incubation for 1 h with agarose-coupled goat anti-rabbit IgG at 4°C on a rotary mixer, followed by centrifugation at 13,000 × g for 1 min to pellet the agarose. To ensure efficient protein depletion, the supernatant was subjected to three rounds of depletion. Samples were resolved by SDS-PAGE using 9% gels under nonreducing conditions, unless otherwise stated, and transferred electrophoretically (80 V for 50 min) onto either polyvinylidene difluoride or nitrocellulose membranes (Millipore). Membranes were blocked overnight in TBS containing 0.1% Tween 20 before probing with Abs. Binding of anti-protein S Ab was detected with HRP-conjugated goat anti-rabbit Igs together with ECL (GE Healthcare).

Flow cytometry

All incubations were performed on ice to prevent internalization of Ab. Adherent MDMϕ were detached by incubation in HBSS without divalent cations containing 0.1% BSA and 3 mM EDTA. After washing with ice-cold HBSS containing 2% FBS, cells (10^5/assay) were incubated with saturating concentrations of mAb for 30 min. Cells were then washed twice in HBSS containing 2% FBS before incubation with FITC-conjugated F(ab')$_2$ goat anti-mouse Igs for 30 min before analysis using a FACScan flow cytometer (29).

Analysis of results

Results are presented as mean $\pm$ SEM, and n = number of independent experiments using macrophages from different donors. Results were analyzed by repeated measures one-way ANOVA with a Bonferroni posttest.

Results

Glucocorticoid-augmented clearance of apoptotic neutrophils by macrophages is serum dependent

Previous studies relating to glucocorticoid-enhanced phagocytosis of apoptotic cells used monocyte-derived macrophages and apoptotic cell targets that had been cultured in the presence of serum (6, 7). To evaluate the potential role of serum opsonization in promoting apoptotic neutrophil clearance, human blood monocytes were cultured for 5 days in the absence or presence of Dex, and neutrophils were rendered apoptotic by overnight culture in serum-free conditions (Fig. 1A). Neutrophil populations cultured in serum-free conditions for 20 h exhibit a slightly higher percentage (63–70%, n = 35, 95% confidence limit) of annexin V$^+$/propidium iodide$^-$ (apoptotic) cells when compared with neutrophils cultured in the presence of serum (50–60%, n = 10), consistent with the presence of a survival factor(s) in human serum. In addition, there were significantly higher percentages of annexin V$^+$/propidium iodide$^+$ (secondarily necrotic) neutrophils in serum-free cultures (18–26%, n = 35) when compared with neutrophils cultured in the presence of serum (8–17%, n = 10).

When we determined the proportion of untreated MDMϕ and Dex-MDMϕ that were capable of phagocytosis of neutrophils, we were surprised to find that there was no significant augmentation of phagocytosis of serum-free apoptotic neutrophils observed for Dex-MDMϕ (Fig. 1, C and E). In contrast, in the presence of 10% autologous serum, we observed increased phagocytic capacity for Dex-MDMϕ (Fig. 1, D and E). The presence or absence of serum also had a small, but statistically significant stimulatory effect upon phagocytosis of apoptotic neutrophils by untreated MDMϕ (Fig. 1E). The effect of serum on phagocytosis by Dex-MDMϕ was concentration dependent and reached significance at 1% (data not shown). The possibility that the presence of serum acts to promote phagocytic activity of MDMϕ directly was excluded in a series of experiments in which preincubation of apoptotic neutrophils with serum was found to confer augmentation of Dex-MDMϕ phagocytic capacity (data not shown), raising the possibility that a serum factor binds to the apoptotic neutrophil surface to promote phagocytosis by Dex-MDMϕ.

Augmentation of phagocytosis by serum is independent of the presence of necrotic neutrophils

Neutrophils undergo apoptosis in a relatively heterogeneous manner during in vitro culture (Fig. 1A), making it difficult to determine whether enhanced phagocytosis following opsonization depends upon the presence of apoptotic or secondarily necrotic cells. We therefore treated neutrophils with roscovitine, a cyclin-dependent kinase inhibitor that induces neutrophil apoptosis rapidly and uniformly without induction of secondary necrosis (4). Neutrophil populations cultured with 20 μM roscovitine in serum-free conditions for 4 h exhibit a high percentage (80–90%) of annexin V$^+$/propidium iodide$^-$ (apoptotic) cells with less than 3% annexin V$^+$/propidium iodide$^+$ (secondarily necrotic) cells (Fig. 1B). Serum-dependent enhancement of phagocytosis of roscovitine-treated apoptotic neutrophils by Dex-MDMϕ confirmed that opsonization of early apoptotic cells was required (33.7 $\pm$ 9.3% and 51.8 $\pm$ 6.3% for Dex-MDMϕ in the absence and presence of serum, respectively). Data are mean percentage phagocytosis $\pm$ SEM, n = 3 (**, $p < 0.01$).
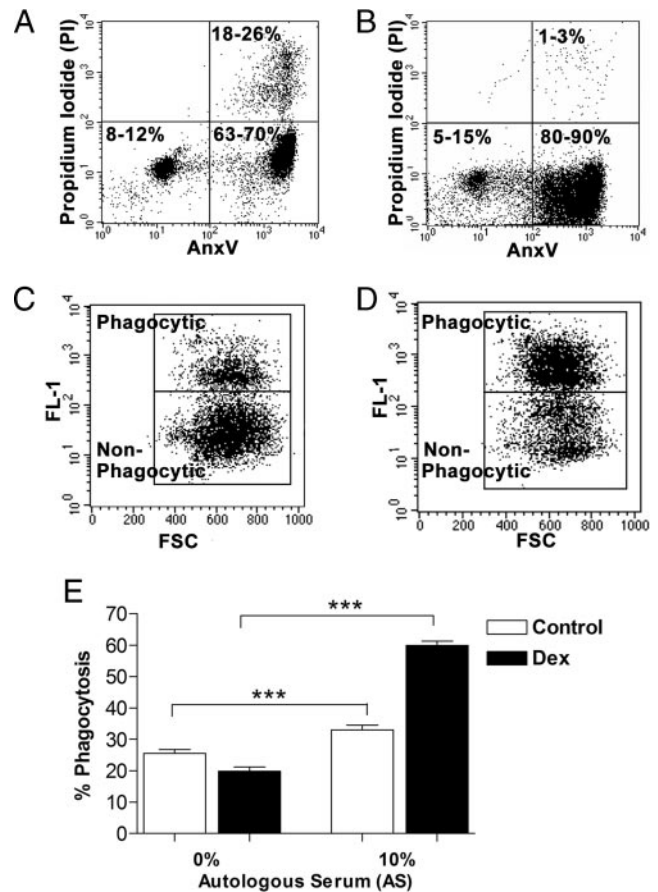

FIGURE 1. Augmentation of phagocytosis of apoptotic neutrophils by glucocorticoid-treated macrophages is serum dependent. Staining with annexin V (AnxV)-FITC (FL-1) and propidium iodide (FL-2) was used to determine the proportion of apoptotic (AnxV$^+$/propidium iodide$^-$) and secondarily necrotic (AnxV$^+$/propidium iodide$^+$) cells present in neutrophil populations used for phagocytosis assays. Representative two-parameter histograms of flow cytometric data are shown for *A*, neutrophils cultured for 20 h in serum-free conditions (n = 35), and *B*, neutrophils cultured for 4 h in serum-free conditions in the presence of 20 μM roscovitine (n = 3). Mean data for the proportion of viable, apoptotic, and secondarily necrotic cells present for each treatment are shown in the appropriate quadrant together with 95% confidence intervals. Phagocytosis of fluorescently labeled neutrophils was determined by flow cytometry using forward scatter and fluorescence to define FL-1^{high} phagocytic and FL-1^{low} nonphagocytic MDMϕ populations. Representative dot plots for Dex-MDMϕ incubated with CMFDA-labeled neutrophils in the absence (*C*) or presence (*D*) of 10% autologous serum are shown. FSC, forward light scatter. *E*, Percentage of phagocytosis ($\pm$SEM) recorded for untreated MDMϕ ($\square$) and Dex-MDMϕ ($\blacksquare$) following incubation with neutrophils for 30 min is shown. Phagocytosis of apoptotic neutrophils by Dex-MDMϕ was significantly augmented by 10% autologous serum (AS). The presence of serum also had a small, but statistically significant stimulatory effect upon phagocytosis by untreated MDMϕ. Data are mean percentage phagocytosis $\pm$ SEM, n = 75. ***, $p < 0.001$.

Serum-dependent enhancement of phagocytosis of apoptotic neutrophils does not require complement activation

Down-regulation of complement regulatory molecules CD55 (decay-accelerating factor), CD46 (membrane cofactor protein), and CD35 (CR1) on the surface of human apoptotic neutrophils (31) may allow complement proteins present in serum to bind, and hence promote their removal by phagocytes (11). We found that addition of commercially available C1q-depleted human serum
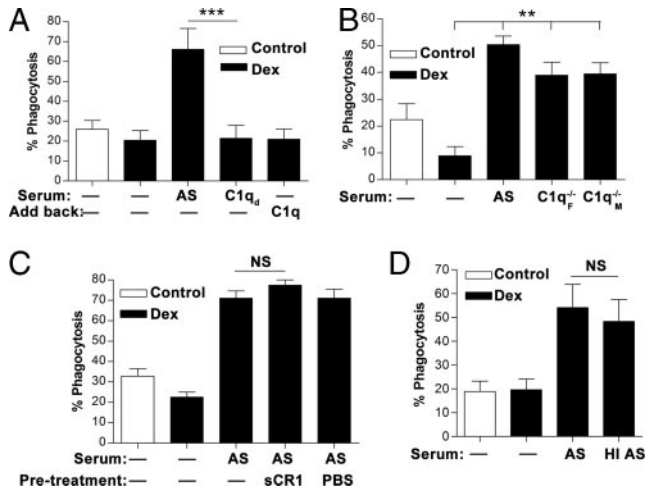
FIGURE 2. Glucocorticoid-enhanced phagocytosis of apoptotic cells does not require complement activation. Phagocytosis of apoptotic neutrophils by untreated MDMϕ (□) and Dex-MDMϕ (■) was assessed in a 30-min assay by flow cytometry. *A*, Phagocytosis of apoptotic neutrophils by Dex-MDMϕ in the presence of serum was not augmented by C1q-depleted serum (C1q$_d$), whereas addition of 70 μg/ml C1q failed to restore augmentation of phagocytosis ($n = 3$; ***, $p < 0.001$ compared with Dex-MDMϕ in the presence of serum). *B*, Serum derived from either male (M) or female (F) C1q-deficient mice restored serum-dependent augmentation of phagocytosis of apoptotic neutrophils by Dex-MDMϕ ($n = 3$; **, $p < 0.01$ compared with Dex-MDMϕ in the absence of serum). *C*, Autologous serum (AS) pretreated with 250 μg/ml soluble human rCR1 to inhibit C3 activation or with PBS as a control for 10 min did not affect phagocytosis of apoptotic neutrophils by Dex-MDMϕ in the presence of serum ($n = 4$; NS), and *D*, heat inactivation of autologous serum (HI AS; 56°C for 30 min) failed to affect phagocytosis of apoptotic neutrophils by Dex-MDMϕ ($n = 4$; NS). Data are mean percentage phagocytosis ± SEM.

failed to confer augmentation of phagocytosis (Fig. 2*A*), suggesting that C1q was the serum opsonin binding to apoptotic neutrophils. However, addition of 70 μg/ml human C1q alone (Fig. 2*A*) or to C1q-depleted serum (data not shown) did not restore phagocytosis by Dex-MDMϕ to levels observed in the presence of autologous serum. In a series of experiments examining the effects of sera from different species, we noted that augmentation of phagocytosis by Dex-MDMϕ was also observed when apoptotic neutrophils were incubated in serum obtained from mice, allowing us to use specific knockouts to define serum components (data not shown). We found that serum derived from either male or female C1q-deficient mice was able to significantly augment phagocytosis of apoptotic neutrophils by Dex-MDMϕ, demonstrating that C1q was not the serum opsonin required (Fig. 2*B*). We next inhibited complement activation and subsequent deposition of C3b on the surface of apoptotic neutrophils by pretreating autologous serum with 250 μg/ml C3 inhibitor for 10 min before the assay. The effects of C3 inhibitor-treated serum were indistinguishable from control PBS-treated serum in enhancement of Dex-MDMϕ phagocytosis (Fig. 2*C*). Further confirmation of a lack of requirement for complement activation and opsonization of targets was made through use of heat-inactivated serum (Fig. 2*D*).

Identification of a serum component required for augmentation of apoptotic neutrophil phagocytosis by Dex-MDMϕ

A number of serum factors have been reported to modulate apoptotic cell phagocytosis by macrophages, ranging from small molecules to very large protein complexes. A series of experiments using size fractionation of serum indicated the serum component to

Table I. *Effect of sera and serum proteins on Dex-MDMϕ phagocytosis*[a]

Add Back (during phagocytosis)	Phagocytosis by Dex-MDMϕ (as percentage of phagocytosis by Dex-MDMϕ in the absence of serum)
Autologous serum	301.4
Ultracentrifuged autologous serum	293.8
Murine serum	491.1
Bovine serum	241.8
IgG	109.8
Pentraxin-3	113.4
Fibronectin	97.9
Platelet releasate	84.7

[a] Data are shown as percentage of phagocytosis relative to that recorded for Dex-MDMϕ in the absence of serum (equivalent to 100%) for at least three independent experiments.

be greater than 100 kDa (data not shown). We initially sought to use an "add back" approach to evaluate the role of well-characterized serum proteins in the observed opsonization phenomenon. This strategy eliminated a role for IgG, pentraxin-3, fibronectin, platelet-derived factors, and immune complexes (Table I). Annexin I and lipoxin A4 are anti-inflammatory mediators that are regulated by glucocorticoids and can act to stimulate phagocytosis of apoptotic cells through the formyl-peptide receptor-like 1 (32). However, pretreatment of Dex-MDMϕ with 10 μM WRW4 (a formyl-peptide receptor-like 1 antagonist) for 1 h before assessment of phagocytosis of apoptotic neutrophils in the presence of 10% autologous serum showed no inhibitory effects (50.5 ± 9.1% and 42.5 ± 6.5% for Dex-MDMϕ with or without pretreatment; mean percentage phagocytosis ± SD, $n = 3$). Similarly, comparison of phagocytosis of apoptotic cells in the presence of either control or annexin I-deficient mouse serum demonstrated that this pathway is not used by Dex-MDMϕ for recognition of apoptotic neutrophils (80.4 ± 6.3% and 79.3 ± 3.23% for Dex-MDMϕ in the presence of wild-type and annexin I knockout serum, respectively; mean percentage phagocytosis ± SD, $n = 3$).

Identification of a serum fraction with phagocytic activity

Preliminary experiments showed that the serum factor could be bound to Q Sepharose in a 50 mM HEPES buffer at pH 7.0 or above and eluted with 0.2 M NaCl (data not shown). Because fewer proteins would bind at pH 7.0, we ran subsequent separations at this pH to facilitate identification of the factor. Further fractionation of the 0.2 M NaCl eluate using Sephacryl S-300 column yielded two partially overlapping peaks of protein with descending size (Fig. 3, *A* and *B*), as might be expected for a crude protein fraction with the first peak, representing high molecular mass proteins (>300 kDa), able to confer augmentation of phagocytosis (Fig. 3*C*).

Identification of the serum component using mass spectrometry

Mass spectrometry analysis of the major proteins present in the high molecular mass fraction revealed that the principal proteins present were IgM, α_2-macroglobulin, and C4-binding protein (C4BP), most likely in complex with protein S (33). The presence of protein S in the high molecular mass fractions isolated from gel filtration chromatography was confirmed by immunoblotting analysis (Fig. 3*D*). Previous work had eliminated a role for IgM in the augmentation of phagocytosis of apoptotic cells by Dex-MDMϕ (data not shown). Phagocytosis of apoptotic neutrophils by Dex-MDMϕ in the presence of 20 μg/ml α_2-macroglobulin was not augmented, suggesting that α_2-macroglobulin was not involved either (Fig. 4*A*), whereas addition of purified protein S during the
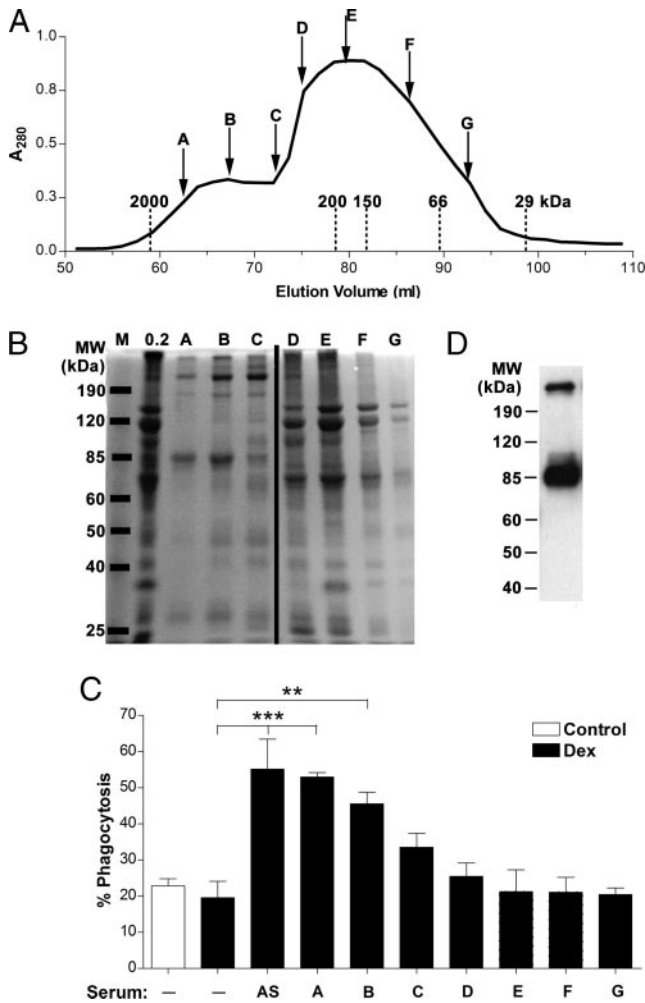
FIGURE 3. Identification of a high molecular mass factor required for augmentation of phagocytosis using anion-exchange chromatography and gel filtration. Serum proteins were fractionated using a combination of anion-exchange chromatography (Q Sepharose) together with gel filtration (Sephacryl S-300) of a 0.2 M NaCl eluate from the Q-Sepharose column. A, Protein elution profile of a typical gel filtration separation (of five that were performed) determined by measurement of absorbance at 280 nm (A_{280}) reveals two partially overlapping peaks of protein. B, Gel filtration samples (labeled A–G) were separated by SDS-PAGE on a 9% gel under reducing conditions and stained with 0.5% Coomassie blue (M, molecular mass marker; 0.2, 0.2 M NaCl eluate). C, The effect of different protein fractions on phagocytosis of apoptotic neutrophils by Dex-MDMϕ ($\blacksquare$) was determined using a 30-min phagocytosis assay. Samples were standardized for protein content, and phagocytosis of apoptotic neutrophils by untreated MDMϕ ($\square$) is shown for comparison. Significant augmentation of Dex-MDMϕ phagocytosis of apoptotic neutrophils was observed for two fractions. Data are mean $\pm$ SEM; $n = 3$. ***, $p < 0.001$, and **, $p < 0.01$ compared with Dex-MDMϕ in the absence of serum. D, The presence of protein S in the high molecular mass gel filtration fraction (fraction A) was confirmed by immunoblotting, as described in Materials and Methods.

FIGURE 4. Protein S, but not α_2-macroglobulin, stimulates phagocytosis of apoptotic neutrophils by Dex-MDMϕ. The effect of addition of either 20 μg/ml α_2-macroglobulin (A) or different concentrations of protein S (B) on phagocytosis of apoptotic neutrophils by Dex-MDMϕ ($\blacksquare$) was assessed in a 30-min assay. Phagocytosis of apoptotic neutrophils by untreated MDMϕ ($\square$) is shown for comparison. Addition of α_2-macroglobulin failed to restore augmented phagocytosis by Dex-MDMϕ, whereas protein S significantly augmented macrophage phagocytosis at concentrations of 250 ng/ml or higher. Data are mean $\pm$ SEM; $n = 3$. ***, $p < 0.001$, and *, $p < 0.05$ and NS, compared with Dex-MDMϕ in the absence of serum.

assay restored phagocytosis of apoptotic neutrophils by Dex-MDMϕ to levels similar to those observed in the presence of 10% serum (Fig. 4B). We therefore tested whether immunodepletion of protein S from the 0.2 M NaCl eluate affected Dex-MDMϕ phagocytosis of apoptotic neutrophils. As shown in Fig. 5A, immunodepletion did not result in the nonspecific removal of proteins from the 0.2 M NaCl eluate as assessed by total protein staining. Confirmation of the depletion of protein S from the 0.2M NaCl eluate containing the prophagocytic activity was made by immu-
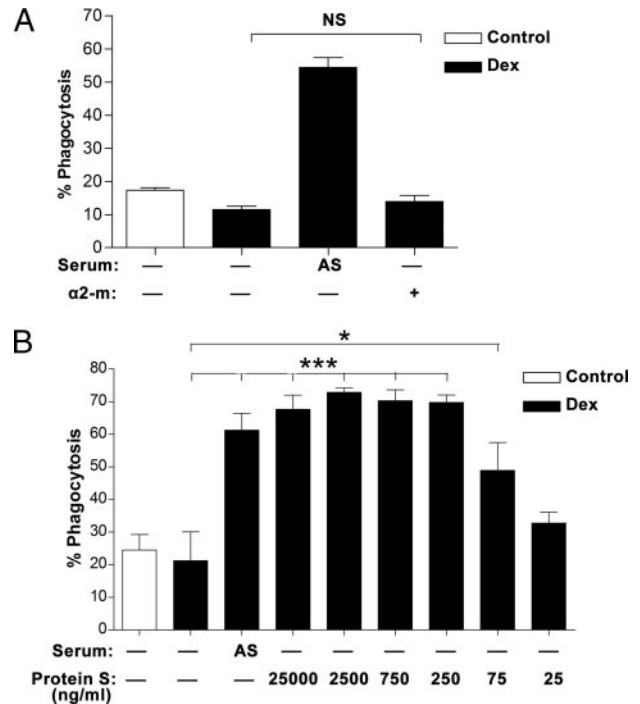
noblotting (Fig. 5B). In contrast to a mock Ab depletion of the 0.2 M NaCl eluate, protein S-depleted 0.2 M NaCl eluate failed to confer augmentation of phagocytosis by Dex-MDMϕ (Fig. 5C). Together with data presented in Fig. 3C, these data suggested that protein S, possibly complexed with C4BP, was required to confer full phagocytic capacity of Dex-MDMϕ. Interestingly, addition of 250 ng/ml purified human protein S (equivalent to the concentration of protein S present in 1% serum) to protein S-depleted 0.2 M NaCl eluate from ion-exchange chromatography fully restored Dex-MDMϕ phagocytosis (Fig. 5C). These data raised the possibility that protein S acts as an opsonin, binding to the surface of apoptotic neutrophils to specifically promote clearance by Dex-MDMϕ.

Protein S binds to apoptotic neutrophils in a calcium-dependent manner to mediate their removal by Dex-MDMϕ

To confirm that protein S was able to opsonize apoptotic neutrophils, we preincubated serum-free apoptotic neutrophils with either the high molecular mass fraction from gel filtration chromatography or 2.5 μg/ml human protein S before washing in IMDM with or without the addition of 5 mM EDTA. Binding of protein S to neutrophils in a Ca^{2+}-dependent manner could be detected by flow cytometry using anti-protein S Ab together with CD16 staining to define apoptotic and nonapoptotic cells (29). The possibility that anti-protein S Abs were binding nonspecifically through FcR-mediated interactions was excluded by use of a rabbit Ig control (Fig. 6A) and by mAb blockade of FcγRIIa (data not shown). In the presence of Ca^{2+}, protein S binds to apoptotic (CD16 low-expressing) and also nonapoptotic (CD16 high-expressing) cells, but the
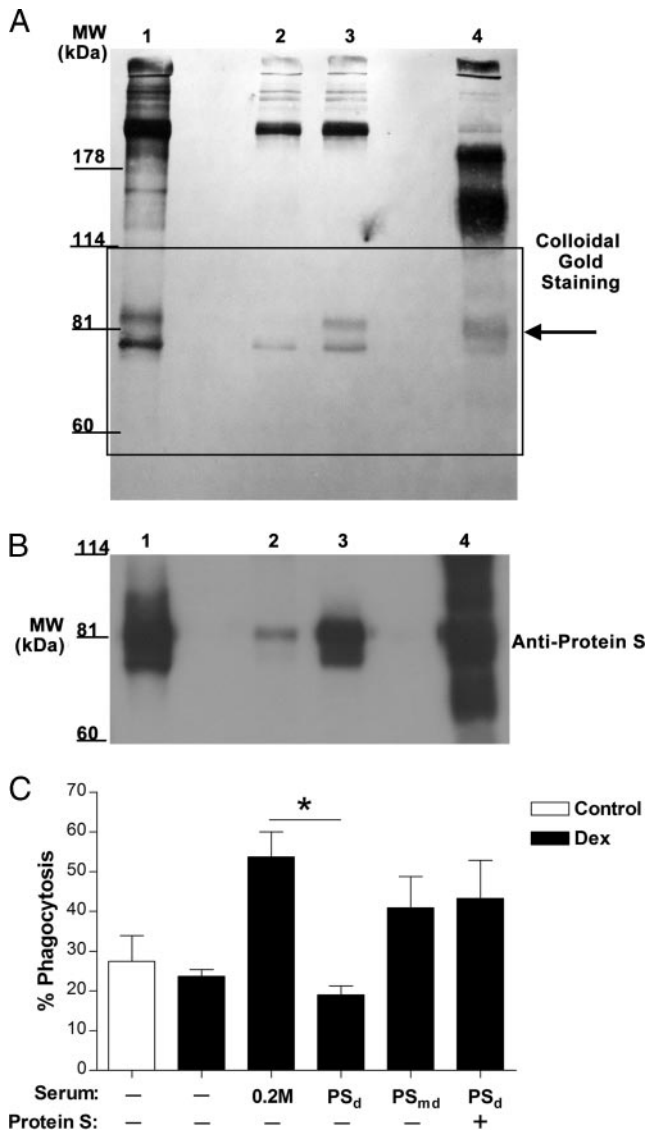
FIGURE 5. Protein S depletion from a 0.2 M NaCl elution fraction from Q-Sepharose abolished augmentation of phagocytosis by Dex-MDMφ. Immunodepletion of protein S was achieved by three rounds of depletion using a polyclonal rabbit anti-protein S Ab and agarose-coupled goat anti-rabbit IgG, as detailed in *Materials and Methods*. A mock depletion was performed using agarose-coupled goat anti-rabbit IgG alone. Samples were separated by SDS-PAGE on a 9% gel under nonreducing conditions and then transferred to nitrocellulose. *A*, The presence of protein S (∼85 kDa) in the 0.2 M NaCl eluate from anion-exchange chromatography and in depleted fractions was confirmed by colloidal gold labeling of transferred protein (*lane 1*, 0.2 M NaCl eluate; *lane 2*, protein S-depleted 0.2 M NaCl eluate; *lane 3*, mock depletion of 0.2 M eluate; *lane 4*, anti-protein S-immunodepleted material, to confirm the presence of protein S). *B*, Specific depletion of protein S in the samples shown in *A* was confirmed by immunoblotting, as described in *Materials and Methods*. In view of the presence of Ab in the immunodepleted sample (*lane 2*), only the outlined section of the membrane in *A* is shown. *C*, The effects of protein S depletion upon phagocytosis of apoptotic neutrophils by Dex-MDMφ (■) were assessed in a 30-min assay. Phagocytosis of apoptotic neutrophils by untreated MDMφ (□) is shown for comparison. In contrast to a mock depletion of protein S (PS$_{md}$), protein S depletion from a 0.2 M NaCl fraction (PS$_d$) abolished augmentation of phagocytosis by Dex-MDMφ, an effect that was restored by addition of 250 ng/ml human protein S (equivalent to level in 1% autologous serum). Data are mean ± SEM; $n = 3$. ∗, $p < 0.05$ compared with Dex-MDMφ in the presence of 0.2 M NaCl eluate.
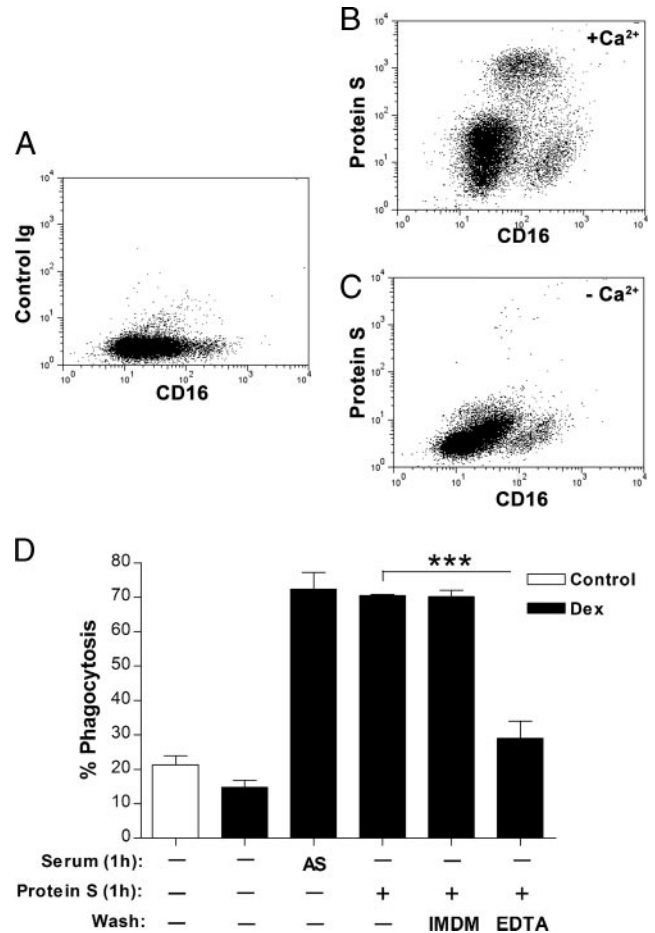

FIGURE 6. Protein S binds to neutrophils in a calcium-dependent manner and confers augmentation of phagocytosis by Dex-MDMφ. Neutrophils cultured for 20 h in serum-free conditions were preincubated with 1% autologous serum for 30 min in the presence of 1.5 mM CaCl$_2$. During subsequent steps, incubations were performed in TBS in either the presence or absence of 1.5 mM CaCl$_2$ throughout. Neutrophils were washed before labeling with either *A*, rabbit Igs (as control), or *B* and *C*, rabbit anti-human protein S for 30 min. Neutrophils were then washed twice, labeled with PE-conjugated CD16 mAb for 20 min, and washed before flow cytometric analysis. Levels of CD16 expression can be used to define apoptotic (low), secondarily necrotic (intermediate), and nonapoptotic (high) neutrophils. Representative histograms for binding in either the presence (*A* and *B*) or absence (*C*) of 1.5 mM CaCl$_2$ are shown. *D*, Calcium-dependent effects of protein S upon phagocytosis of neutrophils cultured for 20 h in serum-free conditions. Preincubation of neutrophils with either 10% autologous serum or 250 ng/ml purified protein S for 1 h, followed by washing in either the presence (IMDM) or absence (5 mM EDTA) of divalent cations before assessment of phagocytosis of apoptotic neutrophils in a 30-min assay by untreated MDMφ (□) and Dex-MDMφ (■). Augmentation of phagocytosis of apoptotic neutrophils by Dex-MDMφ following protein S preincubation was lost when neutrophils were washed in EDTA-containing IMDM before assessment of phagocytosis. Data are mean ± SEM; $n = 3$. ∗∗∗, $p < 0.001$ compared with Dex-MDMφ in the presence of protein S.

level of binding to apoptotic cells was 2.3-fold higher relative to that observed for nonapoptotic cells (Fig. 6*B*). In contrast, protein S binds poorly to cells in the absence of divalent cations (Fig. 6*C*). Interestingly, high levels of protein S binding to secondarily necrotic cells was observed (CD16 intermediate cells; Fig 6*A*), as demonstrated for many other opsonins, including C-reactive protein and C1q (23, 34). Apoptotic neutrophils bind protein S when washed in divalent cation-containing medium, but not when
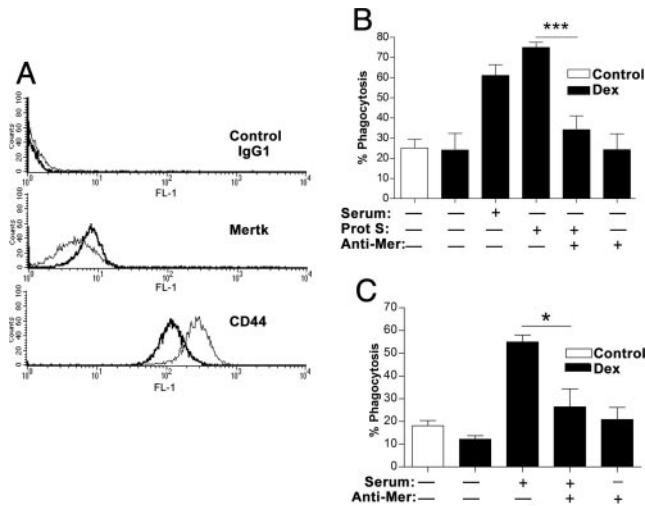
FIGURE 7. Protein S-enhanced phagocytosis by Dex-MDMφ is dependent on Mertk. *A,* Surface expression of Mer on MDMφ was assessed by indirect immunofluorescence together with flow cytometry. Representative overlay histograms show expression of Mertk and CD44 for untreated MDMφ (dotted lines) and Dex-MDMφ (solid lines) compared with binding of control IgG1 mAb. Expression of Mertk (as determined by mean fluorescence intensity) was increased 1.6-fold in five separate comparisons that were made. In contrast, CD44 expression was down-regulated on the surface of Dex-MDMφ, as previously reported (7). Preincubation of Dex-MDMφ (■) with 10 μg/ml anti-Mer Ab for 10 min inhibited subsequent phagocytosis of apoptotic neutrophils in the presence of either 2.5 μg/ml protein S (*B*) or 10% autologous serum (*C*). Pretreatment with anti-Mer alone had no effect on phagocytosis by Dex-MDMφ in the absence of protein S or serum. Data are mean ± SEM; $n = 3$. ***, $p < 0.001$, and *, $p < 0.05$.

washed in EDTA-containing medium, consistent with a calcium-dependent opsonization event (Fig. 6D). However, we also observed low levels of protein S binding to nonapoptotic neutrophils in the presence of divalent cations, suggesting that the prophagocytic effect of protein S on uptake of apoptotic neutrophils by Dex-MDMφ may require additional cell surface signals.

We also tested the effects of preincubation of neutrophils with or without protein S and then anti-protein S Ab before assessment of phagocytosis. In two experiments that were performed, the results for glucocorticoid-treated macrophages were as follows: no serum (18%), 1% serum (56%), and 1% serum plus anti-protein S (68%). One possibility is that binding of anti-protein S Ab to neutrophils (as shown in Fig. 6, *A–C*) may lead to their opsonization with IgG leading to phagocytosis by FcγR-mediated pathways. In the absence of commercially available Fab′ preparations of anti-protein S Ab to test this possibility directly, we used function-blocking mAb, IV.3, to block the interaction of IgG bound to the neutrophil surface with macrophage FcγRII (CD32). Treatment with IV.3 did not influence phagocytosis (65% phagocytosis for Dex MDMφ with 1% serum plus anti-protein S; 62% phagocytosis for DexMDMφ with 1% serum plus anti-protein S in the presence of IV.3; $n = 2$). These data may indicate either that the polyclonal Ab to protein S used in this study does not neutralize the prophagocytic activity or that multiple FcγRs (FcγRIII and/or FcγRI) expressed by MDMφ mediate the uptake of anti-protein S-opsonized neutrophils.

Protein S-enhanced phagocytosis by Dex-MDMφ is dependent on Mertk

Surface expression of Mertk, a potential receptor for protein S (35), was increased (1.6-fold) on Dex-MDMφ compared with untreated MDMφ (Fig. 7A), consistent with previous reports using

oligonucleotide arrays (8). The observed up-regulation of Mertk expression was not due to a nonspecific increase in receptor expression because CD44 was decreased on the surface of Dex-MDMφ relative to untreated MDMφ (Fig. 7A). To assess the contribution of Mertk to protein S-dependent phagocytosis, Dex-MDMφ were pretreated with an anti-human Mer Ab for 10 min before phagocytosis. Although anti-Mer had no effect on phagocytosis in the absence of protein S, anti-Mer significantly inhibited phagocytosis by Dex-MDMφ in the presence of 2.5 μg/ml protein S (Fig. 7B). Similar experiments were undertaken to determine whether Abs to protein S would exert similar inhibitory effects on phagocytosis. However, pretreatment of neutrophils with anti-protein S resulted in an augmentation of macrophage phagocytosis, possibly through an opsonization event (see Fig. 6) leading to FcγR-mediated phagocytosis. In contrast, blockade of Mer also significantly inhibited phagocytosis in the presence of 10% autologous serum, implying that the Mertk pathway is critical for glucocorticoid augmentation of phagocytosis of apoptotic neutrophils (Fig. 7C). We also examined the effects of short-term treatment of MDMφ with Dex upon the protein S dependency of phagocytosis of apoptotic neutrophils. MDMφ that had been cultured in the absence of Dex for 96 h were then treated for 24 h with Dex. Compared with untreated MDMφ (18 ± 5% phagocytosis in the absence of protein S), 96- to 120-h Dex-treated MDMφ had slightly higher basal levels of phagocytosis of apoptotic cells in the absence of protein S (25 ± 6%), but exhibited increased phagocytosis in the presence of protein S (60 ± 8%).

Discussion

In this study, we have examined the mechanisms that underlie the requirement for serum in augmentation of human macrophage phagocytosis of apoptotic neutrophils following treatment with glucocorticoids. We demonstrated that protein S opsonizes early apoptotic neutrophils (induced by treatment with roscovitine) to promote their internalization by Dex-MDMφ, and that the presence of cells that had undergone secondary necrosis was not necessary. This is an important observation because a number of serum opsonins have been reported to bind to late apoptotic or secondary necrotic neutrophils, including C1q, and the pentraxins, C-reactive protein and pentraxin-3 (18, 23, 34). Restoration of phagocytic capacity of Dex-MDMφ by a high molecular mass serum fraction raised the possibility of a requirement for a C4BP-protein S complex, which has been reported to inhibit phagocytosis of apoptotic lymphocyte cell lines (36). Our data showing the presence of protein S in the high molecular mass fraction would imply that the C4BP-protein S complex can augment phagocytosis under some circumstances. Because protein S binding can be demonstrated following incubation of neutrophils cultured in the absence of serum with either the high molecular mass fraction from gel filtration or purified protein S, one possibility is that under certain conditions, protein S can dissociate from C4BP and subsequently oligomerize at the apoptotic neutrophil surface (35). Our data clearly demonstrate that protein S alone is able to confer the augmentation of phagocytosis of apoptotic neutrophils that we observe.

The importance of complement proteins in apoptotic cell opsonization has been highlighted in studies of complement deficiency. In C1q deficiency, impaired clearance of apoptotic cells is thought to contribute to the development of an systemic lupus erythematosus-like autoimmune disease (27). For Dex-MDMφ, C1q did not restore levels of phagocytosis to those observed in the presence of serum even when C1q-binding proteins such as pentraxin-3 or fibronectin (37, 38) were present. Moreover, C1q-deficient mouse serum was able to confer phagocytic activity, demonstrating that

C1q was not required for efficient phagocytosis of apoptotic neutrophils by Dex-MDMφ. We were also unable to demonstrate a role for opsonization of apoptotic neutrophils with C3bi for removal through CR3 and CR4, as reported by Elkon and colleagues (11). Furthermore, the data presented in this study argue against a role for IgG, pentraxin-3, fibronectin, annexin I, platelet-derived factors, and immune complexes in phagocytosis of apoptotic neutrophils by Dex-MDMφ.

We believe that this is the first report demonstrating a switch in the molecular mechanism used by human MDMφ for apoptotic cell clearance. Our observations are clearly different from the induction of phosphatidylserine-dependent recognition of apoptotic murine thymocytes by bone marrow-derived murine macrophages treated with β1,3 glucan reported by Fadok et al. (39). Treatment with β1,3 glucan did not increase phagocytic potential when compared with untreated bone marrow-derived macrophages, but did alter the molecular mechanism used. In contrast, our findings demonstrate that phagocytosis of apoptotic cells by Dex-MDMφ is profoundly augmented by glucocorticoids, promoting a critical switch from a protein S-independent to a protein S-dependent recognition pathway.

The tissue microenvironment has the potential to influence the mechanisms involved in apoptotic cell removal and thus apoptotic cell clearance capacity. The cytokine and matrix composition will determine the differentiation status of phagocyte populations during progression of an inflammatory response. Interestingly, protein S-dependent recognition of an apoptotic B cell line was previously characterized in MDMφ generated by differentiation in M-CSF (35), which promotes the development of M2 macrophages that have anti-inflammatory phenotype properties and respond to TLR stimulation by producing IL-10 (40). We find that when MDMφ were cultured in the presence of autologous serum, apoptotic cell recognition pathways that are used are predominantly protein S dependent-independent (as shown in Fig. 1). In contrast with dex-treated MDMφ, these MDMφ have a more proinflammatory phenotype and release IL-12 in response to TLR stimulation, suggesting that protein S-dependent recognition pathways may be restricted to macrophage phenotypes associated with resolution of inflammation.

The production and release of potential opsonins (complement components, pentraxins, annexins, protein S, etc.) are also regulated during inflammation. A number of reports indicate that inflammation and the coagulation cascade are closely regulated, particularly during the acute-phase response. Protein S is produced in the liver and by endothelial cells (41). Production of both protein S and C4BP in the liver appears to be controlled by inflammatory mediators, including IL-6 (42, 43). Levels of protein S are reduced in patients with ischemic stroke (44) and in patients with sepsis (45), possibly via the effects of TNF on endothelial cells (46). In contrast, glucocorticoids have been reported to elevate levels of protein S (47). Based upon data presented in this work, we propose that a major effect of glucocorticoids on macrophage differentiation is the induction of the capacity to recognize a distinct set of molecular cues that are presented on the apoptotic cell surface. Apoptotic cells display a complex surface molecular signature as a consequence of cell death with altered expression of receptors together with binding (or opsonization) of a number of different proteins. One implication of our observation is that the surface molecular signature of an apoptotic cell may be interpreted differently by different phagocyte populations.

We have also examined the effects of treatment of differentiated MDMφ with Dex for 24 h (6) upon acquisition of the capacity for protein S-dependent phagocytosis of apoptotic cells. Our data suggest that augmentation of phagocytosis observed following short-er-term treatment is also associated with use of a protein S-dependent pathway for recognition of apoptotic cells. Because both untreated MDMφ and Dex-MDMφ populations examined in this study express Mertk, the reason that Dex-MDMφ are enabled to use a protein S-dependent clearance pathway is not clear. One possibility is that the observed up-regulation of Mertk expression on the surface of Dex-MDMφ may be sufficient to confer phagocytic potential. Alternatively, Mertk may interact with other receptors on the cell membrane following glucocorticoid treatment. Ligand-activated Mertk forms dimers in the membrane, resulting in Mertk autophosphorylation and activation (48), and may heterodimerize with other Tyro3/Axl/Mer family receptors or cooperate with other receptors involved in the phagocytic process, such as scavenger receptor A (49) or $\alpha_v\beta_5$ (50). Induction of cooperative action of receptors may allow regulation of phagocytosis of apoptotic cells in response to different environmental cues encountered during the inflammatory response.

Alternatively, glucocorticoids may influence engagement of downstream signaling pathways critical for Mertk-dependent phagocytosis. We have previously demonstrated that glucocorticoid-treated MDMφ exhibited reduced phosphorylation and localization of paxillin and pyk2 to podosome-like adhesion structures, together with increased Rac activity (7). Interestingly, Rac guanine nucleotide exchange factor Vav1 is activated downstream of Mertk (51). Mertk has also been reported to induce FAK phosphorylation and recruitment to $\alpha_v\beta_5$ and formation of p130Cas/CrkII/Dock180 complex (50, 52). One possibility is that phosphorylation of Mertk at Tyr[867] in the absence of assembly of adhesion structures promotes MDMφ phagocytic activity (52).

Induced expression of Mertk and protein S by glucocorticoids may promote acquisition of a negative-feedback pathway to both switch off proinflammatory cytokine production (53) and enhance phagocytic capacity for apoptotic cells. The efficacy of glucocorticoids in treatment of autoimmune diseases such as systemic lupus erythematosus that are characterized by impairment of apoptotic cell clearance may be due, in part, to engagement of these pro-resolution mechanisms. Manipulation of the Mertk pathway may represent a novel approach to engage aspects of glucocorticoid action that favor resolution of inflammation without promoting deleterious side effects.

Disclosures

The authors have no financial conflict of interest.

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