

Glucocorticoid Modulation of Macrophage Function

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DECLARATION

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

Sarah Jane Heasman, 2005

ABSTRACT

Macrophages have a central role in immune responses. They are important effector cells, binding and phagocytosing invading microorganisms, and producing reactive oxygen species and proteases involved in tissue remodelling. In addition, they exert immunoregulatory activity via presentation of antigen to T cells and through production of cytokines. Macrophage phagocytic clearance of apoptotic neutrophils is a process that is central to tissue homeostasis and for the resolution of inflammation. Failure to remove apoptotic cells results in necrotic cell death and the release of histotoxic intracellular contents, with the potential for exacerbating inflammation thus contributing to the pathogenesis of inflammatory and autoimmune diseases.

In this thesis, I have examined the effects of glucocorticoid-treatment of peripheral blood monocytes which has previously been demonstrated to markedly augment phagocytic capacity for apoptotic cells, an effect which may contribute to anti-inflammatory actions of glucocorticoids. Within the inflammatory site, the cytokine environment governs the differentiation and function of infiltrating leukocytes. I have investigated the effects of combinatorial treatment of monocytes with the principal Th1 and Th2 cytokines, IFN- γ and IL-4 respectively. I have demonstrated that whilst glucocorticoids exert a dominant effect over those of IFN- γ in terms of cell morphology and cell surface receptor expression, glucocorticoid-augmented phagocytosis of apoptotic neutrophils is inhibited by IFN- γ . These findings suggest that the effectiveness of glucocorticoids in promoting a highly phagocytic macrophage phenotype is crucially dependent on the cytokine milieu at inflammatory sites.

Cellular migration is an important determinant for the initiation of inflammatory responses and for the resolution phase, where macrophages migrate to draining lymph nodes. My results provide evidence for an alteration in the adhesion and migration of macrophages following glucocorticoid treatment. I have demonstrated changes in cytoskeletal organisation and assembly/engagement of Rho family

GTPase signalling pathways. These changes may influence macrophage migration patterns that are important for the progression of inflammatory responses.

Finally, I present novel studies which separate binding and the subsequent internalisation of apoptotic cells for the first time. Critically, I have demonstrated that glucocorticoid-treated macrophages have an enhanced ability to bind apoptotic neutrophils in a divalent cation independent manner, when compared to untreated macrophages. In terms of phagocytic mechanism, I also show that internalisation requires the presence of divalent cations and can be attenuated by blocking phosphatidylserine-mediated uptake.

Together, the studies presented in this thesis suggest that glucocorticoids exert profound effects upon macrophage cytoskeletal organisation that influences both phagocytosis and migration and may also cause a switch in apoptotic cell recognition mechanisms.

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LIST OF ABBREVIATIONS

11 β -HSD	11 β -hydroxysteroid dehydrogenase
ABC1	ATP binding cassette transporter
ACTH	Adrenocorticotrophic hormone
AP-1	Activation protein-1
AS	Autologous serum
BMDM Φ	Bone marrow derived macrophages
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
Dex	dexamethasone
DMEM	Dulbecco's minimal essential medium
DNA	deoxyribonucleic acid
dNTP	generic term for the 4 deoxyribonucleotide triphosphates: dATP, dCTP, cGTP, cTTP
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
ERK	extracellular regulated kinase
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
Fc	fragment crystallisable
FITC	fluorescein isothiocyanate
FMLP	N-formyl-methionyl-leucyl-phenylalanine
FS	forward scatter
GCs	glucocorticoids
GEF	guanine nucleotide exchange factor
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
HBSS	Hanks' balanced saline solution
(H)MDM Φ	(human) monocyte-derived macrophages
HPA	hypothalamus-pituitary axis

I κ B- α	inhibitor of nuclear factor κ B
ICAM-1	intercellular adhesion molecule-1
IFN- γ	interferon-gamma
IgG	immunoglobulin
IL-4	Interleukin-4
IP	Interperitoneal
ITAM	Immunoreceptor tyrosine-based activation motif
LPS	Lipopolysacharide
MHC	Major Histocompatibility complex
MLCK	Myosin Light Chain Kinase
MMP	Matrix Metalloproteinases
mRNA	messenger RNA
NF κ B	Nuclear factor κ B
NO	Nitric Oxide
p160ROCK	Rho-associated coiled-coil forming protein kinase
PAK	p21 activated kinase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PI 3-kinase	Phosphatidylinositol 3 kinase
PI	Propidium Iodide
PKC	Protein Kinase C
PMA	Phorbol-12-Myristate-13-Acetate
PMN	Polymorphonuclear leukocytes
PRP	Platelet rich plasma
PS	Phosphatidylserine
PSR	Phosphatidylserine Receptor
RGDS	Arg-Gly-Asp-Ser peptide
RNA	Ribonucleic acid
Rpm	revolutions per minute
RT	Room temperature
RT-PCR	Reverse Transcriptase PCR

SEM	Scanning Electron Microscopy
SRA	Scavenger Receptor class A
SS	Side Scatter
TBS	Tris-buffered Saline
TNF- α	Tumour Necrosis Factor- alpha
WASp	Wiskott-Aldrich Syndrome Protein

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CHAPTER 1: INTRODUCTION

Inflammation

Inflammation is the response mounted by the innate immune system in reaction to infection, tissue injury or toxins. This normally self-resolving process, results in the destruction of the invading micro-organism and healing of the damaged tissue.

Within tissues, bacteria are recognised by tissue resident phagocytes via pattern recognition receptors such as the mannose receptor and CD14, which bind to conserved motifs of bacterial products (pathogen-associated molecular patterns or PAMPs) (Janeway, 1989) or following opsonisation by immunoglobulin (Anderson et al., 1990; Ravetch and Bolland, 2001) or complement components (Ehlers, 2000; Plow and Zhang, 1997). The subsequent secretion of chemokines and cytokines by leukocytes and tissue resident cells such as fibroblasts and smooth muscle cells (Garcia-Ramallo et al., 2002), instigates the inflammatory response. The adherence of the inflammatory cells to the blood vessel endothelial cells and ensuing migration into the tissues is prompted by exposure to the secreted cytokines (Garcia-Ramallo et al., 2002).

Neutrophils are the first cells recruited in large numbers into inflamed tissue (Doherty et al., 1988; Tsai et al., 2000), where they identify, bind and phagocytose the bacteria either via pattern recognition receptors, or following the triggering of the complement cascade and deposition of C3b or C3dg on the pathogen surface (Underhill and Ozinsky, 2002). Neutrophil synthesis of a range of cytotoxic compounds products occurs via the enzyme NADPH oxidase and includes the reactive oxygen species hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and nitric oxide (NO), which are released into pathogen-containing phagocytic vacuoles (Karlsson and Dahlgren, 2002; Lofgren et al., 1999; Roos et al., 2003). NADPH oxidase-induced Ca^{2+} release results in the opening of BK K^+ channels and the generation of a hypertonic, alkaline and K^+ rich phagosome environment within

the neutrophil, activating proteases necessary for the killing of enclosed bacteria (Ahluwalia et al., 2004). Phagocytosis of bacteria by neutrophils then accelerates the onset of apoptosis (Kobayashi et al., 2003) which is accompanied by a down-regulation in pro-inflammatory cytokine production by the neutrophil and the up-regulation of pathways that synthesise antioxidant products in favour of toxic metabolites (Kobayashi et al., 2003).

A second wave of leukocyte recruitment follows with the infiltration of monocytes from the blood vessels. Under the influence of the tissue cytokine environment, monocytes differentiate into macrophages and can perform key roles in the progression and ultimately in the resolution of inflammatory responses. In the presence of pro-inflammatory cytokines and bacterial products such as IFN- γ and LPS, monocytes differentiate into 'classically activated' macrophages, equipped for the phagocytosis and killing of pathogen. Like neutrophils, macrophages are capable of engulfing and subsequently killing pathogen and can therefore aid the clearance of pathogen. Alternatively, differentiation of monocytes in the presence of anti-inflammatory cytokines and mediators such as IL-4 and glucocorticoids results in an 'alternatively activated macrophage' with reduced capacity for pathogen uptake but increased capability for wound healing and inflammatory resolution (Goerdt and Orfanos, 1999).

The Resolution of Inflammation

The induction of apoptosis in the large numbers of recruited neutrophils must be followed by their efficient removal for the successful resolution of inflammation (See Figure 1.1). A second important function of monocyte-derived macrophages is therefore the recognition and phagocytosis of the apoptotic cells via receptors binding 'eat me' signals displayed by the apoptotic cell. Phagocytosis of the apoptotic cell not only down-regulates the secretion of pro-inflammatory cytokines by the macrophage, but also increases the release of anti-inflammatory cytokines (Fadok et al., 1998a; Meagher et al., 1992). Because cytokines define the behaviour of the infiltrating cells such as macrophages, changes in the cytokine profile can act

as a switch in the transition from the pro-inflammatory responses required for elimination of the pathogen, to the anti-inflammatory responses required for the resolution phase. The interaction between monocytes and apoptotic neutrophils in the presence of the gram negative bacterial product LPS, has been shown to deactivate the monocytes, turning off secretion of the pivotal pro-inflammatory cytokine TNF- α in favour of the anti-inflammatory cytokine IL-10 (Byrne and Reen, 2002).

A failure in either the induction of apoptosis, or the clearance of the apoptotic neutrophils, will result in neutrophil necrosis accompanied by the release of intracellular components that have the potential to generate autoantibody production and potentially toxic contents into the surrounding tissue, exacerbating the inflammatory process (Haslett, 1997). Accordingly, failure in the clearance of apoptotic cells would be predicted to be associated with inflammatory disease and autoimmunity (Beutler, 2001; Haslett, 1997; Haslett, 1999; Savill et al., 1989b). The pro-inflammatory consequences of disrupting induction of apoptosis have been exploited by the disease causing pathogens *Anaplasma phagocytophilum* and *Staphylococcus aureus*, which delay apoptosis or induce neutrophil necrosis ((Kobayashi et al., 2003) and references therein). Whilst the issue of whether phagocytosis of necrotic cells by macrophages does in fact cause the release of pro-inflammatory cytokines is contentious (Brouckaert et al., 2004; Fadok et al., 2001), lysis of the neutrophil and release of pro-inflammatory contents, coupled with the lengthened time required for the phagocytosis of a necrotic cell could be responsible for an increase in the inflammatory response (Brouckaert et al., 2004). In addition, uptake of apoptotic cells fails to stimulate antigen presentation by macrophages, whilst uptake of necrotic cells is associated with up-regulation of CD40 and co-stimulation of T cells (Barker et al., 2002).

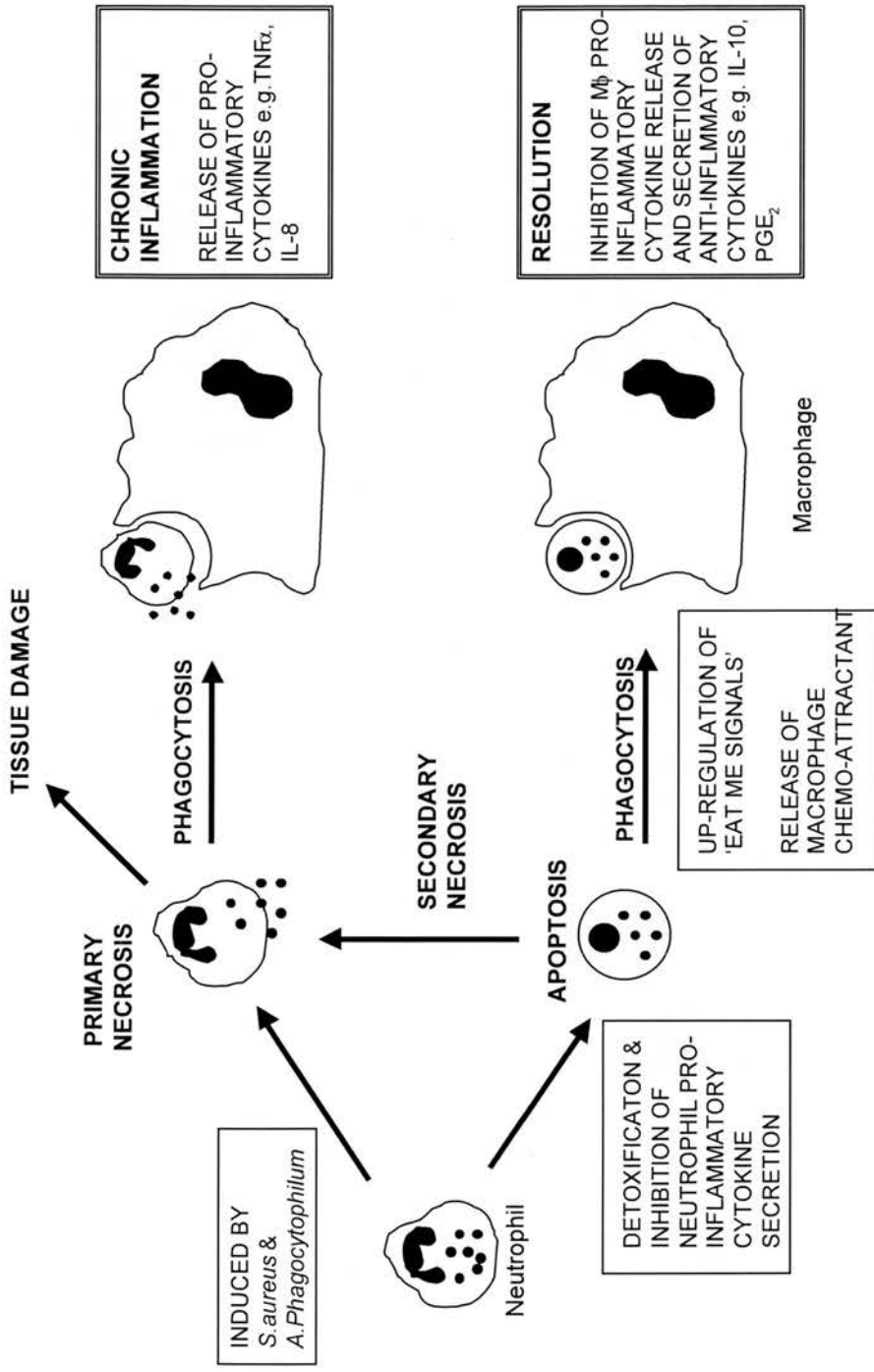


Figure 1.1 Diagrammatic representation of the resolution of inflammation. Phagocytosis of pathogen induces apoptosis in the neutrophil, resulting in the safe disposal of the apoptotic cell by macrophage phagocytosis. A failure in this process results in a necrotic cell, which releases toxic contents into the surrounding tissue, exacerbating inflammation.

Apoptosis

Apoptosis is a highly conserved form of programmed cell death, which results in the safe packaging of the cell for disposal by phagocytic cells. By contrast to necrotic cell death, apoptotic cells retain an intact plasma membrane with the result of the containment of potentially damaging cytosolic enzymes (Searle et al., 1982). Apoptosis induces a co-ordinated sequence of biochemical events which serves to destroy the nuclear and cytoplasmic mechanisms which control cellular responses, resulting in an unresponsive cell.

Caspases are a highly conserved family of proteinases, which play a significant role in the majority of cell apoptosis. They are retained in the cytoplasm in a non-active form (pro-caspases), and are activated by cleavage of the pro-domain from the protein, which is achieved by two main pathways. The first involves the formation of a death-inducing signalling complex (DISC) at the intracellular tail of TNF family receptors. The recruitment of pro-caspases to the DISC triggers caspase activation. The second mechanism follows the initiation of mitochondrial membrane permeabilisation (MMP), which triggers a surge of pro-apoptotic effects, including the release of stores of caspases, caspase activators and proteins including cytochrome-c and apoptosis-inducing factor (Apaf-1). Cytochrome-c release triggers the assembly of caspase-3 and caspase-9 into an Apaf-1 containing activation complex, termed an 'apoptosome', which activates caspase-9 (Kroemer and Reed, 2000; Reed, 2001).

Activated 'initiator' caspases, such as caspase 8 and 9, then cleave target proteins at aspartate (Asp) residues. These target proteins include other members of the caspase family, which are in turn activated following cleavage of their pro-domain, initiating an amplifying cascade of caspase activation. 'Effector' caspases cleave structural proteins such as lamins and focal adhesion kinase (FAK) that disband the cytoskeleton of the cell and cause loss of cell adhesions. Further they breakdown homeostatic proteins required for protein and nucleic acid synthesis and signalling

and cell repair, irreversibly committing the cell to the apoptotic pathway (Wolf and Green, 1999).

Regardless of the initiating stimulus, apoptosis results in a characteristic morphology (Ellis and Horvitz, 1986; Kerr et al., 1972; Martin et al., 1994), which is distinguishable from that of necrosis. Cytoplasmic shrinkage and membrane blebbing are accompanied by nuclear shrinkage, chromatin condensation and DNA fragmentation (Hebert et al., 1996). Characterised changes associated with apoptosis in neutrophils includes the loss of functions including phagocytosis, respiratory burst, degranulation and following down-regulation of FMLP receptor on the cell surface, ability to migrate and shape change (Whyte et al., 1993). Loss of surface receptors such as Fc γ RIII (Dransfield et al., 1994) and adhesion associated molecules such as L-selectin (Dransfield et al., 1995) have also been reported, and together may 'functionally isolate' the neutrophil prior to phagocytosis (Hart et al., 2000; Haslett, 1997).

Failure of cells to undergo apoptosis may contribute to oncogenesis, whilst inappropriate levels of apoptosis can lead to a range of inflammatory or degenerative diseases such as Alzheimers, and thus agents which allow manipulation of apoptosis have been an area of active research (Reed, 2001; Ward et al., 1999). A number of agents have been described which alter granulocyte apoptosis, including Fas-Fas ligand (Matsumoto et al., 1995), glucocorticoids (Meagher et al., 1996), TNF- α (Murray et al., 1997), NF- κ B inhibitors and cytosolic calcium concentration (Rossi et al., 1995) (for review see (Ward et al., 1999)), although many have been shown to have disparate effects on eosinophil and neutrophil granulocytes.

Macrophage Phagocytosis of Apoptotic Cells

Receptor mediated phagocytosis of apoptotic neutrophils

It has been proposed that the expression of ligands, so called 'eat me' signals, on the surface of the apoptotic cells, signals for their recognition and phagocytosis by macrophages, and to date many phagocytic receptors have been described for ligands on the apoptotic cell (see Figure 1.2). The list includes the $\alpha v\beta 3$ /CD36/thrombospondin complex (Savill et al., 1990), the phosphatidylserine receptor (Fadok et al., 2000), scavenger receptors such as SRA-1 (Platt et al., 1996) receptors for OxLDL (Chang et al., 1999), pattern recognition receptors such as CD14 (Devitt et al., 1998) and lectin receptors (Duvall et al., 1985). Many of the receptors involved in the recognition of apoptotic cells also have a dual role in the binding by antigen-presenting cells of conserved regions of pathogens (pattern recognition motifs). For example, CD14 is involved in LPS binding and also has a role as an apoptotic cell receptor. Studies examining the involvement of CD14 in apoptotic cell recognition demonstrated that, despite the fact that LPS and the apoptotic cell ligand bind to the same receptor, LPS binding resulted in the release of pro-inflammatory cytokines, whilst they were not detected following binding of apoptotic cells to macrophages via CD14. This implies that the ligand determines the response which follows from the macrophage (Devitt et al., 1998).

Despite the description of a large list of phagocytic receptors, very few partner ligands on the surface of the apoptotic cell have been discovered. Profiling the surface of different apoptotic cell types (Hart et al., 2000) (Morris et al., 1984) has identified very few surface changes by which the apoptotic cell can be distinguished from the viable cell. ICAM-3 (Moffatt et al., 1999) and phosphatidylserine (PS) (Fadok et al., 1992a) have been explicitly identified, although more subtle changes

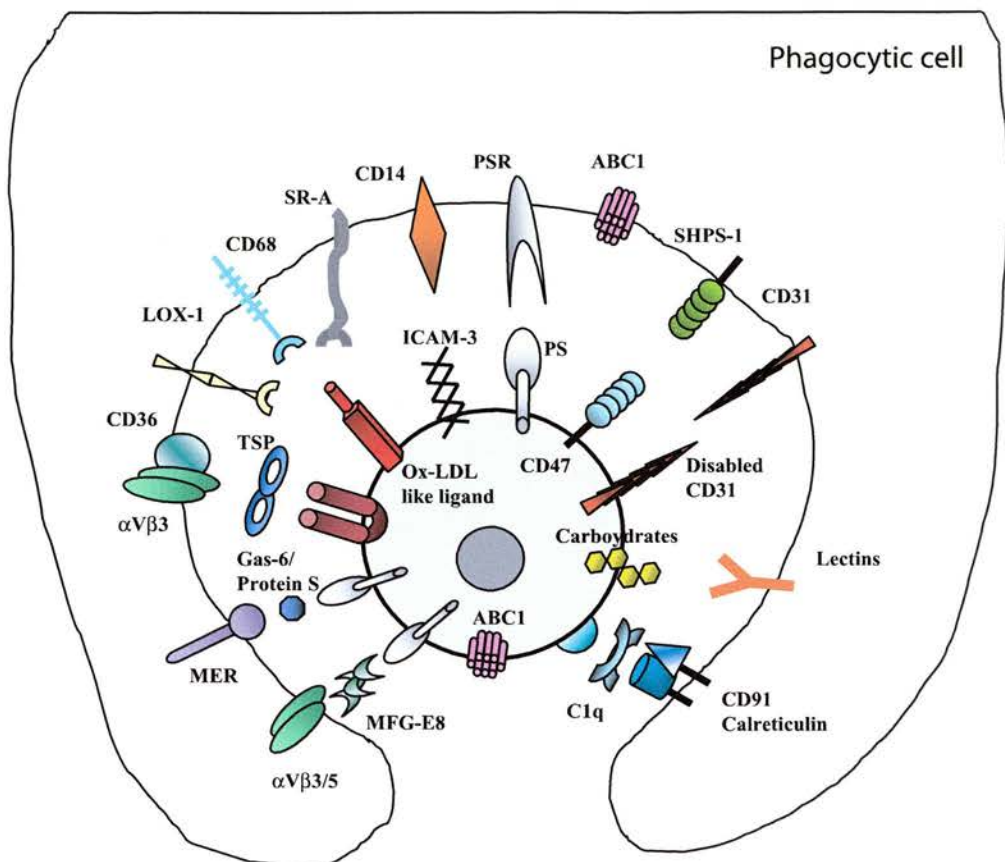


Figure 1.2 Phagocytic recognition of apoptotic cells

Adapted from Gregory et al. (2004)

Putative receptors, bridging molecules and ligands implicated in the recognition and phagocytosis of apoptotic cells (grey target cell in centre of figure). Clockwise the abbreviated molecules are: MFG-E8, milk fat globule epidermal growth factor-8; MER, myeloid epithelial reproductive tyrosine kinase; Gas-6, growth arrest specific gene-6; TSP, thrombospondin-1; Lox-1, oxidised low density lipoprotein receptor 1; SR-A, Scavenger receptor A; PS, phosphatidylserine; PSR, phosphatidylserine receptor; ABC, ATP-binding cassette transporter A1; SHPS-1, Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1; C1q, complement component.

such as alterations in surface carbohydrates have also been implicated (Duvall et al., 1985). In fact, many of the surface molecules profiled are down-regulated on the surface of the apoptotic cell, concurrent with the loss of plasma membrane surface area (Morris et al., 1984) and, coupled with macrophage engulfment of inert particles, this argues for a loss of signal model for phagocytosis of apoptotic cells.

Changes in surface charge density on the apoptotic cell following cleavage of surface proteins and desialylation have also been described (Morris et al., 1984) and provide an attractive explanation for the increased interaction between phagocyte and apoptotic cell, whereby repulsive encounters between identically charged cells may be overcome. Indeed, studies showing the inhibition of macrophage recognition of apoptotic neutrophils by cationic monosaccharides and amino acids occurred in a charge and pH dependent fashion (Savill et al., 1989a) suggesting recognition requires an anionic charge on the apoptotic neutrophil surface.

Exposure of PS on the outer cell surface is a feature common to all apoptotic cells (Fadok et al., 1992c; Martin et al., 1995). Healthy cells maintain the asymmetrical distribution of PS on the inner surface of the plasma membrane by active transport via the enzyme aminophospholipid translocase (Verhoven et al., 1995). PS surface expression is a characteristic of early apoptosis (Martin et al., 1995), and requires both the loss of aminophospholipid translocase activity and activation of the calcium dependent flip flop of phospholipids in the plasma membrane, (Bratton et al., 1997), aided by scrambalases, flippases (Zhou et al., 1997) and polyamines (Bratton et al., 1999). In addition, the ATP-binding cassette transporter 1 (ABC1), previously implicated in phagocytic uptake of apoptotic cells, (Luciani and Chimini, 1996) has been demonstrated to be involved in the transport of PS to the outer leaflet of the plasma membrane (Hamon et al., 2000). Specific recognition of PS by the recently cloned PS receptor (PSR) (Fadok et al., 2000) causes subsequent engulfment of the apoptotic cell (Fadok et al., 1992a), although recently published evidence demonstrating nuclear localisation signals in the 'PSR' contradict its suggested role as a phagocytic receptor (Cikala et al., 2004; Cui et al., 2004). The generation of a PSR^{-/-} mice by Bose and colleagues has further challenged the hypothesis that the

cloned PSR is an apoptotic cell receptor. The PSR^{-/-} mice showed severe defects in embryonic developmental and organ differentiation, with no observed defect in the clearance of apoptotic cells during embryonic development (Bose et al., 2004). Bridging molecules act to link the receptor on the phagocyte to ligands on the apoptotic cell, increasing the efficiency of phagocytosis. Many of these bridging molecules bind to PS, including MFG-E8 (Hanayama et al., 2002), β 2-glycoprotein I (Balasubramanian and Schroit, 1998), Protein S (Anderson et al., 2003), and Gas-6 (Ishimoto et al., 2000)(see Figure 1.2). MFG-E8 binds to α v β 3 on the phagocytic cell (Hanayama et al., 2002), whilst Gas-6 bridges PS to the receptor tyrosine kinase, Mer (Scott et al., 2001). Annexin I is expressed on the surface of apoptotic Jurkat cells following caspase activation, and co-localises with PS, potentially increasing phagocytic uptake by the PSR (Arur et al., 2003).

One of the major receptor mechanisms in human monocyte-derived macrophage (MDM Φ) recognition of apoptotic neutrophils is the divalent cation-dependent α v β 3 integrin 'vitronectin receptor'/CD36/thrombospondin complex which binds an as yet undiscovered ligand on apoptotic cells (Savill et al., 1990). The requirement of the vitronectin receptor and CD36 has been demonstrated using both blocking antibodies and the RGDS peptide. Subsequently, thrombospondin has been shown to bind to both the vitronectin receptor and CD36, acting as a bridging molecule between the macrophage and the apoptotic neutrophil (Savill et al., 1990).

Scavenger receptors have also been implicated in uptake of phagocytic cells. The class A macrophage scavenger receptor (SR-A) is a trimeric membrane glycoprotein, capable of binding a range of ligands including acetylated LDL and polyionic molecules. The phagocytosis of apoptotic thymocytes by mouse peritoneal macrophages was partially inhibited by blocking SR-A. This was demonstrated using an antibody against SR-A, using competitive ligands such as fucoidan, or using macrophages from SR-A^{-/-} mice (Platt et al., 1996). A second scavenger receptor, the OxLDL receptor, is also important in mouse peritoneal macrophage phagocytosis of apoptotic cells. Studies showing that OxLDL inhibited the binding of PS liposomes to mouse peritoneal macrophages, suggest that OxLDL and PS compete to bind this

receptor (Sambrano and Steinberg, 1995). In fact, many of the macrophage receptors which recognise OxLDL e.g. CD36, CD68 and LOX-1 also bind PS liposomes or apoptotic cells (discussed in (Chang et al., 1999)).

Inhibition studies using CD14 specific blocking antibodies 61D3 and MEM-18 demonstrated the involvement of the glycoprotein receptor CD14 in the interaction between human MDM Φ and apoptotic lymphocytes (Devitt et al., 1998). The ligand on the apoptotic cells which CD14 binds to is unknown. Following the demonstration that CD14 bound to phospholipids including PS, and that 61D3 inhibited the interaction between PS and macrophages (Pradhan et al., 1997), CD14 was investigated as a putative PS receptor. Results showed that PS binding to macrophages did not demonstrate specificity for CD14 and that PS-liposomes did not inhibit CD14-mediated phagocytosis (Devitt et al., 2003), suggesting that CD14 is not a PS receptor in human MDM Φ . In addition, ICAM-3 present on the apoptotic lymphocyte has been demonstrated to mediate an interaction with the phagocyte in an as yet undefined CD14-dependent manner (Moffatt et al., 1999).

The reported inhibition of mouse peritoneal macrophage binding of apoptotic thymocytes by N-acetyl glucosamine supports the presence of a lectin-like receptor on the macrophage, which recognises modified carbohydrates on the surface of the apoptotic cell (Duvall et al., 1985). Early experiments described an increased binding of apoptotic thymocytes compared with viable thymocytes at 4°C, to monolayers of peritoneal macrophages (Morris et al., 1984). Later work by Brown and colleagues demonstrated, under low shear at 37°C, a mechanism for the detachment of viable but not apoptotic leukocytes following binding to macrophages via a CD31 homophilic interaction, providing the first evidence for a 'detachment' signal from non-apoptotic cells (Brown et al., 2002).

Despite PS being universally displayed by apoptotic cells, different combinations of mechanisms are responsible for uptake of apoptotic cells by different macrophage populations. Unstimulated macrophages, such as human MDM Φ and murine bone marrow-derived macrophages (BMDM Φ) have been suggested to rely mainly on the 'vitronectin receptor'/CD36/thrombospondin mechanism, with minor roles for the

scavenger and lectin receptor mechanisms. However, activated macrophages, such as elicited peritoneal macrophages or β -glucan stimulated-BMDM Φ and -human MDM Φ , have been reported to utilise the PS-PSR mechanism in favour of the α v β 3/CD36/ thrombospondin receptor complex (Fadok et al., 1992b) (Fadok et al., 1998b). Pradhan and colleagues further showed that inhibition by sugars also fell into two groups; with glucosamine, galactosamine and arginine inhibiting non-activated macrophages whilst N-acetylglucosamine inhibited activated macrophages. Pradhan and colleagues propose from their data that two recognition complexes exist; PSR and α v β 3/CD36/thrombospondin on unactivated macrophages and a lectin-like receptor and PSR complex on activated macrophages (Pradhan et al., 1997).

As research progresses, commonalities are being found between receptors initially thought of as disparate, e.g. CD36 is associated with phagocytosis via the vitronectin receptor, PS recognition and OxLDL receptor. There are also data supporting the possibility of multiple receptors for single ligand, for example, PS binding to the OxLDL receptor, CD36 and the recently described PS receptor. Finally, most inhibition studies have failed to fully account for all macrophage phagocytosis, implying that further recognition systems remain to be discovered.

Opsonin-mediated uptake of apoptotic cells

Opsonins are serum constituents or locally secreted proteins that are able to bind pathogens, enhancing uptake by phagocytic cells. Complement systems are activated by one of three pathways; the classical pathway, the alternative pathway and the lectin pathway, which converge with the cleavage of C3 into C3a and C3b and deposition of C3bi on the surface of the pathogen. Phagocytes such as macrophages and neutrophils have receptors such as CR1, CR3 (CD11b/CD18) and CR4 (CD11c/CD18) for C3bi, allowing uptake of complement opsonised pathogen. Phagocytes also express receptors for immunoglobulin subclasses e.g. macrophages express three receptors for the Fc portion of IgG: Fc γ RI, II and III. Thus, the generation and binding of antibodies specific for bacterial antigens allow recognition of the bacteria via the Fc portion of the antibody. There is accumulating evidence

demonstrating a role for antibodies and complement in the uptake of apoptotic cells, illustrating a dual role for classical receptors of pathogens.

Complement

As discussed above, inhibition of the macrophage receptors for apoptotic cell 'eat me' signals, have failed to completely inhibit and therefore fully account for the recognition of apoptotic cells. It is notable that many studies have been carried out in serum-free conditions and a role for opsonisation by serum proteins was proposed following a three fold induction in binding and phagocytosis upon the addition of serum (Mevorach et al., 1998). Complement components Factor B, C1q and C3 present in the serum were shown to mediate the induction of phagocytosis (Mevorach et al., 1998). The well characterised PS exposure on the surface of the apoptotic cells permits C3bi binding and subsequent recognition by macrophage CR3 and CR4 (Mevorach et al., 1998).

Further evidence of the involvement of complement has been provided by the identification of C1q as a major genetic risk factor for the autoimmune disease systemic lupus erythematosus (SLE), characterised by the formation of auto-antibodies and immune complexes (Petry, 1998). C1q initiates the classical complement pathway by binding to IgG- or IgM-antigen complexes on the cell surface, and was shown to bind to keratinocyte and endothelial apoptotic cell blebs (Korb and Ahearn, 1997) (Navratil et al., 2001). In addition, C1q knockout mice present with a defect in clearance of apoptotic cells and develop an SLE-like phenotype (Botto et al., 1998), with further validation of a role for C1q in apoptotic cell uptake from *in vitro* studies. Calreticulin was identified as the receptor for C1q and mannan-binding lectin and C1q/MBL bound to the surface of apoptotic cells, are recognised by calreticulin in combination with CD91 (Ogden et al., 2001).

Antibodies

Recently, a novel role for antibodies has been proposed to mediate recognition of apoptotic cells bearing Fc γ RII. Hart and colleagues using an *in vitro* model have recently suggested that immune complexes bind to apoptotic neutrophils. Further studies revealed that immune complex opsonised cells could be recognised via an Fc-mediated mechanism (Hart et al., 2003).

Anti-phospholipid syndrome is characterised by deep vein thrombosis and the presence of autoantibodies, raised against apoptotic cell phospholipid-associated proteins such as β 2 glycoprotein I (β 2-GPI) (Balasubramanian et al., 1997). *In vitro* studies have shown that antibodies derived from patients with anti-phospholipid syndrome recognised β 2-GPI only when bound to apoptosis associated-phospholipids (Pittoni et al., 2000). Further studies have shown that β 2-GPI antibody opsonisation of apoptotic cells and PS vesicles increased engulfment by immature dendritic cells and macrophages respectively (Rovere et al., 1998) (Balasubramanian et al., 1997).

The uptake of apoptotic cells by Fc-mediated phagocytosis may be expected to be pro-inflammatory. However, in situations where large numbers of apoptotic cells require clearance, perhaps owing to an over-whelming infection, the fast and efficient process of Fc-mediated phagocytosis as an apoptotic clearance mechanism may outweigh the prospective pro-inflammatory cytokine release by preventing induction of necrosis in the apoptotic cells.

Many of the opsonins, such as Protein S (Anderson et al., 2003), β 2-glycoprotein I (Balasubramanian et al., 1997) and MFG-E8, which are secreted by activated macrophages and mediate apoptotic cell uptake via α v β 3 integrins (Hanayama et al., 2002), recognise and bind to PS, as discussed earlier. PS exposure is an early characteristic of apoptosis, suggesting opsonisation may stimulate uptake of early apoptotic cells. In contradiction to this, it has been suggested that complement binding to necrotic lymphocytes provides a mechanism for the removal of cells which have failed to be cleared by other means (Gaipl et al., 2001).

Intracellular Mechanisms Required for Cellular Engulfment

Following binding of an apoptotic cell to specific phagocyte surface receptors, a series of signalling events are initiated which result in the formation of an F-actin rich phagocytic cup (Aderem and Underhill, 1999). Continued membrane extension around the apoptotic cell, followed by generation of contractile forces allows internalisation of the cell (Chavrier, 2002). Multiple intracellular signalling pathways may become engaged during phagocytosis. For example, an apoptotic cell expresses multiple ligands on its surface, some or all of which may interact with their receptors. Furthermore, there may be heterogeneity in the expression of apoptotic 'eat me' signals or in the degree of opsonisation of apoptotic cells that are bound to a single phagocyte, and interpretation of the inputs from the different cells is required to generate the appropriate response. For example, it is not clear whether concomitant binding of an opsonised cell and an unopsonised apoptotic cell results in the secretion of pro- or anti-inflammatory cytokines. The points where different signalling pathways intersect are described as nodal points according to Prehoda and Lim (Prehoda and Lim, 2002). Multiple signals are processed together and specific outputs made, allowing for a single combined response to complex multiple inputs (Prehoda and Lim, 2002). One of the future challenges in the unfurling of phagocytic signalling pathways will be the understanding of the outputs at nodal points in signalling pathways following multiple receptor binding.

Signalling associated with Fc-mediated phagocytosis

The signalling events involved in phagocytosis have best been studied in Fc-mediated phagocytosis which, like apoptotic cell uptake, requires actin polymerisation (May and Machesky, 2001). A number of distinct Fc receptors are involved in phagocytosis (Indik et al., 1994; Park et al., 1993; Tuijnman et al., 1992), and receptor ligation triggers phosphorylation of tyrosine residues within immunoreceptor tyrosine-based activation motifs (ITAMS) of the cytoplasmic tail of Fc γ RII or the associated γ -subunit of Fc γ RI and IIIA (Isakov, 1997) by Src family

kinases (Ghazizadeh et al., 1994). The phosphorylation events allow docking and activation of the tyrosine kinase Syk (Greenberg et al., 1994), which is followed by phosphorylation of a large number of substrates, including ERK1/2 (Crowley et al., 1997) and paxillin (Greenberg et al., 1994). Informatively, Syk^{-/-} macrophages are still capable of binding to IgG-opsonised particles and form phagocytic cups, but fail to internalise the particles (Crowley et al., 1997). The PI3Kinase inhibitor, wortmannin, also halts phagocytosis at the same stage, suggesting that Syk may instigate PI3Kinase signalling, which is required for internalisation.

Signalling associated with complement-mediated phagocytosis

The process of complement-mediated phagocytosis is morphologically distinct from that of Fc-mediated phagocytosis, with the target 'sinking' into the phagocyte with the minimum of membrane disruption and little or no inflammatory response (Aderem and Underhill, 1999). CR3 is the most widely studied complement receptor and can bind a variety of different ligands such as C3bi (Ehlers, 2000), adhesion molecules such as ICAM-1 (Diamond et al., 1990) and matrix molecules such as fibrinogen (Trezza et al., 1988). Binding of different ligands induces different cellular responses ranging from phagocytosis to cellular spreading and chemotaxis. Phagocytosis requires prior activation of the phagocyte e.g. by cytokines or ECM attachment (Brown, 1991). Ligand binding to CR3 induces 'outside in' signals that act to regulate cytoskeletal organisation and other cellular responses (Giancotti and Ruoslahti, 1999).

C.Elegans Provides a Model Phagocytic System

The nematode *C. elegans* provides a genetically simplified model for identifying new receptors and interpreting the complex and bewildering array of receptors identified in the mammalian system. During the development of *C. elegans* 131 of an original 1090 cells are known to become apoptotic, upon which they are rapidly

phagocytosed by neighbouring cells (Sulston and Horvitz, 1977). Genetic screens have revealed seven genes which, when mutated, result in cell corpse persistence, implicating them in the process of apoptotic cell engulfment. None of the genes are essential for phagocytosis, suggesting a redundancy in the system, which is conserved through to mammalian cells. Elegant mutant studies, using combinations of different gene mutations led to the proposal that the genes fall into two, partially redundant signalling pathways (Ellis et al., 1991); Ced-2, Ced-5, Ced-10 and Ced-12 forming one such group and Ced-1, Ced-6 and Ced-7 the other (Figure 1.3). Cloning and structural studies of these genes and the subsequent discovery of mammalian homologues to all but one of the genes have provided a clearer insight into the mechanisms of apoptotic cell phagocytosis.

Within the first pathway, the mammalian homologues of Ced-2 and Ced-5 are the adaptor proteins CrkII (Reddien and Horvitz, 2000) and Dock180 (Wu and Horvitz, 1998b) respectively, Ced-10 is the Rho-GTPase Rac-1 (Reddien and Horvitz, 2000) and the recently cloned Ced-12 has three homologues, ELMO1, 2 and 3 (Gumienny et al., 2001). Together these proteins form a signalling pathway, initiated by apoptotic cell binding to an as yet undescribed receptor and terminating with Rac-1 activation, which has been shown to be important for apoptotic cell engulfment and cellular migration during development (Zhou et al., 2001a). Studies using crosses of different Ced gene mutants have shown that Ced-2, -5 and -12 form a trimeric complex, localised at the plasma membrane, which is required for the activation of Ced-10 (Gumienny et al., 2001; Wu et al., 2001; Zhou et al., 2001a). The pathway is conserved in mammals where over-expression of either CrkII or Rac-1 in the phagocytic cell line, LR73, enhanced engulfment. Transfections of cells with combinations of CrkII and Rac-1 mutants demonstrated that CrkII functions upstream of Rac-1 (Tosello-Tramont et al., 2001). The trimeric complex of CrkII, Dock180 and ELMO-1 are thought to recruit a speculative GEF (Rho guanine-nucleotide exchange factor), which confers Rac-1 activation by the transfer of GDP for GTP (Gumienny et al., 2001). The role of Rac-1 in mammalian phagocytosis is well documented, with Rac-1 known to orchestrate actin polymerisation, needed for the extension of membrane around the apoptotic cell (Allen et al., 1997).

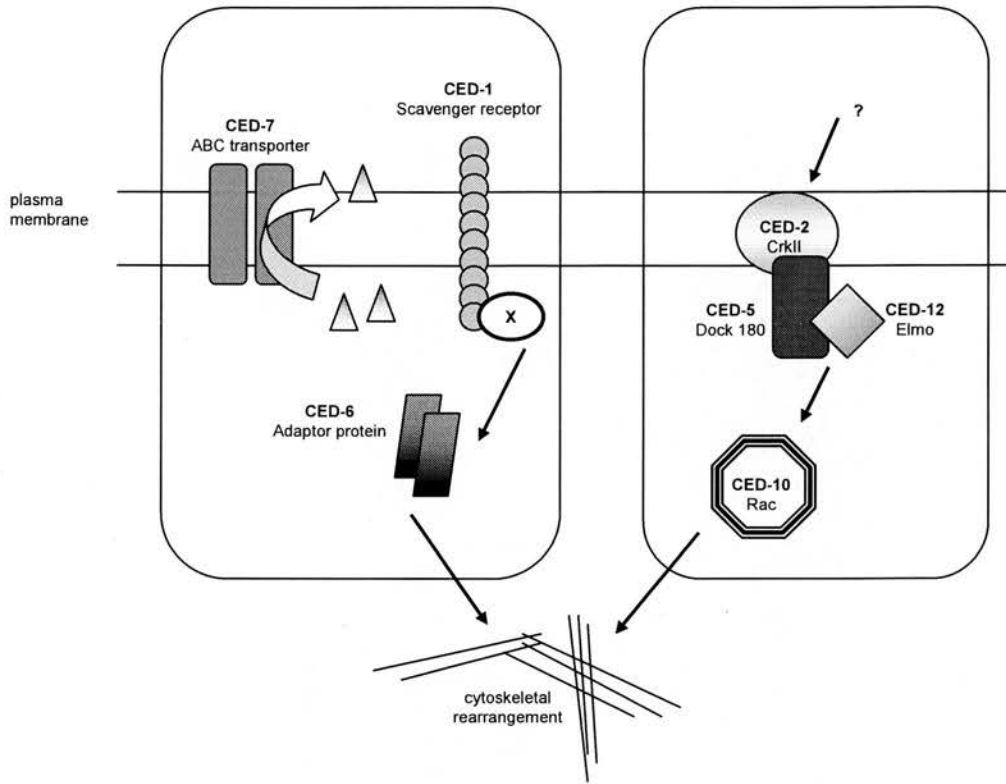


Figure 1.3 *C. elegans* engulfment genes

Genes involved in apoptotic cell engulfment in *C. elegans*, fall into 2 partially redundant signalling pathways: Ced-1, Ced-6 and Ced-7 in one and Ced-2, Ced-5, Ced-10 and Ced-12 in the other. *C. elegans* genes are shown in bold, with the mammalian homologues labelled underneath the respective nematode gene.

In the second pathway Ced-7 encodes a protein similar to the ABC transporters (Wu and Horvitz, 1998a). Ced-7 is a plasma membrane protein required in both the dying cell and the phagocytic cell, where it may function to export lipids through the plasma membrane (Wu and Horvitz, 1998a). The mammalian homologue of Ced-1 is a transmembrane scavenger-like receptor, the only receptor so far cloned in *C. elegans* (Zhou et al., 2001b). Ced-1 protein is expressed on the cell surfaces of many cells and was observed at high levels on membrane facing the apoptotic cell (Zhou et al., 2001b). This may reflect clustering following receptor ligation or in a model proposed by Hengartner, Ced-1 could be distributed into lipid rafts, which require Ced-7 for their formation (Hengartner, 2001). SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains in the cytoplasmic tail of Ced-1 are required for engulfment and may bind an adaptor protein, which confers signalling from the ligand for engulfment. Ced-6 encodes a candidate adaptor protein with PTB binding potential (Liu and Hengartner, 1998) which interestingly has been shown to act downstream of Ced-1 and Ced-7 (Liu and Hengartner, 1998). It was therefore thought to be a likely candidate for interpreting the Ced-1 signal. However, experiments to test this hypothesis failed to demonstrate any interaction between Ced-1 and Ced-6 (Zhou et al., 2001b). Thus, at the present time Ced-6 remains isolated, down-stream from Ced-1 in this signalling pathway.

The same set of Ced genes that have been shown to mediate the engulfment of apoptotic cells, are required for the engulfment of low numbers of necrotic-like cells (Chung et al., 2000). This intriguingly suggests a conservation of mechanism between the uptake of these two cell types, each of which is thought to regulate responses with different consequences in terms of inflammation in the mammalian system (Fadok et al., 2001). Studies in *C. elegans* have also demonstrated an extended role for the engulfment genes Ced-1, -2, -5, -6, -7, -10 and -12 in the execution of apoptosis in dying cells (Hoepfner et al., 2001; Reddien et al., 2001), a function that also appears to hold true for macrophages in some mammalian systems (Diez-Roux and Lang, 1997).

Signalling associated with apoptotic cell phagocytosis

Phagocytosis of apoptotic cells by receptors which bind 'eat me' signals are actin dependent, and share many signalling intermediates with Fc- and complement-mediated uptake (Aderem and Underhill, 1999; Allen and Aderem, 1996; Leverrier et al., 2003). However, the events immediately following receptor ligation are largely unknown, although a role for receptor phosphorylation has been demonstrated (Leverrier and Ridley, 2001). Interestingly, as reported for the Fc-receptors, the *C. elegans* Ced-1 protein receptor has a YXXL motif in the cytoplasmic tail (Zhou et al., 2001b), which could allow coupling to adaptor molecules via Src homology (SH) domains following tyrosine phosphorylation.

Downstream pathways: PI-3Kinases

Distinct roles have been attributed to different classes of the PI-3kinases during phagocytosis (Gillooly et al., 2001); Class I PI-3kinases, and PtdIns(3,4,5)P₃ products are involved in stages of membrane extension and closure but not the initial formation of the phagocytic cup, whereas Class III PI-3 kinases and PI(3)P products are necessary for fusion of the phagosome with late endosomes and lysosomes during phagosome maturation (Vieira et al., 2001).

p110 β is the major PI-3kinase class I isoform involved in phagocytosis and has been shown to be necessary for uptake of apoptotic cells and Fc-opsonised cells by macrophages (Leverrier et al., 2003). In fact, the major role for the class I PI-3kinases appears to be phagosome closure (Araki et al., 1996). PI-3 kinases become asymmetrically distributed in response to asymmetrical activation of chemoattractant receptors (Vanhaesebroeck et al., 1999; Wang et al., 2002) and results in actin polymerisation and migration in the direction of the chemoattractant signal. The signalling role of PI-3kinases in directing membrane extension is thus conserved in phagocytosis. The contractile forces required for the closure of the phagosome during Fc-mediated phagocytosis are dependent on both PI-3kinases and myosin

motor proteins (Swanson et al., 1999). The proposed mechanism has Myosin X linking the extending pseudopodia (which is enclosing the cell being engulfed) with the cytoskeleton. Myosin X binds to both the PI-3 kinase products in the pseudopodia membrane via pleckstrin homology (PH) domains and to actin filaments via the motor head. Movement of Myosin X towards the barbed ends of the actin filaments drags membrane towards the growing ends of the pseudopodia, closing the phagosome (Chavrier, 2002; Cox et al., 2002). Class II PI-3kinase products collect transiently in the phagosomal membrane following closure of the phagosome and associate with a serine/threonine kinase which regulates membrane association with late endosomes and lysosomes (Fratti et al., 2001; Vieira et al., 2001).

Downstream pathways: Rho family GTPases

The small Rho GTPases, Rho, Rac and Cdc42 regulate actin assembly and organisation during processes such as adhesion, phagocytosis and migration. Rho GTPase proteins cycle between an inactive (GDP-bound), and active (GTP-bound) state. Regulation is controlled by GEFs (guanine-nucleotide exchange factors), which activate Rho GTPases by driving exchange of GDP for GTP and GAPs (GTPase-activating proteins), in turn enhancing intrinsic GTPase activity, inactivating the proteins. Rho GTPase proteins direct down-stream signalling via the binding and activation of a range of effector proteins to drive actin polymerisation (Allen et al., 1997). In the Bac1 macrophage cell line, Rac-1 was shown to induce the formation of migration-associated lamellipodia and membrane ruffles, whilst Cdc42 regulated filopodia formation (Allen et al., 1997). Inhibition of the Rho GTPases with *Clostridium difficile* toxin inhibited apoptotic cell-, Fc- and complement- mediated phagocytosis. Further definition of the roles of different Rho GTPases in phagocytosis was established by microinjection of inhibitory or dominant negative Rho, Rac-1 and Cdc42. The inhibition of Rho, Rac-1 and Cdc42 had no effect on apoptotic cell binding, but inhibition of Rho via a down regulation in Rho-kinase signalling led to an increase in phagocytosis (Leverrier and Ridley, 2001; Tosello-Tramont et al., 2003), whilst inhibition of both Rac-1 and Cdc42 inhibited apoptotic cell phagocytosis (Leverrier and Ridley, 2001). Furthermore, activation of Rac-1 has

been demonstrated during dendritic cell phagocytosis of apoptotic cells, following signalling initiated by the ligation of the integrin adhesion receptor $\alpha\text{v}\beta\text{5}$ (Albert et al., 2000). Differential involvement of Rho GTPases was observed following triggering of Fc or complement receptors. Actin polymerisation necessary for phagocytosis was directed by Cdc42 and Rac-1 during Fc-mediated phagocytosis but Rho during complement-mediated phagocytosis (Caron and Hall, 1998). However, despite differential Rho GTPase activation in the different receptor-mediated phagocytosis, the down-stream mechanism implementing Rho family GTPase directed actin polymerisation was demonstrated to require the Arp2/3 complex in both Fc- and complement-mediated phagocytosis (May et al., 2000).

Downstream Pathways: Protein Kinase C

Protein Kinase C (PKC) family kinases are activated by calcium, diacylglycerol (DAG) and phorbol esters, and are involved in both the signalling pathways for Fc- and complement mediated phagocytosis and in the ingestion of apoptotic cells (May and Machesky, 2001). Apoptotic thymocyte binding to mouse peritoneal macrophages via receptor tyrosine kinase Mer (Scott et al., 2001), was associated with tyrosine phosphorylation of the phagocytic receptor, in common with Fc-receptor activation. This was followed by the association and phosphorylation of the PKC activator PLC- γ2 , demonstrating activation of PKC during apoptotic cell phagocytosis (Todt et al., 2004). Studies examining Fc-mediated phagocytosis by human monocytes showed that following the binding, but preceding the ingestion of IgG opsonised erythrocytes, PKC translocates from the cytosol to the plasma membrane where it is activated. Following internalisation, PKC then localises to the phagosome (Zheleznyak and Brown, 1992). PKC has also been shown to regulate the early process of apoptotic cell binding. The predominant binding receptor for non-inflammatory macrophages is the integrin $\alpha\text{v}\beta\text{3}$, but upon PKC stimulation $\alpha\text{v}\beta\text{5}$ mediated binding is also activated, accompanied by an up-regulation in total binding (Finnemann and Rodriguez-Boulan, 1999). Dissection of the roles of the different PKC isoforms has demonstrated that PS-mediated phagocytosis of apoptotic cells by mouse peritoneal macrophages requires the PKC βII isoform. This is in contrast to

isoforms utilised in other forms of phagocytosis (Todt et al., 2002). Down stream targets of the PKC kinases include plekstrin, MARCKS (myristoylated alanine-rich C kinase substrate) and MacMARCKS (macrophage enriched MARCKS). The latter two are actin-filament crosslinking proteins, linking PKC activation to the cytoskeletal membrane (May and Machesky, 2001).

Phagosome Maturation

'Pinching off' portions of the membrane, which partially surrounds the apoptotic cell or bacteria bound to the phagocyte surface, forms the nascent phagosome (Swanson et al., 1999). Maturation of the phagosome follows its interaction with the endocytic pathway, during which it undergoes dramatic membrane and vacuolar changes which confer ability to kill micro-organisms or breakdown apoptotic cells. The nascent phagosome sequentially associates with, and takes on characteristics of early endosomes, late endosomes and lysosomes, by a 'kiss and run' interaction permitting transfer of contents between the phagosome and endocytic vesicles without fusion of membranes. This complex maturation process is governed by microtubules and kinesin motor proteins, allowing the movement of the nascent phagosome from regions adjacent to the plasma membrane through the cytoplasm. Further, Annexins I, II and VI together with actin, localise to the surface of the maturing phagosome, perhaps mediating interaction with the cytoskeleton, and aiding movement and association with the endosomes and lysosomes (Vieira et al., 2002).

Mediators of Macrophage Phagocytosis

For therapeutic strategies for treatment of inflammatory disease via induction of apoptosis by external mediators to be successful, there is a requirement for strategies which increase the phagocytosis of such generated apoptotic cells. The regulation of phagocytic ability, like the induction of apoptosis, is amenable to exogenous

stimulation. This has been demonstrated by the short term exposure of macrophages to pro-inflammatory cytokines such as GM-CSF, which increases both the percentage of macrophages that phagocytose and the number of apoptotic cells phagocytosed by an individual macrophage. Stimulation with pro-inflammatory cytokines is independent of changes to surface receptors, suggesting alteration instead of signalling pathways required for actin polymerisation (Ren and Savill, 1995). However, the involvement of immuno-modulatory cytokines in defining macrophage phenotype and functions, such as phagocytosis of apoptotic cells, has not been fully investigated.

Rapid modulation of phagocytic ability was also observed by ligation of extracellular matrix receptors such as CD44 (Hart et al., 1997) or prostaglandin receptors (Rossi et al., 1998), or by macrophage adhesion to fibronectin (McCutcheon et al., 1998). Elevation of cAMP by exogenous mediators or by the binding of PGE₂, specifically inhibited the phagocytosis of apoptotic cells whilst disrupting cytoskeletal arrangements, resulting in loss of adhesion structures and rounding up of the macrophages in a PKA-dependent manner (Rossi et al., 1998). In contrast, cross-linking of the CD44 receptors rapidly and specifically up-regulated macrophage phagocytosis of apoptotic neutrophils (Hart et al., 1997) and, like lipoxin-mediated apoptotic cell phagocytosis, may also be associated with cytoskeletal alteration (Maderna et al., 2002). Cumulatively, these data suggest a connection between the processes of adhesion and phagocytosis. Phagocytosis and adhesion have cytoskeletal components in common, e.g. the adaptor protein paxillin, present in podosome adhesion structures and in the extending phagocytic cup during complement-mediated phagocytosis (Allen and Aderem, 1996) and the cytoskeletal regulators, Rho-GTPases which are critical to both processes (Allen et al., 1997; Leverrier and Ridley, 2001). The unfolding phagocytic signalling pathways, defined by studies in *C.elegans*, also demonstrate a link between adhesion, migration and phagocytosis. Ced-5, Ced-2, Ced-12 and Ced-10 and their mammalian homologues DOCK180, CrkII, ELMO and Rac-1 form a signalling pathway during phagocytosis (Gumienny et al., 2001), initiated by the ligation of the integrin adhesion receptor, $\alpha\text{v}\beta\text{5}$, in dendritic cells (Albert et al., 2000). In fact, many of the receptors implicated

in phagocytosis of apoptotic cells are also adhesion receptors e.g. CD36 and $\alpha v\beta 3$. One possibility is that ligation of these receptors may modulate adhesion, releasing or sequestering cytoskeletal proteins necessary for phagocytosis.

Effects of Glucocorticoids on inflammation

Glucocorticoids (GCs) are the most effective and widely used compounds for the treatment of diseases such as rheumatoid arthritis, autoimmune diseases and asthma, owing to their associated anti-inflammatory properties (see Figure 1.4). Synthetic GCs such as dexamethasone (Dex) and prednisolone, work by mimicking natural glucocorticoids. GCs may mediate their anti-inflammatory effects by increasing the transcription of anti-inflammatory genes e.g. IL-1 receptor antagonist, whilst decreasing transcription of pro-inflammatory genes such as TNF- α (Barnes, 1998). In addition, GCs affect the survival of inflammatory cells, for example by inducing apoptosis in T-cells and eosinophils via down-regulation of the secretion of survival factors. Interestingly, GCs have opposing effects on granulocyte apoptosis; inhibiting neutrophil apoptosis, but accelerating it in eosinophils (Meagher et al., 1996). The mechanisms by which GCs mediate their anti-inflammatory effects are discussed in detail in Chapter 3.

GCs also modulate the ability of macrophages to phagocytose apoptotic neutrophils. Short term exposure of 4 day monocyte-derived macrophages to GCs increased phagocytosis of apoptotic cells but not Fc-mediated uptake of opsonised erythrocytes. Importantly, this augmented uptake did not induce the release of pro-inflammatory cytokines (Liu et al., 1999). Recent studies have shown that exposure of monocytes to GCs for the first 24 hours of 5 day culture resulted in a highly phagocytic macrophage population, without any increase in the expression of any of the putative apoptotic cell receptors. GC-treatment resulted in a homogeneous population of small, rounded and weakly adherent macrophages with profound alterations to the cytoskeleton. Loss of podosomes-like adhesion structures was accompanied by a down-regulation in phosphorylation and recruitment of paxillin

and Pyk2 to adhesions. A down-regulation in the adaptor protein p130Cas, but a marked up-regulation in levels of active Rac-1 were also observed (Giles et al., 2001). Based upon these findings, it was suggested that the observed decrease in the adhesion associated protein p130Cas, normally found in a complex with DOCK180, CrkII and ELMO, may release the other complex proteins to sites of phagocytosis. Certainly, activity in the downstream effector Rac-1 is up-regulated in these cells and may drive the membrane extension facilitating the high levels of phagocytosis.

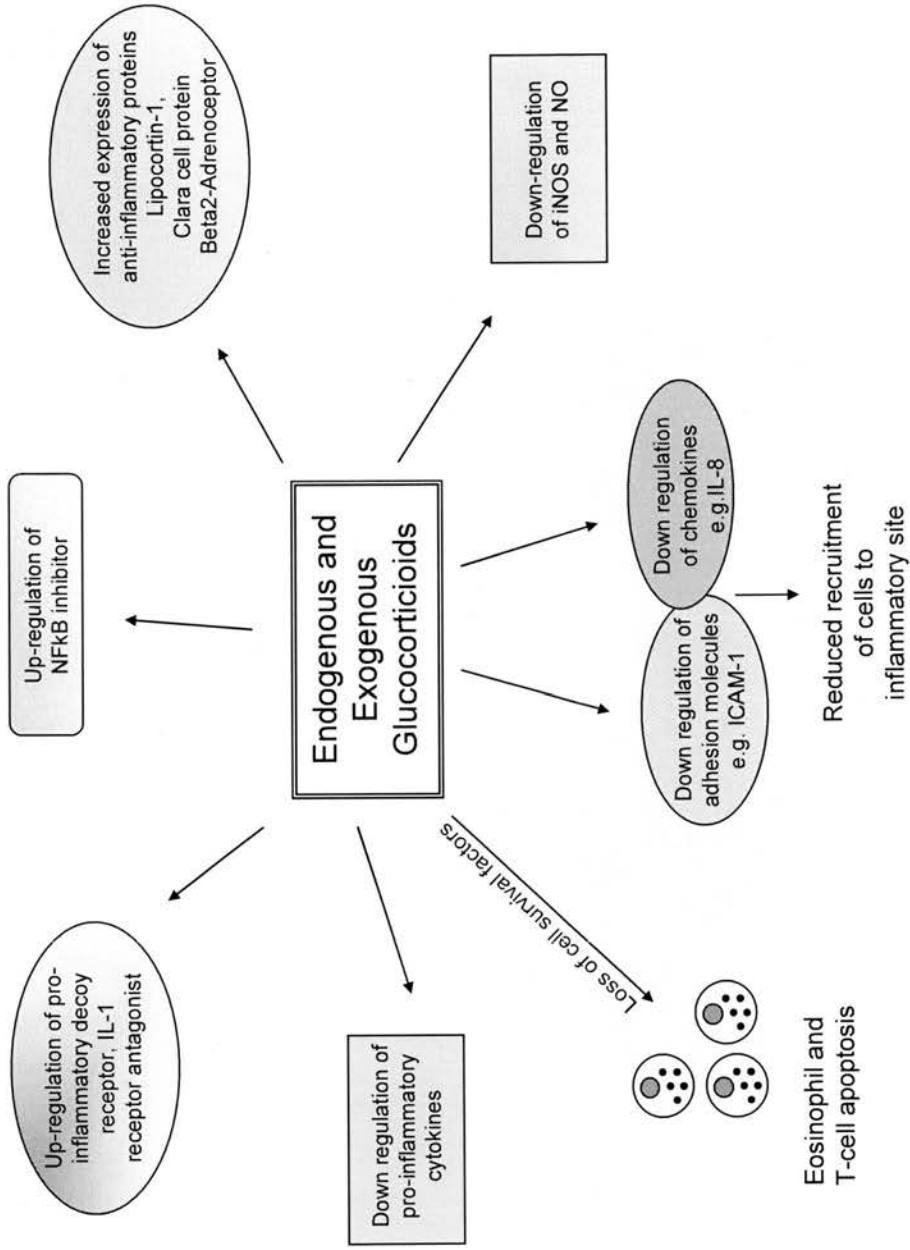


Figure 1.4 Effects of endogenous and exogenous glucocorticoids on inflammation

AIMS

The primary aims of this study were to investigate the mechanism by which long term glucocorticoid treatment of peripheral monocyte-derived macrophages causes *in vitro* augmentation of phagocytosis of apoptotic cells. Previous studies have demonstrated an altered macrophage cytoskeletal morphology following Dex treatment. Considering the central role that cytoskeletal regulation plays in macrophage function, I also sought to examine changes to macrophage adhesion and migration caused by Dex treatment. Specifically, I wished to examine the following hypothesis:

1. Glucocorticoid treatment of MDM Φ alters the utilisation of receptors required in the binding and/or internalisation of apoptotic cells.
2. Glucocorticoid treatment of MDM Φ induces altered cytoskeletal regulation that changes adhesion and migration responses in macrophages.
3. The cytokine environment of MDM Φ would directly modulate glucocorticoid-mediated differentiation of macrophages.

CHAPTER 2: MATERIALS AND METHODS

Antibodies and other reagents

Reagents were obtained from Sigma (Poole, UK) unless otherwise stated. Percoll and Dextran were from Amersham Pharmacia Biotech (Birmingham, UK) Iscove's DMEM was obtained from Invitrogen (Paisley, UK) and Dexamethasone (Dex) from Organon Laboratories Ltd (Cambridge, UK).

Antibodies, murine monoclonal, unless otherwise stated, were used at saturating concentrations as determined by titration in indirect immunoassays and flow cytometry as follows: HLA-DR (clone WR18 IgG2a, used at 1/100, Serotec, Oxford, UK), CD11a (clone 38 IgG2a, used at 1/100, Serotec) (clone WAC70 IgG2a, used at 1/100) CD11b (clone 44 IgG1, used at 1/100, Serotec) (clone LM2 IgG1, used at 1/50), CD14 (clone UCHM1 IgG2a, provided by Dr. Peter Beverley, Edward Jenner Institute for Vaccine Research, Compton, UK), CD14 (clones 61D3 and 63D3, provided by Professor Gregory, Centre for Inflammation Research, University of Edinburgh), CD16 (clone 3G8 IgG1, used at 1/500, provided by Dr Unkeless, Mount Sinai Medical School, New York), CD18/ β 2 (clone TS1/18 IgG1, used at 1/5), CD29/ β 1 (clone 12G10 IgG1, used at 1/50, Serotec) CD31 (clone Hec7.2 IgG2a, used at 5 μ g/ml, Endogen, Woburn, USA), CD32 (clone IV3 IgG2b, cell culture supernatant used neat), CD45 (clone 2D1 IgG1, used neat), CD54/ICAM-1 (clone 15.2 IgG1, used at 1/100, provided by Dr. Hogg, Cancer Research London, UK), CD49d (clone 44H6 IgG1, Serotec), CD49e (clone JBS5 IgG1, Serotec), CD62L (clone SK11 IgG2a, used at 1/50), CD64 (clone 10.1 IgG1, used at 1/100, provided by Dr. Hogg), CD66 (clone KAT4C IgG1, used neat), CD86 (clone BU63 IgG1, used at 1/50, Caltag, UK), CD51/ α V (clone 13C2 IgG1, used at 1/500, provided by Dr Horton, UCL, London), CD51CD61/ α h α v β 3 (clone 23C6 IgG1, used at 1/100, provided by Dr Horton), CD163 (clone Bermac IgG1, used at 1/35, Dako, Oxford, UK), mouse SRA-1 (clone 2F8 rat IgG2b, Serotec) IgG1 control (MOPC

IgG1, plasmacytoma, obtained from ECACC, UK), IgG2a (clone OX34, Serotec), Phosphor-c-Jun (ser63)II (rabbit polyclonal, Cell Signalling, Beverly, USA) PAK-1 (rabbit polyclonal, used at 1/500, Cell Signalling), ROCK-1 (clone 46 IgG1 used at 1/250, BD Transduction Laboratories, UK), Rac-1 (clone 102 mouse IgG2b, used at 1/1000, BD Transduction Laboratories), I κ B- α (rabbit polyclonal, Cell Signalling), Paxillin (clone 177, used at 1/400, BD Transduction Laboratories), Phospho-p44/42 MAPK (mouse IgG, used at 1/1000, Cell Signalling), Rhodamine phalloidin (used at 1/800, Molecular Probes, Leiden, Netherlands).

Secondary antibodies and directly conjugated antibodies were as follows: Goat anti-mouse IgG 430, 488 and 568 (used at 1/400, Molecular Probes), F(ab')₂ goat-anti mouse IgG FITC (used at 1/50, DAKO, High Wycombe, UK) and rat anti-mouse F4/80 FITC (clone CI:A3-1 IgG2b, Serotec).

Mononuclear and polymorphonuclear leukocyte isolation

Human peripheral blood was drawn from healthy volunteers and collected into tubes containing 4ml sodium citrate (Phoenix Pharmaceuticals Ltd., Gloucester, UK) to a final volume of 40ml and then centrifuged at 350 x g for 20 minutes. All steps following venepuncture were carried out at room temperature unless stated otherwise. The resulting upper platelet-rich plasma (PRP) layer was removed and autologous serum (AS) was prepared by adding 10ml of PRP to 220 μ l of 1M CaCl₂ and incubating for 30 minutes at 37°C in glass tubes. Erythrocytes were sedimented out of the lower cell suspension layer by the addition of 2.5ml of dextran T500 (500,000 Mwt.) per 10ml of cells and reconstituted to 50ml with 0.9% saline and then left for 30 minutes at room temperature. The resulting leukocyte-rich upper layer was then collected and spun at 350 x g for 6 minutes to pellet the leukocytes. Percoll was made isotonic by preparing a 90% solution using 10x PBS. The isotonic Percoll was then used to make 72.9%, 61.2% and 49.5% layers using 1x PBS without CaCl₂ or MgCl₂. Gradients were prepared by layering 3ml of 61.2% onto 3ml of 72.9% Percoll. The cells pellets were then mixed with the 49.5% layer (pellet from

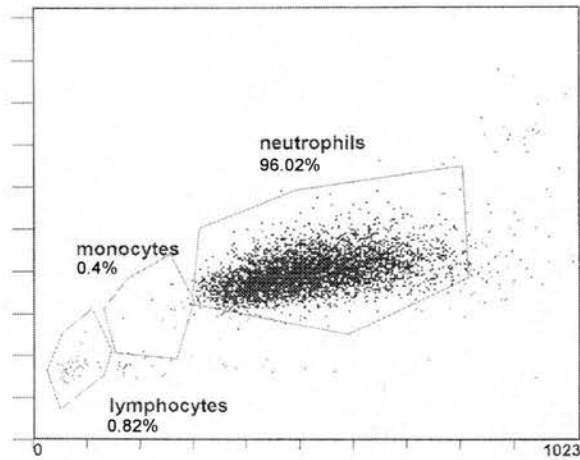
80ml blood /3ml of 49.5% Percoll) and layered onto the gradient, before centrifuging at 720 x g for 20 minutes. Mononuclear leukocytes were removed from the 49.5/61.2% interface whilst the polymorphonuclear leukocytes were removed from the 61.2/72.9% interface. Leukocytes were then washed twice in PBS without CaCl₂ or MgCl₂ before cell culture.

Quality control

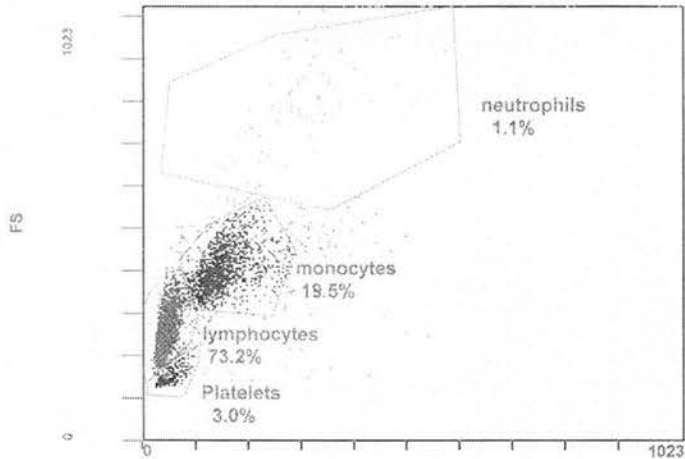
Cells collected from each layer of the gradient were subject to flow cytometry analysis by forward (FS) and side scatter (SS). The PMN's used were >95% pure, with the mononuclear cells typically comprising 15-20% monocytes and 80-85% lymphocytes (Figure 2.1).

Cell culture

Neutrophils, resuspended at 20×10^6 /ml in Iscove's DMEM were labelled with 2 μ g/ml (final concentration) CellTracker green 5-chloromethylfluorescein diacetate (Molecular Probes, Leiden, Netherlands) for 20 minutes at 37°C. Following labelling, the cells were pelleted by centrifugation at 220 x g for 5 minutes and the pellet was then resuspended at 4×10^6 /ml in Iscove's DMEM + 10% autologous serum and incubated for 20 hours at 37°C to induce apoptosis. The resultant population typically consisted of 45-65% apoptotic cells with 15-20% necrosis, as determined by morphological or flow cytometry examination. Alternatively, neutrophils were cultured without labelling, in which case they were incubated at 4×10^6 /ml in Iscove's DMEM + 10% AS for 20 hours. Cultured neutrophil preparations are hereafter referred to as overnight 'aged' neutrophils.



SS



SS

Figure 2.1 Flow Cytometry profiles for PMN- and monocyte-rich layers following percoll gradient isolation

Mononuclear and polymorphonuclear (PMN) cells were isolated from peripheral blood and separated by Percoll gradients. Representative flow cytometry FS and SS profiles are shown for (A) the PMN fraction and (B) the mononuclear cell fraction.

Neutrophil apoptosis was also induced by treatment with TNF- α and gliotoxin for 2 hours. Following isolation, the neutrophils were centrifuged at 220 x g for 5 minutes to pellet the cells and then re-suspended at 4×10^6 /ml in Iscove's DMEM + 10% AS + 0.1 μ g/ml (final concentration) TNF- α + 1 μ g/ml (final concentration) of gliotoxin, and incubated at 37°C, 5% CO₂ for 2 hours, following which the levels of apoptosis (typically 85-100% apoptosis and no necrosis) were assessed as described below.

Monocytes (typically 15-20% of the mononuclear cells) were cultured by re-suspending the mononuclear cells at 4×10^6 /ml in Iscove's DMEM and culturing in tissue culture dishes for 1 hour. Monocytes were enriched by selective adherence to tissue culture plastic followed by 4 x washes in Hanks Balanced Salt Solution to remove the non-adherent lymphocytes. The monocytes were then cultured for 5-7 days in the presence of Iscove's DMEM + 10% AS, during which time they differentiated into monocyte-derived macrophages (MDM Φ).

Dex (final concentration of 125nM or 1 μ M) and/or cytokines (final concentration of 10ng/ml) were added to the culture media following washing, unless otherwise stated in individual experiments. Working concentrations were established by examining MDM Φ phagocytosis of apoptotic neutrophils at different concentrations (see Figure 2.2).

Characterisation of neutrophil apoptosis by morphology

220 μ l of unlabelled apoptotic neutrophils at 4×10^6 /ml were placed into a cytospin chamber and centrifuged onto glass slides at 300 x g for 3 minutes. Following air drying, the cells were fixed for 1 minute in 100% methanol, stained for 1 minute each with haematoxylin and eosin (Diffquik, Baxter Healthcare, Glasgow, UK) and a coverslip added before examination by light microscopy under oil using a 63x objective lens. 500 cells were counted per slide and apoptosis was defined as a cell containing a condensed nucleus, distinct from the multi-lobed nucleus of a non-apoptotic neutrophil (see Figure 2.3A).

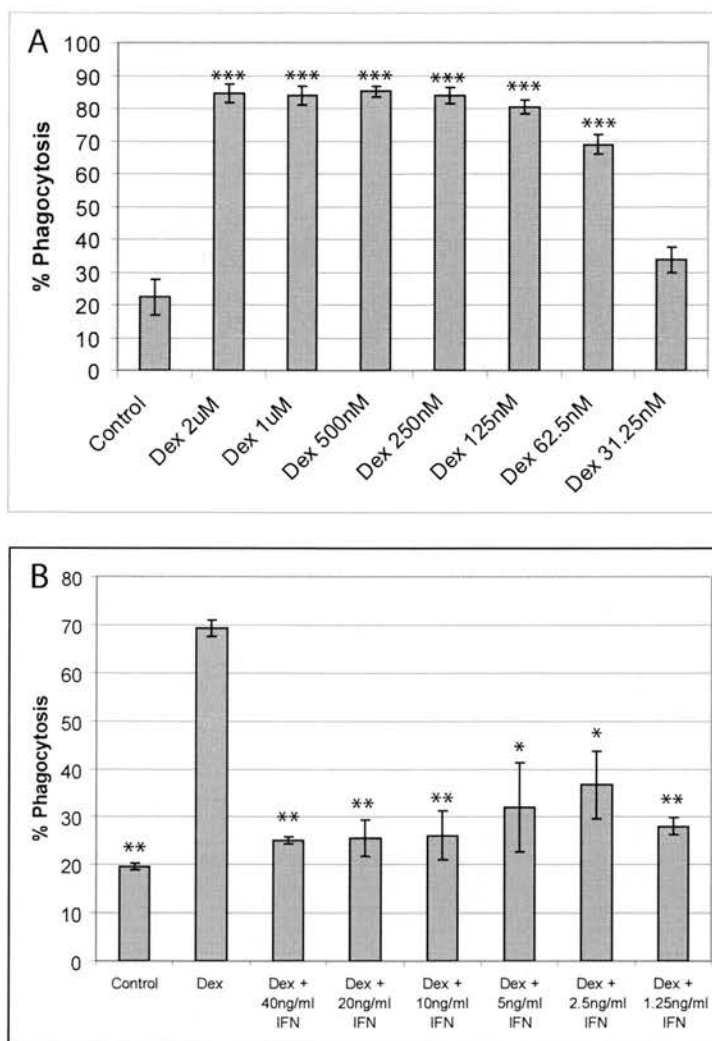


Figure 2.2 Concentration curves for Dex and IFN- γ

(A) The effect of diluting concentrations of Dex on MDM Φ phagocytosis of apoptotic neutrophils was determined. Concentrations between 2 μ M and 125nM augmented phagocytosis equally. ***, $p < 0.001$ vs. control MDM Φ , Tukey Kramer repeated ANOVA with post-test performed on transformed data (n=3).

(B) The concentration of IFN- γ which inhibited Dex-augmented MDM Φ phagocytosis was investigated. 10ng/ml IFN- γ was chosen as the concentration to use. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. Dex-treated MDM Φ , Tukey Kramer repeated ANOVA with post-test performed on transformed data (n=2).

Characterisation of neutrophil apoptosis by flow cytometry

Following treatment, neutrophils were dual labelled for annexin V and propidium iodide (PI). Phosphatidylserine (PS) is asymmetrically expressed on the inner membrane of viable cells, but following apoptosis the asymmetry is lost and PS becomes exposed on the outer leaflet where it can be detected by the specific binding of annexin V. Because PS is also expressed on the outer leaflet of necrotic cell membranes, PI, which specifically labels necrotic cells, was used to distinguish between apoptotic and necrotic cells. 2µl of fluorescein isothiocyanate (FITC)-conjugated annexin V (Roche, East Sussex, UK) (final concentration 5µM) was added to 1ml of annexin V binding buffer (HBBS, 5mM CaCl₂). 280µl of annexin V + buffer was then added to a tube containing 20µl of neutrophils at 4x10⁶/ml and incubated on ice for 10 minutes. Immediately prior to analysing the sample, 1µl of 1mg/ml PI was added to the cells. Annexin V and PI were measured using FL1 and FL2 detectors respectively (see Figure 2.3B).

Assessment of macrophage phagocytosis by flow cytometry

5 day MDMΦ, differentiated in 48 well plates, were washed once in HBSS and the media removed. 500µl of overnight 'aged', CellTracker green 5-chloromethylfluorescein-labelled neutrophils (labelling as described in Cell Culture section), at 4x10⁶/ml in Iscove's DMEM were added to each well and the plate returned to the 37°C incubator for 1 hour. Following incubation the media was removed and replaced with 250µl of pre-warmed trypsin/EDTA, and incubated at 37°C for 15 minutes and then on ice for 15 minutes. The cells were then vigorously pipetted to remove them from the plastic, transferred to flow cytometry tubes and then analysed on a FACS Calibur flow cytometer (Becton Dickinson). The macrophages were gated using FS and SS, and non-phagocytic, low FL1 macrophages then separated from the phagocytic high FL1

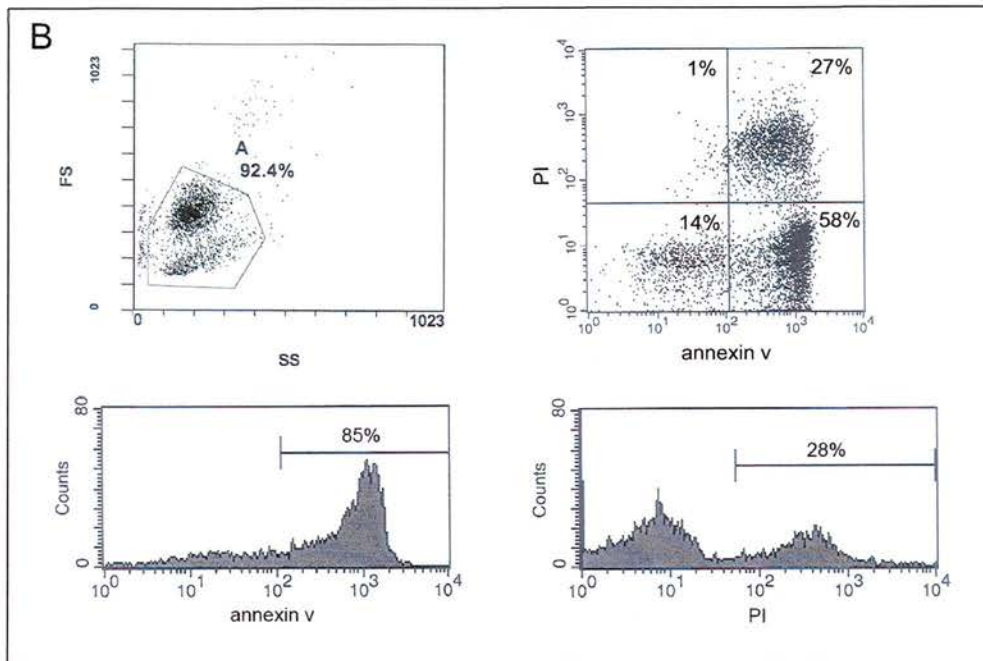
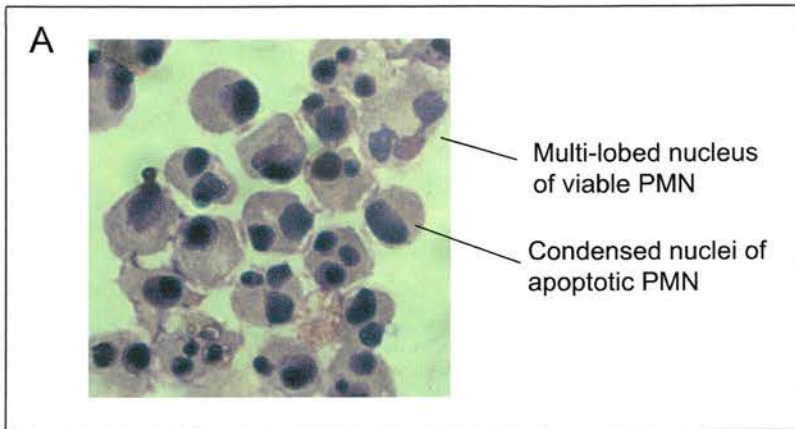


Figure 2.3 Assessment of neutrophil apoptosis

Following 20 hours culture in Iscove's DMEM + 10% AS, viability was determined by morphological assessment and by flow cytometric analysis of annexin V binding and PI exclusion.

(A) Overnight 'aged' neutrophils: apoptotic nuclei appear condensed whilst viable cells have multi-lobed nuclei.

(B) Representative flow cytometry profiles. Both the apoptotic and necrotic neutrophils are annexin V positive, whilst only the necrotic neutrophils are PI positive. In this example approximately 14% of the neutrophils are viable (annexin V and PI negative), 58% apoptotic (annexin v positive) and 27% necrotic (annexin V and PI positive).

macrophages (Jersmann et al., 2003). 6000 events were collected for each sample and the percentage of phagocytic macrophages in each population calculated (see Figure 2.4).

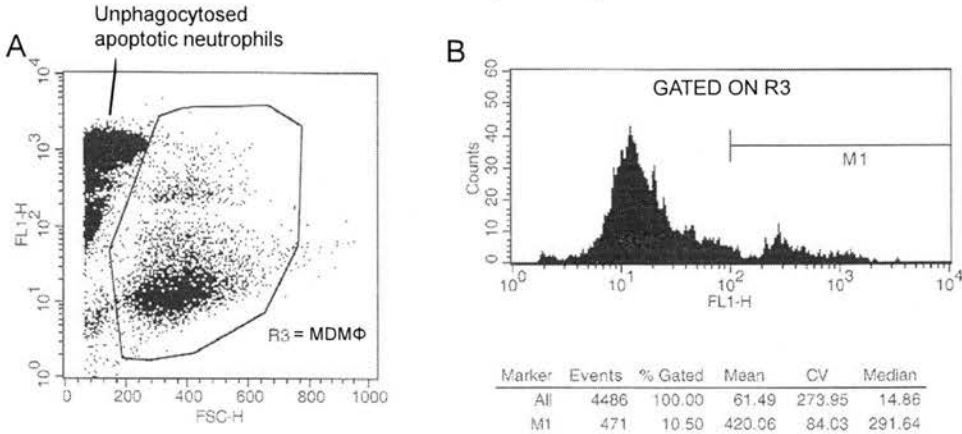
Assessment of macrophage phagocytosis by plate assay

5 day MDM Φ , differentiated in 24 well plates, were gently washed once in HBSS and the media then replaced with 1ml of unlabelled overnight 'aged' neutrophils. The plate was then incubated at 37°C for 1 hour. Following incubation, the cells were washed 3 times with PBS without CaCl₂ or MgCl₂ to remove all bound, non-internalised neutrophils. The cells were then fixed in 2.5% glutaraldehyde for 20 minutes at RT. Following 3 washes in PBS without CaCl₂ or MgCl₂ to remove the fixative, the neutrophils were stained for myeloperoxidase activity with 0.1mg/ml dimethoxybenzidine and 0.03% (v/v) hydrogen peroxidase for 20 minutes. The percentage of macrophage phagocytosis was quantified microscopically by counting a minimum of 500 cells per well, and an average between the duplicate wells calculated. Phagocytic index was calculated as (average number of neutrophils phagocytosed per macrophage) x (% of macrophages that had phagocytosed one or more neutrophil).

Phagocytosis inhibitor/activator studies

MDM Φ were incubated with: neuraminidase (Sigma) diluted to 0.1U/ml in PBS for 20 minutes at 37°C, or phorbol-12-myristate-13-acetate (PMA) diluted in Iscove's DMEM to 30nM, or RO 31-8220 diluted to 1 μ M or 4- α -PMA diluted to 80nM (optimal concentrations determined in previous studies in the laboratory) for 1 hour at 37 °C, or 13C2 antibody (α_v) diluted 1/500 or 23C6 antibody ($\alpha_v\beta_3$) diluted

Untreated (Control) MDM Φ



Dex-treated MDM Φ

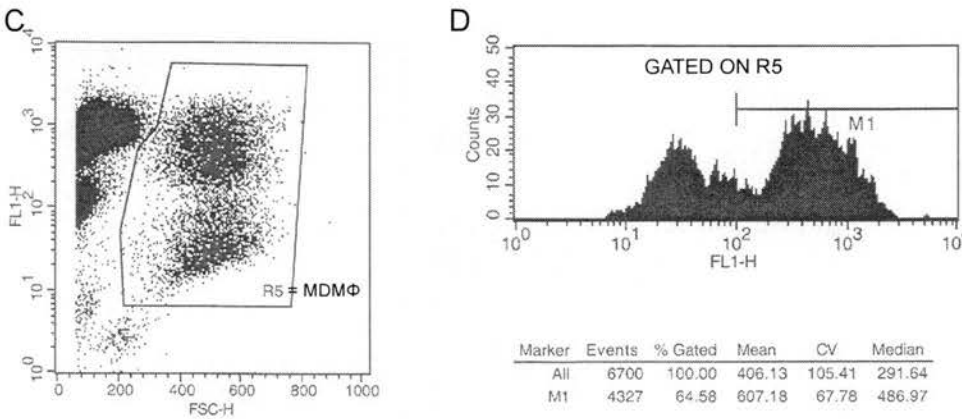


Figure 2.4 Flow Cytometric analysis of MDM Φ phagocytosis of apoptotic neutrophils

(A) and (C) show representative flow cytometry dot plots (FS vs FL-1) following a phagocytosis assay. The FL-1 labelled, unphagocytosed neutrophils are labelled in (A) The MDM Φ were gated in the Control and Dex-treated MDM Φ plots (R3 and R5 respectively)

(B) and (D) show FL-1 spread for the gated MDM Φ population, allowing the MDM Φ which have phagocytosed to be identified (gate M1) and the percentage of phagocytic cells calculated.



1/100 in Iscove's DMEM for 30 minutes at 37 °C. The inhibitor/activator was then replaced by apoptotic neutrophils and the flow cytometry phagocytosis assay carried out as described.

Overnight 'aged' neutrophils were resuspended at 4×10^6 /ml in HBSS + 5mM CaCl₂ + 1/50 annexin V-biotin (Roche) and incubated on ice for 20 minutes. The cells were then pelleted and resuspended in Iscove's DMEM at 4×10^6 /ml and used in a flow cytometry phagocytosis assay as previously described.

Assessment of macrophage binding of apoptotic neutrophils

In order to assess the binding ability of apoptotic neutrophils by different populations of macrophages, MDMΦ in 24 well plates were gently washed once in HBSS and the media then replaced with 1 ml of unlabelled 'aged' neutrophils at 4×10^6 /ml and the plate incubated on ice for 1 hour. Following incubation, the plate was placed onto a plate shaker (Eppendorf) rotating at 300rpm for 5 seconds. The media was then replaced with PBS without cations and again placed on the mixer for a further 5 seconds. This was repeated again to give a total of 3 washes. The cells were then fixed in 2.5% glutaraldehyde on ice for 20 minutes. Following three washes in PBS without CaCl₂ or MgCl₂ the neutrophils were stained with 0.1mg/ml dimethoxybenzidine and 0.03% (v/v) hydrogen peroxidase for 20 minutes. 500 macrophages were counted per well, with duplicate wells for each treatment, and the percentage of macrophages with 1+ neutrophils bound recorded, as well as the number of neutrophils bound to each macrophage. Binding index was calculated as (average number of neutrophils bound per macrophage) x (% of macrophages that had bound one or more neutrophil).

Binding inhibition studies

5 day MDM Φ were incubated with 61D3 or 63D3 (anti-CD14) diluted to 20 μ g/ml in Iscove's DMEM for 30 minutes at 37° C or Hec7.2 (anti-CD31) or IgG2a control antibody diluted to 5 μ g/ml in Iscove's DMEM for 10 minutes at room temperature. The media was then replaced with 'aged' or TNF/gliotoxin-treated neutrophils and the binding assay carried out as described above.

Flow cytometry for profiling

MDM Φ , cultured for 5-7 days, were removed from the tissue culture plastic by replacing the culture media with sufficient dissociation buffer (PBS without cations, 5mM EDTA and 0.2% serum) to cover the bottom of the well. The plate was incubated on ice for 15 minutes followed by removal of the macrophages using a rubber cell scraper. 50 μ l of cells were then added to each well of a 96 well plate and the plate centrifuged at 200 x g for 5 minutes to pellet the cells. The buffer was then removed by flicking the plate and the cells re-suspended by gentle vortexing. 100 μ l of flow buffer (PBS without cations, 0.2% BSA, 0.1% sodium azide) was then added to each well and the plate centrifuged at 200 x g for 2 minutes at 4°C, followed by removal of the buffer and re-suspension of the cells. To block Fc receptors, 5 μ l of rabbit Ig antibody (1/50) was added to each well and the plate incubated for 10 minutes on ice. 50 μ l of primary antibody was then added to each well and the plate incubated on ice for 20 minutes, followed by 2 washes in flow buffer. 50 μ l of FITC goat anti-mouse IgG (1/50) was then added to each well and the plate incubated for 20 minutes. Following two washes, the cells in each well were re-suspended in 120 μ l of flow buffer and transferred to tubes before analysis on a Coulter EPICS XL Flow Cytometer.

Cytokine bead assay

Following 5 day's culture, MDM Φ culture supernatant was removed and centrifuged at 10,000 x g for 10 minutes to pellet any cellular debris. The supernatants were then stored at 80°C until analysis.

The method for cytokine measurement was as described by the manufacturer (BD Biosciences). The cytometric bead array system allows single supernatant samples to be simultaneously analysed for the presence of IL-2, IL-4, IL-6, IL-10, TNF- α and IFN- γ . The kit contains 6 bead populations, each with a distinct FL-3 intensity, that have been coated with capture antibodies for the 6 proteins named above. The capture beads are then incubated with the supernatant samples whilst the vial of inflammatory cytokine standards (Becton Dickinson) were reconstituted with 0.2ml of assay diluent to make a 10x bulk standard and left to equilibrate for 15 minutes at room temperature. 12 standards were then made by diluting the bulk standard; 900 μ l of assay diluent was added to the first tube and 300 μ l to the remaining 11 tubes. 100 μ l of 10x bulk standard was then added to the first tube and mixed. A serial dilution was then performed.

10 μ l of each capture bead (x number of assay tubes) was added to a single 'mixed capture beads' tube and the beads mixed by vortexing. 50 μ l of mixed beads were then transferred to each of the assay tubes, followed by the addition of 50 μ l of PE detection reagent. 50 μ l of standard dilutions and test samples were then added to the correct tubes and incubated for 3 hours at RT. 1ml of wash buffer was then added to each tube and centrifuged at 200 x g for 5 minutes. The buffer was then carefully aspirated off and 300 μ l of wash buffer added to each tube to resuspend the pellet. Analysis was performed on a BD FACS Calibur flow cytometer with a 488nm laser that can detect and distinguish emissions at 576 and 670nm. Each sample was vortexed for 3-5 seconds prior to analysis.

Electron microscopy

Monocytes seeded onto glass cover slips in 12 well plates were differentiated in the Iscove's DMEM + 10% AS ± 125nM Dex. After 5 days the cells were washed once in HBSS and then fixed for 3 hours in 3% glutaraldehyde in 0.1M sodium cacodylate buffer pH7.4, on a rocking platform. The subsequent preparatory work was carried out by Steven Mitchell, College of Medicine and Veterinary Medicine Electron Microscopy Services. Following three, 20 minute washes in 0.1M sodium cacodylate buffer pH7.4 the cells were placed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer pH7.4 for 2 hours. The cells were dehydrated by washing through graded acetones for 10 minutes each wash; 50%, 70%, 90%, 100%, 100%, 100% acetone. The cells were then critical-point dried with carbon dioxide in a poloron E3000 SII CPD and a conductive coating of 20nM gold/palladium (60/40) added using an Emscope SC500 sputter coater.

Cells were viewed and imaged using a Philips 505 scanning electron microscope.

Immunofluorescence

Mononuclear cells were plated at 4×10^6 /ml in Iscove's DMEM onto sterile cover slips placed in the wells of 12 well plates. After one hour incubation at 37°C the cells were washed and cultured as described in the cell culture methods section.

Alternatively, neutrophils were cultured and cytopun onto glass slides as described previously.

The cells were fixed in 3% PFA at room temperature for 20 minutes, and then washed 3 times in PBS. The MDMΦ were then permeabilised in 0.1% Triton X 100 for 4 minutes at RT, followed by a further 3 washes. To block non-specific antibody binding, cells were then incubated in heat-inactivated human serum diluted 1/10 in PBS for 10 minutes at RT, followed by three washes as before. The primary antibody was diluted in PBS and the cells stained for 30 minutes on ice, followed by 3 washes in PBS. The secondary antibody was then diluted in PBS and added into the wells of

the plate containing the cover slips for 30 minutes on ice. The cells were then washed a further 3 times and the coverslips were placed onto slides containing the mounting medium mowiol and left to dry overnight in the dark. Slides were examined by using a Zeiss Axiovert 5100 fluorescent microscope, with images captured using AppleMac OpenLab image capture system.

Time lapse microscopy

MDM Φ were cultured for 5-7 days in slide flasks (Nunc). The cells were washed once in HBSS to remove non-adherent cells and the media replaced with Iscove's DMEM + 10 % AS. For experiments where macrophages were treated with the MAP kinase inhibitor, PD98059, the culture media was replaced with Iscove's DMEM containing the inhibitor diluted to 20 μ M and the MDM Φ returned to the 37°C incubator for 1 hour before continuing as described. The monolayer of macrophages was then wounded using a rubber cell scraper which enabled a consistent width of cells to be removed without damaging any underlying ECM. Non adherent cells were removed by replacing the Iscove's DMEM + 10% AS. The migration of cells back into the wound was then examined by phase contrast microscopy with an image captured every 5 minutes for 20 hours. Data was analysed by opening the initial image taken at time 0 and placing an acetate sheet over the computer screen and defining the area of the wound. Subsequent images were then opened and the number of cells in the 'wounded area' recorded. From this information the migration of the cells can be presented graphically and the rate of migration for different stages recorded.

Western blotting

MDM Φ were cultured for 5 days in 6 well plates, and washed once in HBSS without cations before replacing media with 200 μ l of lysis buffer (25mM Tris pH7.4, 1%

NP40, 150mM NaCl and 1/100 Sigma protease inhibitor cocktail). The plate was then placed on ice for 30 minutes before using a rubber cell scraper to ensure all cells were removed from the plate. The supernatant was then moved into tubes and centrifuged at 10 000 x g for 10 minutes at 4°C to pellet cell debris.

The protein concentration of the supernatants was then determined using a Bradford Assay kit (Pierce). Standards were made using a 10mg/ml stock of bovine serum albumin (BSA) and performing a serial dilution to provide a 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16 and 0 mg/ml. 10µl of either standard or supernatant were added to wells of a 96 well plate in duplicate, to which the colourmetric detection buffers were added. The 96 well plate was incubated at 37°C for 30 minutes and then analysed on a spectrophotometer with a measurement filter of 450nm.

Protein gels of various concentrations (12%, 9%) were made as follows: for 20ml of a 9% Tris/glycine SDS-Polyacrylamide running gel, 5ml 1.5M Tris pH8.8, 6ml 30% bis-acrylamide, 200µl of 10% sodium dodecylsulphate (SDS), 60µl 1,2-Bis (dimethylamino) ethane (TEMED), 120µl of 10% ammonium persulphate (APS) and 8.62ml of water were mixed and then poured between glass plates on the Biorad Mini Protein II electrophoresis kit, and overlaid with butanol saturated water and left to set. Following this, the butanol saturated water was removed and 4.5% stacking buffer overlaid on the running gel (0.5 TRIS pH6.8, 30% acrylamide/bis, 10% SDS, 0.3% TEMED, 0.6% of 10% APS and made to volume by the addition of water).

Supernatants were diluted in 4x sample buffer and 30µl of each sample was pipetted into each well. The samples were run at 80V through the stacking gel and 120V through the running gel.

Following electrophoresis, the gels were electroblotted onto nitrocellulose by forming a sandwich of blotting paper, gel, nitrocellulose membrane and blotting paper within a cassette. The proteins were then transferred from the gel to the nitrocellulose by passing a current of 80V through the gel to the membrane in transfer buffer (25mM Tris, 0.2M glycine, 20% methanol) for 1 hour.

The membrane was then incubated in blocking buffer (1 x TBS, 0.1% Tween 20, 5% w/v nonfat dry milk) at room temperature for 1 hour. The buffer was then replaced with the primary antibody, diluted in blocking buffer and incubated at 4°C overnight on a rocking platform. Following three, 10 minute washes in 1 x TBS, 0.1% Tween 20, the membrane was incubated with the secondary antibody diluted in blocking buffer for 1 hour at room temperature. Following three further washes the membrane was incubated with ECL reagents (Amersham Biosciences) for 5 minutes, exposed to X-ray film (Kodak) and developed using an X-ray imaging system, compact x4.

Assay for detection of activated Rac

MDM Φ were cultured for 5 days as detailed above. The cells were rinsed gently with HBSS and then the media was replaced with 200 μ l/well of ice-cold RIPA buffer (50mM Tris pH7.2, 500mM NaCl, 10mM MgCl₂, 1% TritonX-100, 0.5% sodium deoxycholate and 0.1% SDS). The plates were put on ice for 30 minutes. PAK-1 agarose beads (a gift from Neil Hotchin, Birmingham or Upstate, New Jersey, USA) were prepared by using 1 vial per well of cells and adding 50 μ l of cold RIPA lysis buffer and mixed thoroughly. The supernatants were then removed from the plates into tubes on ice, and then centrifuged at 10 000 x g for 15 minutes. 200 μ l of supernatant were then placed into clean tubes on ice, with 10 μ l kept back to run as whole cell lysate. The total volume of beads was divided by the number of supernatants and the appropriate volume transferred to the 190 μ l lysates. The lysate/bead mixes were then mixed on a rotary agitator at 4°C for 60 minutes, followed by 3 washes with Buffer B (50mM Tris pH7.2, 150mM NaCl, 10mM MgCl₂ and 1% TritonX-100) with centrifugation at 10 000 x g for 2 minutes. 20 μ l of 2.5x reducing sample buffer was then added to each pellet and 10 μ l added to each of the 10 μ l whole cell lysates, followed by a brief vortex. The samples were then heated for 15 minutes at 95°C followed by centrifugation for 2 minutes at 10000g. 20 μ l of each sample were loaded onto and run on 12% polyacrylamide gels, blotted and proteins detected as described in western blotting section.

RNA isolation and RT-PCR

MDMΦ cultured for 24 hours or 5 days in the wells of 6 well plates were washed once in HBBS to remove non-adherent cells and the media then replaced with 0.5ml of Trizol. Following 5 minutes incubation at RT the Trizol lysed cells were then transferred to 1.5ml Eppendorf tubes. 0.2ml of chloroform per ml of trizol used to lyse the cells was added and the tubes shaken vigorously by hand for 15 seconds, followed by an incubation at RT for 3 minutes. The tubes were then centrifuged at 12,000g for 15 minutes at 4°C. The supernatants settled out into an upper RNA rich phase, an interphase of protein and a lower phase of DNA and protein. The upper phase was collected into fresh tubes and 0.25ml of isopropanol was added to each tube and the RNA incubated on ice for 10 minutes. After centrifugation at 12,000g for 10 minutes at 4°C, a pellet formed which was washed once in 75% ethanol, with a centrifugation at 7,500g for 5 minutes at 4°C. The ethanol was then removed and the pellet air dried for 10 minutes, after which it was dissolved in 20µl of RNase free water and incubated at 55°C for 10 minutes.

RNA was then DNase treated to remove any contaminating DNA using a DNase kit (Ambion). 0.1 volume (of the RNA) of 10x DNase1 buffer and 1µl of DNase 1 enzyme was added to the RNA, and mixed gently and incubated at 37°C for 30 minutes. 5µl DNase inactivation buffer beads were then added to each tube of RNA and incubated at 37°C for 2 minutes and centrifuged at 10,000g for 1 minute to pellet the inactivation buffer beads. The supernatant containing the RNA was then transferred to fresh tubes and stored at -80°C

The isolated RNA was then reverse transcribed to generate cDNA. The RNA was initially assessed for purity and yield by measuring absorbance at 260 and 280λ. The concentration of RNA = (total A260) x (40µg/ml) 40µg/ml = one A260 unit of RNA. The yield of RNA = (Total sample volume) x (concentration), whilst purity was calculated by A260 / A280.

1µl of oligo DT and 2µg of RNA was made to a final concentration of 12µl by the addition of dH₂O and incubated at 70°C for 10 minutes. 4µl of 1st strand buffer, 2µl of

0.1M DTT, 0.5µl of 20mM dNTP and 0.5µl of RNAse inhibitor mix was then added and incubated at 42°C for 2 minutes to equilibrate the mix, after which 1µl of superscript enzyme was added and the mix incubated at 42°C for 50 minutes and then at 70°C for 15 minutes. The cDNA was stored at 20°C until required.

PCR

PCR reactions were carried out by making the following reaction mix for each 25µl reaction: 19µl distilled water (dH₂O), 2.5µl of 10x reaction buffer (Invitrogen), 0.5µl of 10mM dNTP (Promega), 0.75µl of 50mM MgCl₂ (Invitrogen), 0.5µl of x concentration of forward and 0.5µl of reverse primer (MWG, Germany), 1µl of cDNA and 0.25µl of Taq polymerase (Invitrogen). Each time a PCR reaction was run, a negative control reaction without template cDNA was also run, to confirm that any product was derived from the cDNA.

The Primer sequences used as detailed below:

Caveolin 1 Forward Primer 5' → 3' AGC|TGA|GCG|AGA|AGC|AAG|TG

Caveolin 1 Reverse Primer 5' → 3' CGG|TGT|GGA|CGT|AGA|TGG|AA

p160 Rock Forward Primer 5' → 3' TTG|ATG|ACT|TGG|AAG|AAG|ATA|AAG|G

p160 Rock Reverse Primer 5' → 3' AGC|TGT|GAA|TTC|TGA|CTG|ACT|TTC|TT

PAK1 Forward Primer 5' → 3' TAT|GAT|TGG|AGT|CGG|CAG|CA

PAK1 Reverse Primer 5' → 3' AAT|TGT|AAT|CCT|CAG|CTG|AC

ICAM1 Forward Primer 5' → 3' ATC|ACC|ATG|GAG|CCA|ATT|TC

ICAM1 Reverse Primer 5' → 3' TGT|ATA|AGC|TGG|CCG|GCC|AC

β-Actin Forward Primer 5' → 3' CCA|CCA|ACT|GGG|ACG|ACA|TG

β-Actin Reverse Primer 5' → 3' GTC|TCA|AAC|ATG|ATC|TGG|GTC|ATA

For each gene a curve of product versus cycle number was produced. Following optimisation of each pair of primers, a PCR was set up and a sample removed every 2 cycles between 20 and 34 cycles. The product was run on a 1% TBE agarose gel

and the band intensity measured. The band intensity was then plotted against cycle number and the linear phase of the graph where amount of PCR product increased proportionally to cycle number identified (see Figure 2.5). Using cDNA from a minimum of three donors, PCR reactions for β -actin and one of the genes above was run for the number of cycles identified as being in the linear phase. This enables direct comparison of 2 types of cDNA, and thus the amount of RNA present in the cells.

Gene Array

The Atlas human cell interaction cDNA array (Clontec, Becton Dickinson) consisting of 265 genes immobilised onto nylon membranes, was used to investigate potential changes in HMDM Φ gene expression following 24 hours Dex treatment. The array contained genes from a range of gene/protein classifications including ECM proteins, cell surface antigens, cell-cell adhesion proteins, metalloproteinases, protease inhibitors, immune system proteins, intracellular protein phosphatases, GTP/GTP exchange proteins and kinasenetwork members. Purified mRNA from 2 samples were converted to labelled cDNA by specific primers and incubated separately with 2 membranes. Comparison of the membranes bound by labelled cDNA allowed assessment of relative expression of genes between the 2 samples.

Purification of mRNA

Adherence purified peripheral blood monocytes were cultured for 24 hours in the presence or absence of 1 μ M Dex, and were then washed in HBBS to remove non-adherent cells. The monocytes consisted of > 95% CD14-positive cells as assessed by flow cytometry. The media was then replaced with 0.5ml of Trizol. RNA was isolated from the Control and Dex-treated MDM Φ , and then DNAase treated as described in the RT-PCR section. The concentration of the two mRNA samples was calculated, and adjustments made to equalise concentrations in the two samples.

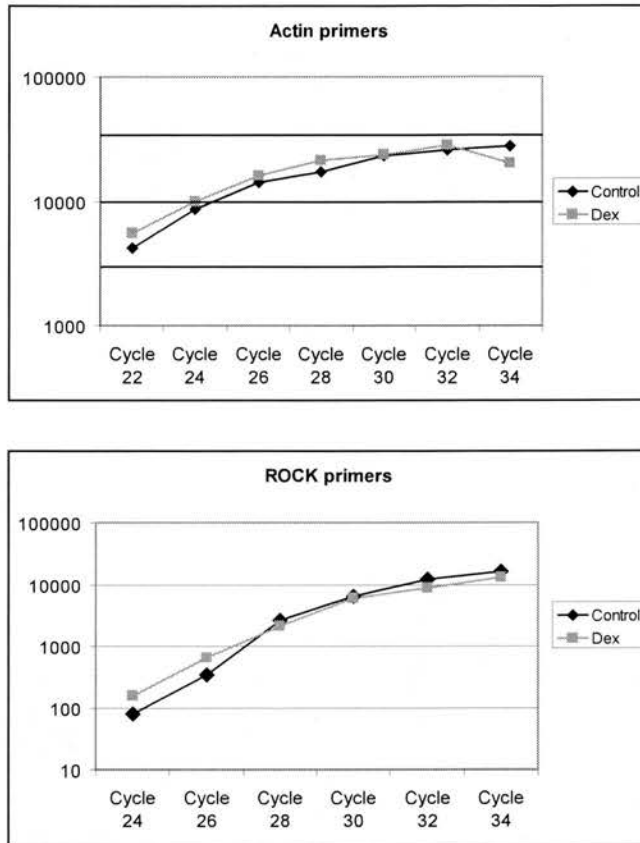


Figure 2.5 RT-PCR curves

In order to ensure that PCR reactions were occurring during the linear phase, i.e. where product amount is directly related to cycle number, product vs. cycle number analysis was carried out for each set of primers. Following PCR reactions for different cycle numbers, the product was run out on an agarose gel and the intensity of the band calculated by densitometry. A curve of product vs. cycle number was drawn as shown above. The cycle number for the p160ROCK and actin PCR reactions was then chosen.

Conversion of mRNA to radioactive cDNA probe

The isolated mRNA was converted to radioactively labelled cDNA. Initially 500-1000ng of control and Dex-treated mRNA were separately pre-incubated with 2µl of Atlas-array specific primer (a mix of primers against all genes on array) for 10 minutes at 70°C followed by 10 minutes at 52°C. 2µl of 10x RT buffer, 2µl of dNTP mix, 3µl of $\alpha^{32}\text{P}$ dATP (2000-4000 Ci/mMol) and 2µl of nuclease free water were then added and all components mixed and incubated for 5 minutes at 52°C. 2µl of M-MLV reverse transcriptase was added and the mix incubated at 52°C for 2 hours. Following the conversion of the mRNA to cDNA, unincorporated nucleotides were removed by spinning the mix through Atlas nucleospin columns. The eluted radioactively labelled cDNA probes were measured on a scintillation counter to ensure that the control and Dex-treated cDNA probes were of equal counts per minute (cpm).

Probe hybridisation to array membranes

The two array membranes (one for the control MDMΦ sample and one for the Dex-treated MDMΦ sample) were washed in 0.5% SDS for 30 minutes at 80°C and then incubated with the hybridisation buffer for 30 minutes at 68°C. The buffer was removed and replaced with 50µl of heat denatured salmon sperm (to lower cross hybridisation) and fresh hybridisation buffer. 1×10^6 cpm of heat denatured labelled cDNA control MDMΦ probe was added to one membrane and 1×10^6 cpm of heat denatured labelled cDNA Dex-treated MDMΦ probe to the other and hybridised overnight at 68°C. The buffer and cDNA probe was removed and the membranes washed twice in low stringency buffer and twice in high stringency buffer for 30 minutes at 50°C.

Analysis

The membranes were wrapped (DNA side up) in Saran wrap and exposed to phosphorimager screen for 36 hours. The screen was then imaged on a STORM 860 phosphorimager, the background subtracted from around each gene array spot, and the intensity of each spot recorded. For each gene, a pair of cDNA spots are immobilised on the membrane and an average intensity reading was therefore generated for each gene. An average intensity of the housekeeping genes on the control MDM Φ membrane (Con Housekeeping average) and Dex-treated MDM Φ membrane (Dex Housekeeping average) were used as correction factors for the other genes on the control and Dex-treated MDM Φ arrays respectively (Con Gene 1/ Con Housekeeping average, Dex Gene 1/ Dex Housekeeping average). The value calculated for control gene 1 and Dex gene 1 were then compared (corrected Con gene 1/ corrected Dex gene 1). Owing to a small number of genes being regulated following Dex-treatment, those 2 times up- or down- regulated were considered for further investigation.

Rat and Mouse Bone Marrow isolation and macrophage culture

Following a schedule 1 killing of age and sex matched wildtype and knockout mice, or wildtype rats, the femurs were dissected out, with care taken to remove enough tissue to ensure that the femur was not cut into. The extracted femurs were then washed in 70% ethanol and all tissue removed from the bone using a scalpel. Following a further wash in 70% ethanol, the femurs were then transferred to a clean dish. Using a scalpel, both ends of the femur were cut through to expose the marrow. A 10ml syringe with a 25 gauge needle was filled with DMEM F-12, 10% L929 media (M-CSF-rich supernatant obtained from cultured L929 fibroblasts as described below) (Boltz-Nitulescu et al., 1987), 10% FBS and the marrow flushed from the bone into 15ml Falcon tubes.

The extracted bone marrow cells were then resuspended through a 19G wide bore needle and the stem cells then counted. The cells were then pelleted by centrifuged at 220 x g for 5 minutes and re-suspended in DMEM F-12, 10% L929 media, 10% FBS at 4×10^4 stem cells /ml and plated into 24 well plates. Following 2 days culture, the media was replaced with fresh DMEM F-12, 10% L929, 10% FBS \pm 125nM Dex. Cells were then used in experiments at 5-7 days. The scavenger receptor A^{-/-} (SRA^{-/-}) mice were shown not to express SRA on their cell surface, unlike the wildtype controls (see Figure 2.6).

L929 supernatant preparation

L929 murine fibrosarcoma cells spontaneously secrete macrophage-colony stimulation factor (M-CSF). L929 fibroblasts in log growth phase were cultured until confluent in 25ml of DMEM F12 Glutmax, 10% FCS, and 50 units/ml of Penicillin/Streptomycin. The culture media containing the supernatant was then harvested, filtered through 22 μ m nylon filters, aliquoted and stored at -80°C until required.

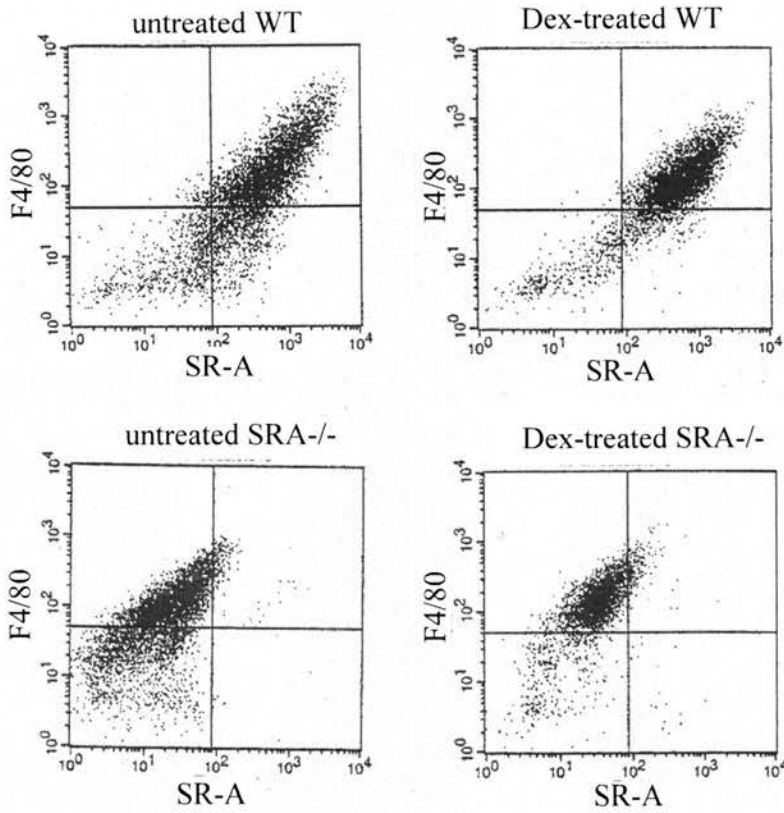


Figure 2.6 Phenotyping SRA knockout and wildtype (WT) mice

Following isolation and differentiation of BMDM Φ from SRA ^{-/-} and WT mice, they were phenotyped to check for SRA expression. BMDM Φ were dual labelled with the mouse macrophage marker F4/80 and SRA. Untreated WT F4/80 positive cells were SRA positive, and SRA expression was not affected by Dex-treatment. The F4/80 macrophages from the SRA^{-/-} mice did not express SRA on their cell surface.

Flow Cytometry software

All flow cytometry data collected was analysed using the Becton Dickinson Cell Quest software package or the EPICS Expo 32 software package.

Statistical Analysis

Data were analysed using the GraphPad InStat statistical package. Data were paired, that is the control and Dex-treated MDM ϕ were from the same donor. However, the data were not normally distributed and was therefore normalised prior to analysis. This was done by transforming the data (calculating log₁₀ of the raw data). This then enabled data to be analysed upon the assumption that the data were paired and from Gaussian distributions.

Data where only two conditions were compared were analysed by a paired t-test (2-tail P value). When more than two conditions were compared, data were analysed by repeated ANOVA with post t-test (Tukey-Kramer Multiple Comparisons Test).

CHAPTER 3: EFFECT OF GLUCOCORTICOIDS ON MDM Φ PHAGOCYTOSIS

Introduction

Endogenous Glucocorticoids

Endogenous glucocorticoids (GC's) are released following infection, the initiation of inflammation or stress. These stimuli act on the hypothalamus to trigger the hypothalamus-pituitary-adrenal (HPA) axis. The hypothalamus releases corticotrophin releasing hormone, which stimulates the synthesis and release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH acts on the adrenal cortex causing release of GCs such as cortisol into the blood stream (Newton, 2000). GCs regulate metabolic processes, for example, the modulation of blood sugar levels, stimulation of gluconeogenesis, and mobilisation of amino and fatty acids in the liver. Importantly, GCs also down-regulate inflammation, an effect mediated in part via changes in transcription. Down-regulation in the transcription of a number of pro-inflammatory mediators occurs, including iNOS, pro-inflammatory cytokines, adhesion molecules and chemokines required for the migration of inflammatory cells. GCs also increase transcription of anti-inflammatory genes such as lipocortin-1, which potentially inhibits production of lipid mediators, and I κ B- α , an inhibitor of the transcription factor NF κ B (Barnes, 1998). Owing largely to the transcriptional down-regulation of survival factors, GCs cause cell type specific induction of apoptosis, for example GCs induce apoptosis in T-cells and eosinophils but not in neutrophils (Amsterdam and Sasson, 2002; Distelhorst, 2002).

GCs mediate their anti-inflammatory and metabolic effects through the glucocorticoid receptor (GR). GCs are believed to passively enter cells via lipophilic regions in their protein structure and once in the cytoplasm they bind to the GR. The GR is held in the cytoplasm via a complex of chaperone proteins which include heat

shock protein-90 (Sanchez et al., 1987). GC binding to the GR induces a conformational change in the GR, which results in the loss of the chaperone proteins and exposure of a nuclear localisation signal.

One mechanism by which neutrophils may evade GC-mediated apoptosis is through the over-expression of the inactive isoform of the glucocorticoid receptor (GR). GC effects are mediated through the binding of the active α -GR isoform and high relative expression of the inactive β -GR isoform in relation to the active α -GR isoform prevents GC- α -GR binding, allowing evasion of apoptosis by neutrophils. The pro-inflammatory cytokine IL-8 acts to up-regulate expression of the β -GR isoform, thereby enhancing neutrophil survival during inflammatory responses (Strickland et al., 2001).

A second level of GC regulation is performed by the enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD). There are two isoforms of this enzyme, 11 β -HSD1 which predominantly converts inactive cortisone to activate cortisol, and 11 β -HSD2 which works in the opposite direction, inactivating cortisol. Therefore the differential expression of the two isoforms, in different tissues, affects the amount of active GCs available to act on cells (Morris et al., 2003).

Mechanisms of Glucocorticoid Action

Transactivation

Ligand-activated GR dimerises and once translocated to the nucleus can bind to 5'-upstream promoter regions of genes at specific DNA sequences called glucocorticoid responsive elements (GRE), promoting gene transcription of anti-inflammatory genes such as lipocortin-1 and β 2 adrenoceptors in a process known as transactivation.

Transrepression

Binding of the GR to negative GRE (nGREs) in promoter regions of genes results in a down-regulation in the transcription of pro-inflammatory genes (Sakai et al., 1988; Zhang et al., 1997). However, very few genes have been shown to contain nGREs and therefore this is not thought to be a common mechanism of action (Barnes, 1998). Transrepression by GCs is predominantly mediated by GR binding directly and indirectly to transcription factors, preventing them from promoting the transcription of pro-inflammatory genes (Adcock et al., 1995; Ray and Prefontaine, 1994).

NF κ B and AP-1 are transcription factors implicated in the up-regulation of a large number of inflammatory genes. The GR-GC complex has been shown to bind directly to, and thus inhibit, the actions of NF κ B. In some cell types, the GR acts to increase transcription of the NF κ B inhibitor, I κ B- α (De Bosscher et al., 1997). I κ B- α binds to and prevents NF κ Bs translocation from the cytoplasm to the nucleus, thus inhibiting transcription of pro-inflammatory genes. The AP-1 transcription factor is a complex of Jun, Fos and other protein families. Pro-inflammatory cytokine activation of the p38 MAPkinase signalling pathway results in the activation of AP-1 via phosphorylation of c-Jun (Shaulian and Karin, 2002). In HeLa cells, TNF- α driven phosphorylation of c-Jun was inhibited by Dex (Gonzalez et al., 2000). The GR also binds to AP-1, resulting in a mutual inhibition of AP-1 and GR activated gene transcription (Barnes, 1998).

GC transrepression effects are also mediated through changes to chromatin structure. DNA is normally tightly associated with histone proteins, forming nucleosomes. Acetylation of the histone residues results in unwinding of the DNA, allowing increased access of transcription factor to gene promoters, and thus increased transcription. Ligand-activated GRs, via co-repressor proteins such as CREB binding protein, cause deacetylation of the histone residues and tighter coiling of DNA. This limits the access of transcription factors, causing a general down-regulation in the transcription of inflammatory genes (Kagoshima et al., 2001).

GC treatment of monocytes results in the production and secretion of an anti-inflammatory agent, thymosin- β 4 sulfoxide. *In vitro* this agent counters neutrophil chemotaxis to FMLP, whilst *in vivo* administration is associated with decreased swelling in a mouse model of inflammation. Together, this data presents a novel anti-inflammatory GC-mechanism by preventing neutrophil recruitment to an inflammatory site during chronic inflammation (Young et al., 1999).

The use of synthetic glucocorticoids in the treatment of inflammation

Synthetic GCs such as dexamethasone (Dex) and prednisolone are the most widely used and effective clinical treatment for a wide range of acute and chronic inflammatory diseases such as rheumatoid arthritis, asthma and multiple sclerosis. Many of these inflammatory disorders are driven by the continued presence of chemokines, pro-inflammatory cytokines and lipid mediators which propagate inflammatory cell recruitment. The anti-inflammatory consequences of GC administration interrupt this inflammatory cycle, abating the effects of the disease (Pelaia et al., 2003). However, their long term use is limited by both the development of GC resistance and severe side effects. The side effects induced by GCs depend on the length of and route of administration of therapy, with the most severe side effects being associated with prolonged systemic treatment. Side effects include atrophy of the skin muscle and adrenal gland, poor wound healing, osteoporosis, glaucoma and cataract, diabetes mellitus, hypertension, thrombosis and peptic ulcers (Schacke et al., 2002).

Dissociated Glucocorticoids

Current consensus is that the severe systemic side effects of long term use of steroids are mainly associated with GC transactivation whilst the beneficial anti-inflammatory and immunosuppressive effects largely associated with transrepression. All currently used therapeutic GCs, including Dex are classed as symmetrical, that is they display activity derived from both transactivation and transrepression.

In order to further examine this theory, Vayssiere and colleagues screened steroids for compounds which displayed reduced transactivation activity. They found three compounds which maintained their transrepression activity but had reduced transactivation activity when compared to Dex. These 'dissociated' glucocorticoids were shown two *in vivo* models to mediate immunosuppressive and anti-inflammatory effects (Vayssiere et al., 1997). A follow-up study demonstrated that these compounds are capable of inhibiting TNF- α induced IL-6 secretion in human Hela and mouse fibroblast cells by a direct interaction with NF κ B gene activity, without altering I κ B α levels (Vanden Berghe et al., 1999). However, a recent study using these dissociated GCs has shown that in *in vivo* models they elicit side effects such as weight loss, thymus involution, and osteopenia of femur growth equally as well as the symmetrical GC budesonide (Belvisi et al., 2001). Another recently identified dissociated GC, which demonstrated full anti-inflammatory properties, was associated with a reduction but not total abolition of side-effects. For example, a decrease in blood glucose levels, a risk factor for Diabetes mellitus, was reduced (Schacke et al., 2004). Seemingly some of the side effects previously coupled to GC transactivation are in fact due to transrepression. Whilst the use of 'dissociated' GCs does not abolish all side effects, their ability to reduce many along with a reduction in risk factors for associated diseases whilst maintaining anti-inflammatory properties may provide additional therapeutic benefit.

In this chapter, data from experiments which examined the mechanism by which Dex treatment of MDM Φ resulted in an augmentation in phagocytosis of apoptotic

neutrophils will be presented. In addition, experiments investigating whether the potential anti-inflammatory process of GC-augmented MDM Φ phagocytosis was a consequence of transactivation or transrepression will also be shown.

Results

Dex augments MDM Φ phagocytosis of apoptotic neutrophils

It has previously been demonstrated that the differentiation of human peripheral blood monocytes in the presence of the synthetic steroid Dex, augments MDM Φ phagocytosis of apoptotic neutrophils (Giles et al., 2001; Liu et al., 1999). To verify this result, human peripheral blood monocytes were differentiated for 5-7 days in Iscove's DMEM + 10% autologous serum (AS) \pm 125nM or 1 μ M Dex and incubated with overnight 'aged' neutrophils as described in Chapter 2, prior to assessment of phagocytic capacity by flow cytometry. Figure 3.1A shows that the Dex-treated MDM Φ s have a significant upregulation in the ability to phagocytose apoptotic neutrophils, compared to the untreated, control MDM Φ .

In view of the paired nature of the data, i.e. one donor's monocytes were differentiated in the presence or absence of Dex, the phagocytosis data is presented as a correlation plot (Figure 3.1B). A best fit line through the data points was drawn and the equation for the line is shown. The R^2 value, however, demonstrates that the data points fit poorly to the best fit line, indicating that the level of control MDM Φ phagocytosis does not serve to predict the level of augmentation following Dex-treatment. However, Dex always increased the phagocytic ability of the MDM Φ s above that of the control MDM Φ s.

Interestingly, a correlation plot of control or Dex-treated MDM Φ phagocytosis versus the percentage of apoptosis in the neutrophil population also yielded a poor correlation - R^2 values for both graphs are near zero (Figure 3.2). Thus, the percentage of apoptotic cells in a population of overnight aged neutrophils does not dictate the level of control or Dex-treated MDM Φ phagocytosis of that population.

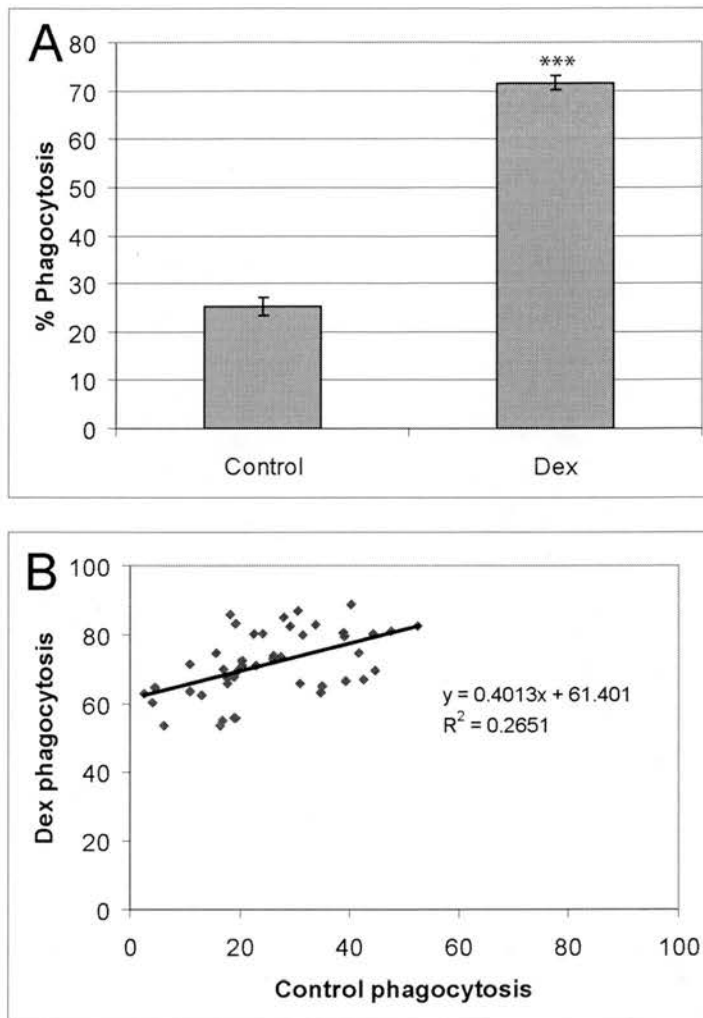


Figure 3.1 Dex augments human MDM Φ phagocytic ability

(A) 5 day control and Dex MDM Φ phagocytosis of overnight 'aged', fluorescently labelled human neutrophils during a 1 hour assay. The phagocytic ability of the MDM Φ was assessed by flow cytometry. Analysis of transformed data by a 2-tail t-test demonstrated that the Dex treatment significantly augmented phagocytosis of apoptotic neutrophils, *** $p < 0.0001$, (n=45).

(B) A correlation plot for the percentage phagocytosis of control vs. Dex MDM Φ was plotted. The R^2 value, which ranges from 0 to 1, predicts how closely the best fit line fits the data, with a value of 1 being the best predictor. For this data $R^2 = 0.27$, suggesting that the extent of Dex-augmentation of phagocytosis is unrelated to the observed level of phagocytosis in untreated, control MDM Φ .

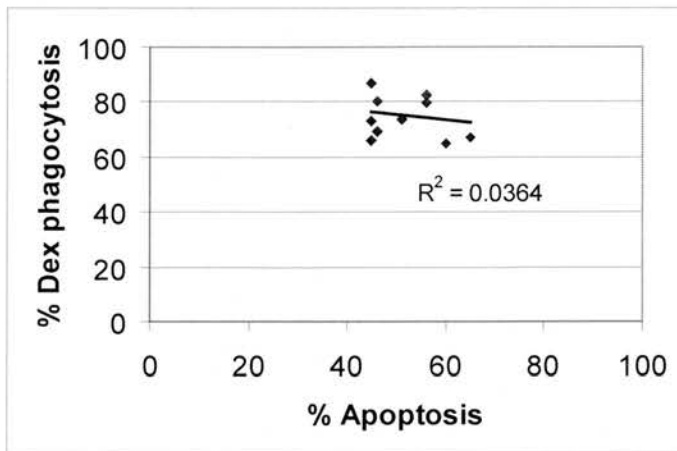
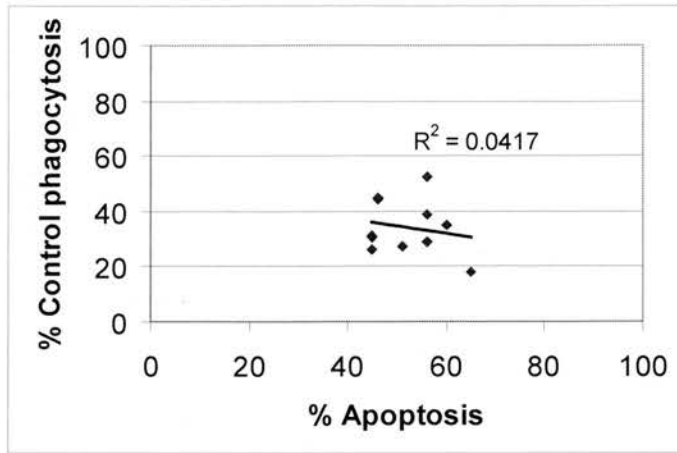


Figure 3.2 The level of apoptosis does not correlate with the level of phagocytosis

The graphs show the correlation of the percentage of control and Dex phagocytosis with the percentage of apoptosis in the neutrophil population, as assessed by morphology. $n=9$ for % control phagocytosis and $n=10$ for % Dex phagocytosis vs % apoptosis.

The R^2 (coefficient of determination) is a value between 0 and 1, which shows how closely the trendline fits the data points. The trendline is most reliable at or near an R^2 value of 1. However the value for both graphs is near to zero, revealing that the best fit line is a poor description of the data points. The percentage of apoptosis does not correlate with the percentage phagocytosis and therefore percentage apoptosis does not appear to limit the extent of phagocytosis.

Monocyte mRNA changes induced by 24 hour treatment with Dex

The phagocytic augmentation by Dex is appreciable following only 24hrs of peripheral blood monocyte culture (Giles et al., 2001; Liu et al., 1999). These data suggest that early changes in gene expression occur that make the Dex-monocytes competent phagocytes. Since GCs mediate anti-inflammatory effects through changes in gene expression, mini cDNA arrays were chosen as a preliminary screen for changes in mRNA following 24 hours culture in control conditions or in the presence of 1 μ M Dex. Appendix 1 shows an image of the scanned gene arrays, whilst the genes on the cDNA array and the changes detected are listed in Appendix 2. Figure 3.3 compares the corrected spot intensity for all genes examined. Points shown above the bisecting line indicate genes which were up-regulated by Dex treatment, whilst those below the line show those down-regulated by Dex treatment. Interestingly, the majority of genes on the array were found to be unaltered by Dex treatment. However more genes showed up-regulated expression than down-regulation following Dex treatment. Owing to the small number of genes investigated, 2 fold changes were considered for further investigation and are presented in a table in Table 3.1.

Verification of changes predicted by the cDNA array

A selection of the Dex-regulated genes (Table 3.1) were chosen for further verification by PCR and either immunoblotting or flow cytometry. Caveolin-1 and p160ROCK (predicted to be up-regulated by Dex) and p21 activated kinase (PAK-1) and ICAM-1 (predicted to be down-regulated by Dex) were chosen because of their involvement in adhesion, migration and phagocytosis. Caveolin-1 was detected by RT-PCR in monocytes and, although the PCR reaction was optimised, the bands were at the limit of detection. Attempts to identify Caveolin-1 by Western blotting repeatedly failed and thus no confirmation of the

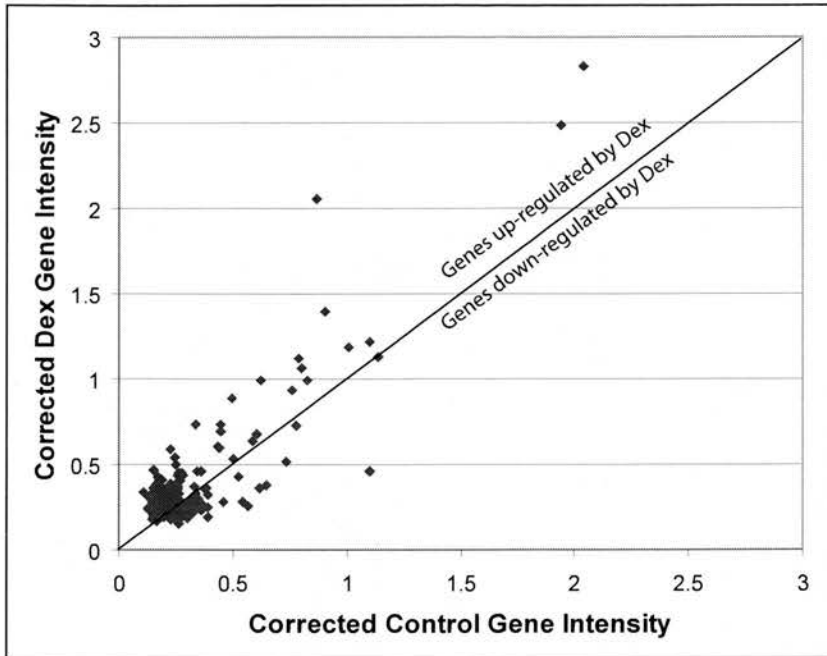


Figure 3.3 Genes up- and down-regulated following 24 hour treatment of peripheral blood monocytes with Dex

Following 24 hours culture of monocytes in the presence or absence of Dex, mRNA was extracted and used to make labelled cDNAs. These were bound to membrane arrays and the relative amount of mRNA from the control or Dex monocytes compared.

The graph shows that more genes were up-regulated than down-regulated by Dex, but that any changes were relatively subtle.

<u>Gene</u>	<u>≥ 2 x up-regulated by Dex</u>	<u>≥ 2 x down-regulated by Dex</u>
Manic fringe homolog	✓	
Opioid-binding protein	✓	
Patched homolog	✓	
Caveolin-1	✓	
Smoothed homolog	✓	
Caveolin-2	✓	
Metastasis associated protein 1	✓	
Matrix Metalloproteinase 18 (19)	✓	
CDC42	✓	
Protein expressed in nonmetastatic cells 4	✓	
Protein expressed in nonmetastatic cells 2	✓	
Focal adhesion kinase 1	✓	
Pyk2	✓	
Prostate specific membrane antigen	✓	
RHOB	✓	
Glycogen synthase kinase 3 alpha	✓	
Cell surface glycoprotein MUC18 (CD146)	✓	
Zinc finger DNA binding protein	✓	
Corneodesmosin	✓	
WNT10b	✓	
CDC42 GTPase activating protein	✓	
Nucleoside diphosphate kinase A	✓	
P160 ROCK	✓	
ELAV like neuronal protein 1	✓	
Matrix metalloproteinase 9		✓
P21 activated kinase		✓ (1.5 x)
ICAM-1		✓

Table 3.1 Genes differentially transcribed following 24 hours Dex treatment

Dex induced up-regulation of Caveolin-1 was possible. In contrast, the up-regulation of p160ROCK was verified at both mRNA and protein levels. cDNA from untreated and Dex-treated monocytes from 4 further donors was used as template for p160ROCK PCR. Actin levels were used as a control for any changes in the efficiency of extraction of mRNA, and in 3 of the 4 donors, an increase in p160ROCK was detected. When control levels were corrected to 100%, those in the four Dex-treated monocytes populations were: 180%, 130%, 80% and 170%. Examination of p160ROCK protein following 24 hours or 5 days culture under either control conditions or Dex-treatment demonstrated that the increased transcription of p160ROCK was translated into protein (Figure 3.4).

PAK-1 was also examined. Whilst the down-regulation in transcription levels following Dex treatment was predicted to be smaller than ICAM-1, PAK-1 expression was of interest because of its role in cellular migration (see chapter 5). Western blotting of 24 hour and 5 day lysates revealed little difference between control and Dex at 24 hours, but an increase in expression following Dex treatment was seen by 5 days, contrary to the effect predicted by examination of the gene array data.

ICAM-1 is known to be regulated by GCs, and surface expression was found to be down-regulated on 5 day Dex-treated MDM Φ (K. Giles, personal communication). Surface expression profiling of 24 hour and 5 day control and Dex-treated MDM Φ showed that the small down-regulation observed at 24 hours, was more exaggerated by 5 days.

In vivo mouse macrophage phagocytosis is not augmented by Dex

Given the *in vivo* use of therapeutic steroids, it was of interest to determine whether the phagocytic augmentation found for Dex-treated MDM Φ s *in vitro* was apparent in an *in vivo* environment. Mice from different strains have inflammatory responses with different predominating cytokine profiles (Mills et al., 2000), so to ensure that any effect seen was not due to strain background the study was carried out in mice of

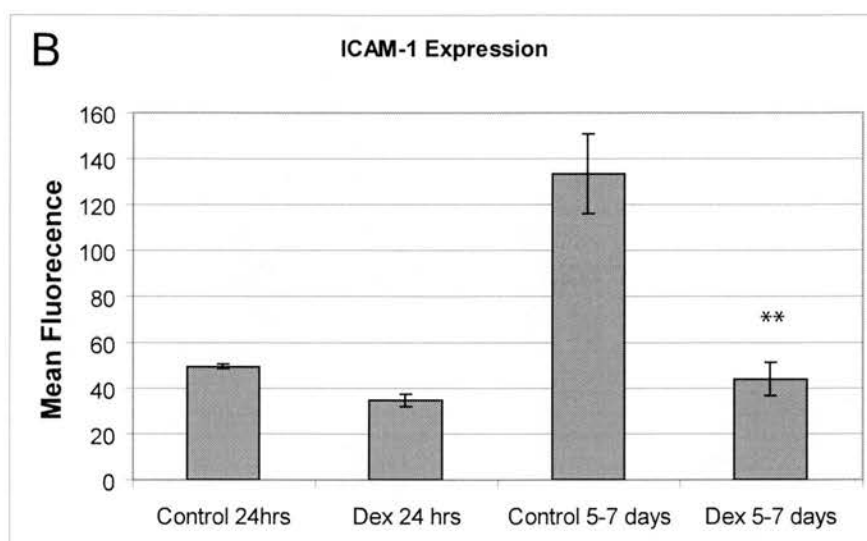
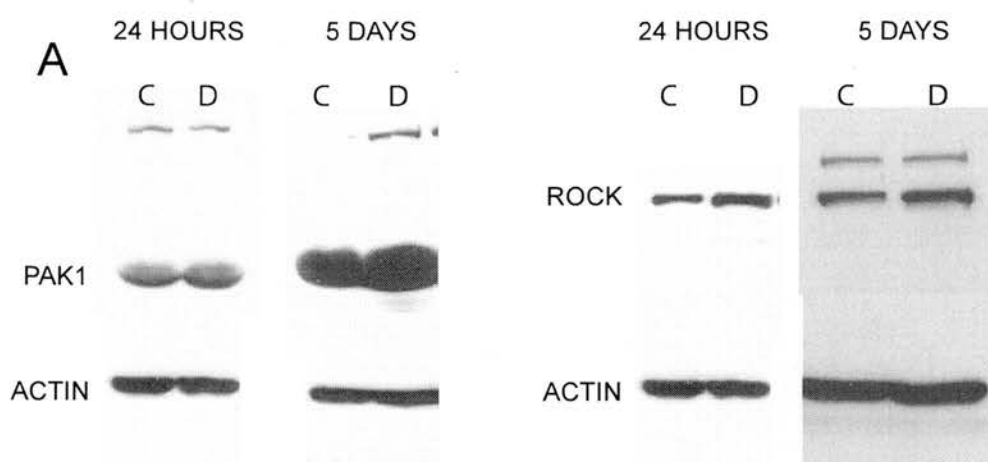


Figure 3.4 Protein expression of molecules predicted by gene array

(A) PAK-1 showed little change at 24 hrs but was upregulated by Dex following 5 days differentiation. p160 ROCK was upregulated in MDM Φ following both 24 hour and 5 day incubation with Dex. A representative blot from 2 experiments for PAK 24 hours, 4 experiments for PAK 5 days and 2 donors for both ROCK experiments is shown.

(B) ICAM-1 expression was profiled at 24hrs and 5 days by flow cytometry. Dex induced a small down-regulation at 24hrs but by 5 days a significant down-regulation was observed. n=4 separate donors. Data analysed by 2-tail t-test, **, p < 0.001 vs. control

two different strains; C57Bl/6 and Balb/c which predominantly have either a Th1 and Th2 cytokine profile respectively. A model of interperitoneal (IP) inflammation was induced by IP injection of thioglycollate into 6-8 week old mice. 24 hours later, at the peak of neutrophilic inflammation (Bellingan et al., 1996), mice were injected interperitoneally with either sterile PBS or 1 μ M Dex. 4 days later the mice were sacrificed and peritoneal cells retrieved by lavage. Interestingly, no significant difference was observed between the number of macrophages/ml lavaged from control and Dex-treated mice from each strain (control BALB/C 1.4x10⁶ macrophages/ml \pm 0.3 SEM; Dex BALB/C 2.4x10⁶ macrophages/ml \pm 0.4; control C57bl/6 2.5x10⁶ macrophages/ml \pm 0.1; Dex C57bl/6 2.3x10⁶ macrophages/ml \pm 0.4). The macrophages were plated into tissue culture plates overnight, prior to assessment of phagocytosis of fluorescently labelled human apoptotic neutrophils. Examination of the FI-1 staining of the F4/80 labelled macrophages enabled phagocytosis to be assessed. Regardless of the strain, the macrophages obtained by peritoneal lavage from mice injected with Dex did not have an augmented phagocytic ability when compared to the mice injected with PBS (Figure 3.5), and thus an *ex vivo* model for macrophage phagocytosis was not generated.

Transrepressor-steroids augment MDM Φ phagocytosis of apoptotic neutrophils

As discussed earlier, GC actions occur through transactivation and transrepression mechanisms. There is evidence supporting the hypothesis that transactivation mediates many of the metabolic side-effects associated with GCs whilst transrepression responses mediate the beneficial anti-inflammatory effects (Barnes, 1998). Whilst Dex-augmented phagocytosis of apoptotic cells is a potentially beneficial and anti-inflammatory mechanism in the resolution of inflammation, it remains unclear how Dex treatment of MDM Φ results in this augmented phagocytosis. It was therefore of interest to determine whether Dex-augmented MDM Φ phagocytosis occurred through a transrepression or transactivation mechanism. Through collaborative links with Glaxo Smith Kline we obtained the rat-

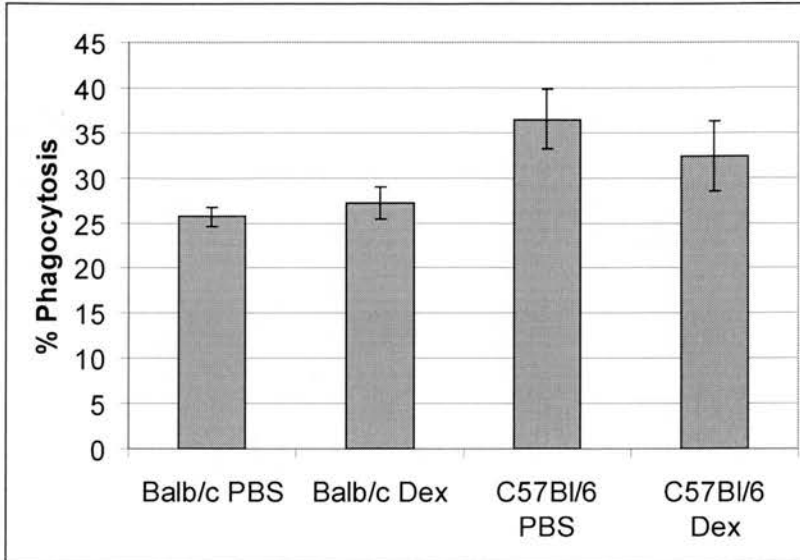


Figure 3.5 Mouse peritoneal macrophage phagocytosis is not affected by Dex-treatment *in vivo*

6-8 week old Balb/c and C57Bl/6 mice were injected interperitoneally (IP) with thio-glycolate. 24 hours later the mice were injected with either PBS or 1 μ M Dex IP. 4 days later the peritoneal was lavaged and the macrophaes plated down in tissue culture plates overnight. Following this, the macrophages were labelled with F4/80 antibody and then used in a 1 hour phagocytosis experiment with FL-1 labelled human neutrophils. The levels of phagocytosis were detected by analysing the FL-1 +ve F480 +ve cells by flow cytometry.

Data shown are mean \pm standard error of the mean (SEM) for 4 separate experiments.

specific GSK transrepressor CC12382 compound. This compound is a specific transrepressor, demonstrated to have much reduced transactivation effects in reporter assays with comparison to symmetrical steroids of similar IC50 such as Dex (S. Farrow, GlaxoSmithKline, personal communication). We therefore used rat bone marrow-derived macrophages (BMDM Φ) cultured in control conditions or either Dex or GSK CC12382 for 7 days. Following differentiation of the BMDM Φ , phagocytic ability was assessed using overnight aged human neutrophils as apoptotic targets. Interestingly, the control BMDM Φ were much more capable phagocytes than either the human MDM Φ or mouse peritoneal MDM Φ s (Figure 3.6). A significant augmentation in phagocytosis was observed following treatment of the BMDM Φ with either Dex or the GSK-transrepressor steroid. This data suggests that GC transrepressor activity alone is sufficient to induce the augmentation in phagocytic ability.

I κ B- α protein levels and phosphorylation of c-Jun are associated with Dex treatment of human peripheral blood monocytes

As discussed in the introduction to this chapter, one of the main ways in which steroid transrepressor action is achieved is by interference with the pro-inflammatory transcription factors, NF κ B and AP-1. In order to examine whether either of these transcription factor pathways was associated with the augmentation of MDM Φ phagocytosis, the levels of I κ B- α , an inhibitor of NF κ B translocation to the nucleus, and the phosphorylation of c-Jun, a component of AP-1, were examined. Lysates of peripheral blood monocytes cultured for 30, 60, 90 minutes or 24 hours either in control conditions or 125nM Dex were prepared and tested for the levels of proteins by western blotting. Protein levels of I κ B- α were higher in 30 minute Dex lysates

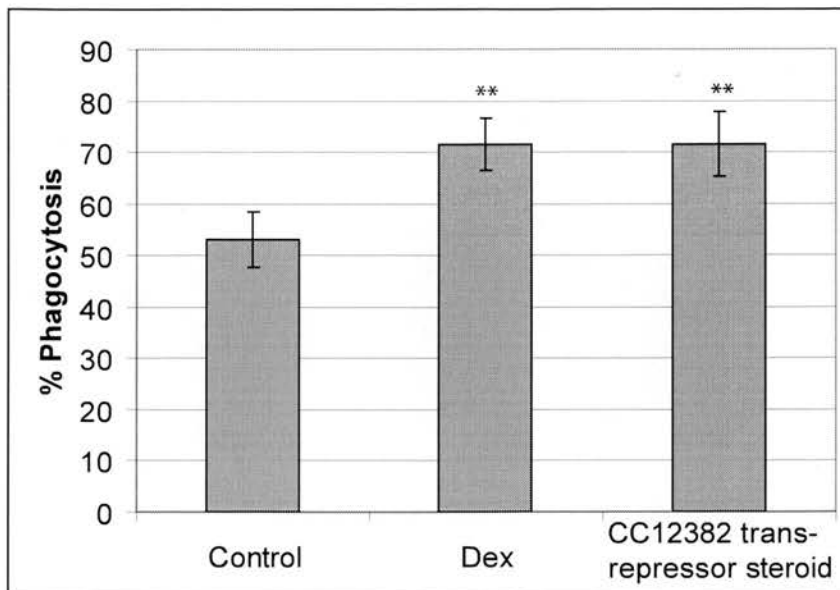


Figure 3.6 Dex- augmented phagocytosis is a result of transrepressor activity

7 day rat BMDM Φ were differentiated *in vitro* in control conditions or 125nM Dex or GSK CC12382 transrepressor steroid. The phagocytic ability of apoptotic neutrophils was assessed in a plate assay. A minimum of 300 cells were counted per well, and each condition was performed in duplicate and the mean value used. Transformed data were analysed by Tukey repeated ANOVA. ** p < 0.01 vs. control. Both Dex and the transrepressor significantly increased phagocytosis levels above that of the control macrophages, demonstrating that the transrepressor element of steroid action is sufficient for the augmentation of phagocytosis. Data shown are mean phagocytosis \pm SEM for 3 bone marrow preparations from separate rats.

than time-matched control lysates, although no differences were detected between control and Dex-treated MDM Φ samples at subsequent time points (Figure 3.7). This suggests that a relatively rapid effect of Dex is either induction of transcription of, or protection against the degradation of I κ B- α protein. This may provide a mechanism for the inhibition of the transcription of pro-inflammatory genes. Phosphorylated c-Jun was only detected once in three experiments although the bands were very faint. The blot where phosphorylated c-Jun was detected showed an up-regulation following 24 hour Dex treatment, but the control and Dex-treated MDM Φ lysates showed identical levels at time points prior to this. This data suggests that total I κ B- α protein levels are controlled by Dex at early stages of monocyte-MDM Φ differentiation, where NF κ B would be predicted to alter early gene expression.

MDM Φ differentiated in the absence of serum have a reduced phagocytic ability

There is increasing evidence that serum components are involved in the phagocytosis of apoptotic cells (Hart et al., 2004). It was therefore of interest to establish whether serum components affected MDM Φ phagocytosis. To this end, peripheral blood monocytes were differentiated in the presence of 10% autologous serum \pm 125nM Dex or in serum-free media + 250U GMCSF \pm 125nM Dex. The differentiation under serum-free conditions or serum-plus conditions did not induce apoptosis in the MDM Φ populations as assessed by morphology (data not shown). The phagocytosis of overnight 'aged', apoptotic neutrophils by the resultant MDM Φ populations was then examined by flow cytometry. Serum-free differentiated MDM Φ exhibited low levels of phagocytic ability either with or without Dex treatment. However, phagocytic capacity was restored by the re-addition of serum on day 0 to the serum-free conditions (Figure 3.8). This data suggests that serum components play an important role, possibly providing factors required for the differentiation of a phagocytic competent MDM Φ .

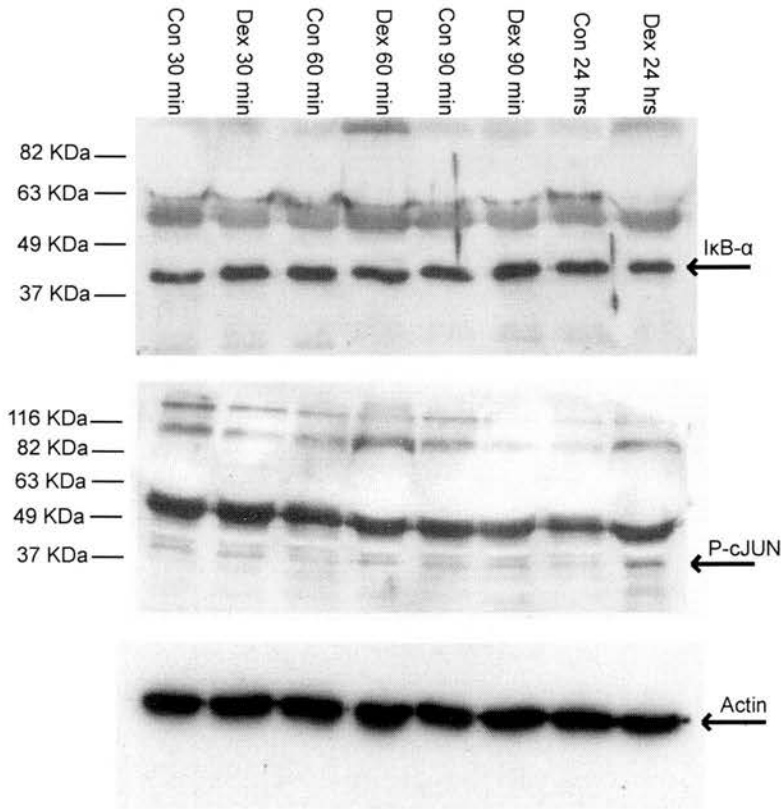


Figure 3.7 Monocyte IκB-α protein levels increase following Dex treatment

Lysates were prepared from peripheral blood monocytes cultured in Iscove's DMEM + 10% AS ± 125nM Dex for 30, 60, 90 minutes or 24 hours. Following 30 minutes culture, IκB-α protein levels were increased in Dex macrophages compared to control macrophages, although levels were equal by 24 hours. Representative gel from 1 experiment of 3 that were performed is shown.

Low level detection of phosphorylated c-Jun was detected in both control and Dex-treated monocytes at 30, 60 and 90 minutes. At 24 hours Dex but not control lysates showed an up-regulation in phosphorylation of c-Jun. Blot is representative of 1 gel.

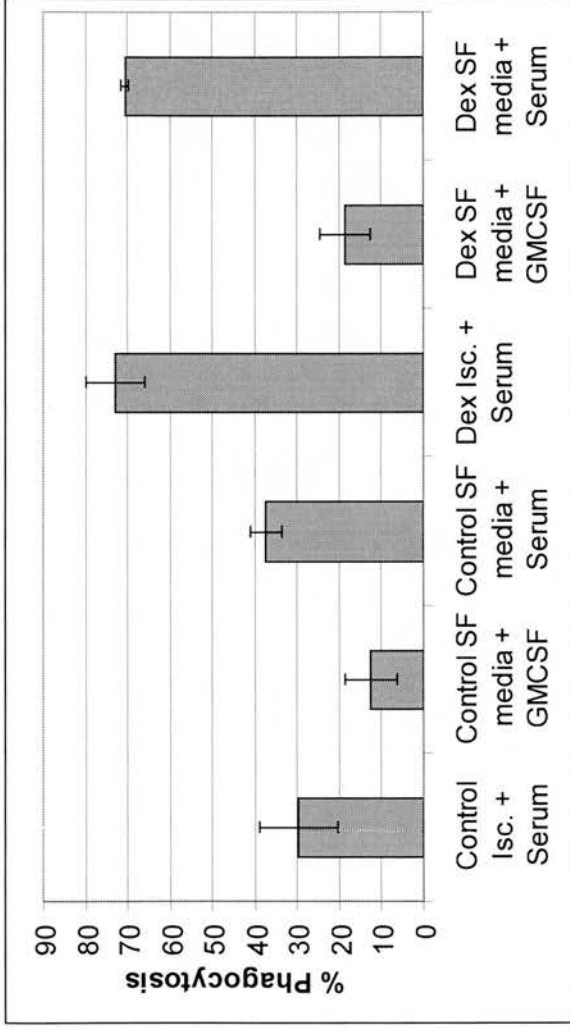


Figure 3.8 Role for a serum component in MDM Φ phagocytosis of apoptotic neutrophils

Peripheral blood monocytes were differentiated for 5 days either in Iscove's DMEM (Isc.) + 10% AS \pm 125nM Dex, or in serum-free media (SF) + GMCSF \pm 125nM Dex or in serum-free media + 10% autologous serum \pm 125nM Dex. The ability to phagocytose apoptotic neutrophils was assessed by flow cytometry. Data shown are the mean % phagocytosis from 3 experiments \pm SEM. Data shown for control and Dex SF media + serum are from 2 experiments.

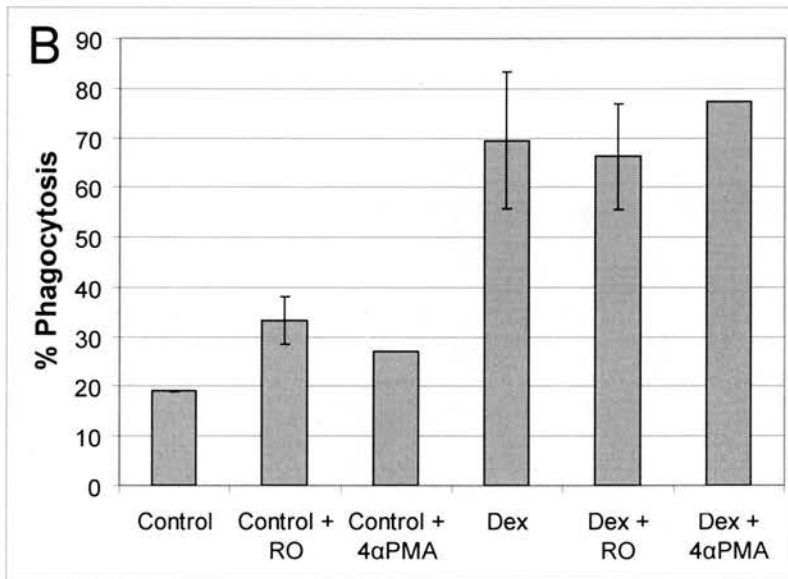
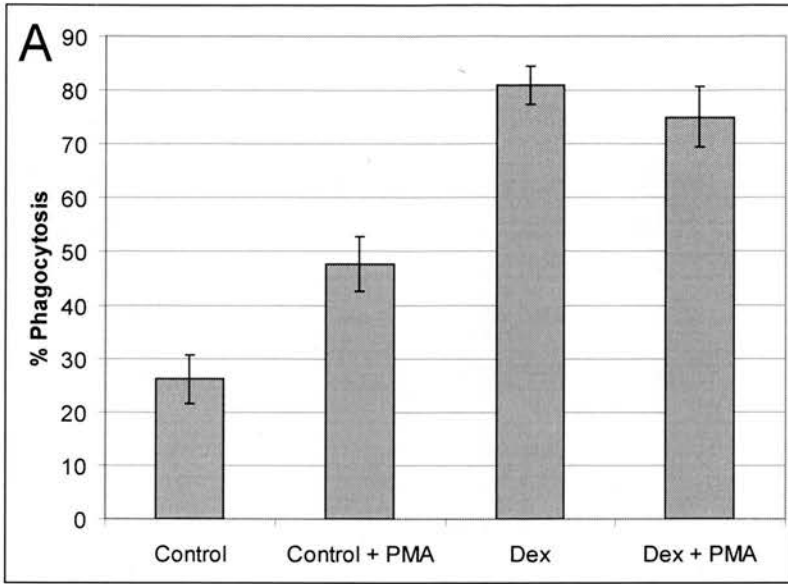
Activation of PKC increases MDM Φ phagocytosis

The PKC signalling pathway has been shown to be required for engulfment following receptor-ligand binding of the apoptotic cell to the MDM Φ (May and Machesky, 2001) and it was therefore of interest to examine the role of PKC during Dex-augmented phagocytosis. Human MDM Φ were differentiated in Iscove's DMEM + 10% autologous serum \pm 125nM Dex for 5 days and then incubated with 30nM PMA (specific PKC activator) for 1 hour prior to phagocytosis of apoptotic neutrophils. Control MDM Φ showed an augmentation in phagocytic ability following treatment with PMA greater than that observed with the inert 4- α PMA, raising the possibility of a role for PKC in the regulation of phagocytosis. However, phagocytosis by Dex MDM Φ was unaffected by PMA treatment, suggesting that Dex-treated MDM Φ either exhibited maximal activation of PKC or that PKC activity was not important in Dex-treated MDM Φ (Figure 3.9A).

To further investigate the role of PKC in MDM Φ phagocytosis, 5 day control and Dex-treated MDM Φ were incubated with the PKC inhibitor, RO 31-8220 compound (Figure 3.9B). Interestingly, inhibition of PKC was also found to augment phagocytosis by control MDM Φ , suggesting that a critical balance of PKC activation may be required. However, the Dex-augmented phagocytosis was unaffected by inhibition of PKC, further suggesting that this pathway is not involved in Dex-augmented phagocytosis.

Figure 3.9 PKC activation augments untreated but not Dex-treated MDM ϕ phagocytosis of apoptotic neutrophils

(A) Untreated and Dex-treated 5 day MDM ϕ were treated with 30nM PMA (PKC activator) or (B) 1 μ M RO 31-8220 (PKC inhibitor) or 80nM 4- α PMA (inert compound) for 1 hour. The ability of the MDM ϕ to phagocytose apoptotic neutrophils was then assessed by flow cytometry. Data shown are mean % phagocytosis for 3 experiments (A) and 2 experiments for RO (B) \pm SEM and 1 experiment for 4- α PMA (B).



Discussion

Dex-augmented phagocytosis of apoptotic cells

My results confirm that there was a large inter-donor variability in the phagocytic ability of the untreated macrophage population (2.7% - 52.6%) and that Dex-treatment consistently up-regulated phagocytic ability, whilst reducing variability in phagocytic ability within the population (53.5% - 87%). However, this decrease in phagocytic 'range' following Dex-treatment may also be explained by the presence of a 'ceiling' in phagocytic ability. Dex-treatment may cause this 'ceiling' in phagocytic ability to be reached for each of the MDM Φ populations and therefore Dex-treatment can only induce a small augmentation in phagocytosis in control MDM Φ with a high baseline phagocytic ability. It might be expected that the baseline phagocytosis in control MDM Φ would dictate the extent of Dex-augmentation if the processes used for control and Dex-treated phagocytosis were the same. Somewhat surprisingly, examination of the phagocytic abilities of untreated and Dex-treated MDM Φ from the same donor revealed that the percentage of apoptotic cells phagocytosed by the untreated macrophages did not predict the extent to which Dex was able to augment phagocytic ability. Furthermore, it was also demonstrated that the percentage of apoptosis in the overnight 'aged' neutrophil populations did not correlate with either control or Dex-treated MDM Φ phagocytosis. Experiments investigating the effect of the concentration of apoptotic neutrophils (8, 4, 2, and 1×10^6 cells per ml) on phagocytosis demonstrated that a reduction in the concentration correlated with a step-wise decrease in both control and Dex-treated MDM Φ phagocytosis for concentrations below 4×10^6 /ml (data not shown). This raises the possibility that the lack of correlation between (morphologically defined) percentage apoptosis and phagocytosis was due to an excess of target cells. A second explanation is that the control and Dex-treated MDM Φ phagocytose a range of apoptotic and necrotic cells and that levels of necrosis need to be taken into account too. This suggestion is in accordance with data

from Devitt and colleagues. They demonstrated that maximal human MDM Φ phagocytosis of apoptotic Mutu I Burkitt's lymphoma cells correlated with later time points in apoptotic cell culture, where the majority of target cells had become necrotic (Devitt et al., 2003). The idea that control and Dex-treated MDM Φ phagocytose both apoptotic and necrotic targets could be investigated by carrying out similar studies to those of Devitt and colleagues, where levels of phagocytosis could be correlated with both the number of apoptotic nuclei and PI uptake at different time points during 20 hour culture of the neutrophils.

Glucocorticoid Modulation of Gene and Protein Expression

I used an 'interaction' array which included cytoskeletal signalling proteins such as Rho GTPases and their effector molecules, extracellular matrix proteins, metalloproteinases, cell-cell adhesion proteins and cell surface antigens to screen for gene changes that might be important for adhesion, migration and phagocytosis. Only a small proportion of the genes included on the array showed a two fold change following Dex-treatment (9% of the genes analysed when compared with untreated monocytes), with the majority of these genes being up-regulated. One of the mechanisms by which steroids mediate anti-inflammatory actions is by deacetylation of histones, reducing transcription factor access to promoter regions of pro-inflammatory genes and thus reducing the transcription of pro-inflammatory genes (Wolffe, 1997a; Wolffe, 1997b). The very small overall up-regulation of genes seen in Figure 3.1, suggests that many of the genes included on the array are not regulated by this mechanism.

The gene array data was verified for a selection of molecules, at the gene or protein level. Interestingly, the changes observed at 24 hours were not always maintained by 5 days. Indeed, ICAM-1, shown to be down-regulated on the gene array following Dex-treatment was detected at reduced levels on the Dex-treated monocyte cell surface at 24 hours compared to control expression. However, the extent of the Dex-mediated inhibition in ICAM-1 expression was increased by 5 days. The gene array

data predicted a down-regulation in PAK-1, but protein studies showed a small up-regulation in expression at 24 hours and a further increase by 5 days following Dex-treatment. The gene array was chosen as a tool to search for changes to gene expression induced by Dex-treatment and whilst mRNA levels matched protein expression for some genes such as p160ROCK, it does not for others such as PAK-1. The use of an antibody-protein array may solve some of these problems but would not account for protein synthesis, turnover, localisation or post-transcriptional modifications such as phosphorylation, all of which affect the function of a particular protein. However, the use of a large scale antibody-protein array would enable a large number of proteins to be screened for differential regulation by Dex, and may therefore prove useful in highlighting changes to key molecules of signalling pathways or structures.

The data showed a two fold or greater change in a range of genes following 24 hours Dex-treatment (Figure 3.4). They included known regulators of migration and actin polymerisation, such as p160ROCK, Pyk2, Cdc42 GTPase activating protein and Cdc42, a Rho GTPase family member which regulates actin polymerisation during migration, adhesion formation and phagocytosis. Cdc42 is necessary for phagocytosis of apoptotic cells (Leverrier and Ridley, 2001) and, during Fc-mediated phagocytosis, Cdc42 appears to be required for pseudopodia extension around the ligand bound cell (Hoppe and Swanson, 2004). Extension of these studies would allow for examination of Cdc42 protein levels and activation. Experiments using agarose-beads coupled to the Cdc42-binding domain of PAK-1 could be used to examine the percentage of active Cdc42 following Dex treatment. Furthermore, immunofluorescence studies may determine if there is increased presence of Cdc42 at the phagocytic cup in Dex-treated MDM Φ .

A recently published gene array experiment, investigating changes in human peripheral blood monocytes following 18 hours Dex treatment confirmed my observed down-regulation in MMP9, and also showed that Dex did not alter transcription of CD36, CD44, MMP 7, 10 and 16 (Galon et al., 2002). My gene array results showed differential regulation of two matrix metalloproteinases (MMP);

MMP18 was up-regulated whilst MMP9 was down-regulated. MMP9 is a terminal member of the protease cascade which degrades extracellular matrix during processes such as wound healing (Opdenakker et al., 2001) and interestingly it is also known to be down-regulated by GCs in rat mesangial cells (Eberhardt et al., 2002) and by anti-inflammatory cytokines such as IL-4 and IL-10 (Lacraz et al., 1994). A down-regulation in MMP9 following Dex-treatment is therefore in agreement with the anti-inflammatory effects of steroids, and with studies demonstrating steroid inhibition of wound healing. Interestingly, both caveolin-1 and -2 were also predicted by the array to be up-regulated by Dex. They are proteins which form a scaffold at the plasma membrane at which signalling molecules assemble. Caveolin proteins therefore concentrate signalling molecules and can also govern the activation of some of the signalling molecules (Harris et al., 2002). One speculation would be that augmentation of phagocytosis by Dex increases the efficiency of assembly of scaffolds containing receptors required for the phagocytosis of apoptotic cells.

The augmentation in MDM Φ phagocytosis of apoptotic neutrophils was observed early during the differentiation process, following only 24 hours of Dex treatment, suggesting that this period was critical for augmenting phagocytosis. The gene array was therefore used to search for early changes to mRNA levels following Dex treatment. However, following the results demonstrating that protein expression can alter later during the differentiation process, it may be interesting to examine changes present in the MDM Φ following 5 day's culture. Adherence purified peripheral blood monocytes were used for the array as they provide a high purity of monocytes with typically only 3-5% of the adherent cells being contaminating lymphocytes. Despite the fact that Dex treatment can selectively induce apoptosis in certain cells such as T cells and eosinophils (Amsterdam et al., 2002), no difference in the number of contaminating lymphocytes was found between the untreated and Dex-treated MDM Φ following 24 hours of culture. The detection of CD4 and FAK on the cDNA array but failure to detect FAK by Western blotting from cells purified by the same technique, suggests that the array is very sensitive to low level contamination. This presents some problems with the interpretation of results obtained from the array and it may therefore be prudent to verify future array results by analysis of protein.

Methods for the removal of lymphocytes from mononuclear cell preparations, such as MACS purification (Miltenyi Biotech), result in 85-90 % monocyte purity (results not shown) and the removal of further contaminating lymphocytes by adhesion purification may help to reduce the total number of contaminating cells. However, based upon my experimental data using purified cells, even if the gene array experiment was to be carried out using 5 day MDM Φ , a significant lymphocyte contamination would be expected, possibly as a result of proliferation.

Transrepression activity in augmentation of phagocytosis of apoptotic cells

Dex-induced gene changes investigated include a down-regulation in the NF κ B-dependent genes ICAM-1 and MMP9 (Eberhardt et al., 2002), suggesting that alterations to the NF κ B transcription factor signalling pathway are induced in monocytes following 24 hours of Dex treatment. Using a rat-specific dissociated glucocorticoid (transrepression activity only), the augmentation of phagocytosis of apoptotic cells was demonstrated to be exclusively a result of transrepression. As discussed in the Introduction, transrepression processes occur via the inhibition of pro-inflammatory transcription factors, NF κ B and AP-1. Further studies investigated changes in these transcription factor signalling pathways, demonstrating a clear up-regulation in protein expression of the NF κ B inhibitory protein, I κ B- α . I κ B- α binds and retains NF κ B in the cytoplasm, preventing NF κ B-promoted gene transcription. It would seem that a down-regulation in the expression of pro-inflammatory genes, ultimately results in changes to the Dex-treated MDM Φ whereby they up-regulate signalling pathways and/or receptor expression necessary for phagocytosis of apoptotic cells. Whilst Dex treatment of MDM Φ has been associated with alterations in Rho GTPase family members, the mechanism by which this is achieved is unknown. Changes to the cytoskeleton and in actin polymerisation are clearly important to phagocytosis, and I have demonstrated that the up-regulation in phagocytosis is a result of transrepression. Therefore, whether directly or indirectly,

changes to the levels and activation status of Rac, Cdc42 and Rho must be a result of this. It would be very interesting to investigate changes in these Rho GTPases after inhibition of either the NF κ B or AP-1 transcription pathways by specific inhibitors.

GCs can inhibit NF κ B activity by induced transcription of I κ B- α (Barnes and Karin, 1997) (Scheinman et al., 1995) and by direct protein-protein interaction between the GR and NF κ B (Marx, 1995). I κ B- α has been shown to bind to NF κ B and prevent its translocation to the nucleus in HeLa cells, lymphocytes and vascular smooth muscle cells (Scheinman et al., 1995) but not in rat mesangial cells or brain cells (Bourke and Moynagh, 1999), suggesting this is a cell-specific mechanism. My data demonstrate very early increases in I κ B- α levels following Dex treatment that were not maintained later during differentiation (24 hours or 5 days). This may suggest that the process of GC transrepression, acting through blockade of NF κ B activity, sets in action an altered monocyte to macrophage differentiation pathway, leading to augmented 5 day MDM Φ phagocytosis of apoptotic cells. However, the transient nature of I κ B- α up-regulation seems unlikely to fully account for all Dex-mediated changes. Further insight into the role of I κ B- α inhibition of NF κ B-promoted gene transcription could be gained. Blocking NF κ B by using specific inhibitors or by over-expressing I κ B- α in control MDM Φ , perhaps by using a TAT- I κ B- α fusion protein, may allow examination of the changes induced by this signalling pathway in MDM Φ . Follow-up studies could also include electrophoretic mobility shift assays (EMSA) to confirm whether the increased protein levels of I κ B- α do in fact inhibit NF κ B binding to DNA in the Dex-treated MDM Φ .

Dex represses AP-1 activity by inhibition of c-Jun phosphorylation (Gonzalez et al., 2000), by the GC-inducible leucine zipper (Mittelstadt and Ashwell, 2001) or by direct protein-protein binding of the GR to AP-1 (Jonat et al., 1990). The result demonstrating an up-regulation in c-Jun phosphorylation following 24 hours Dex treatment, but identical low levels in control and Dex lysates at time points prior to this clearly warrants more detailed examination, but suggests that GC transrepression is not acting via inhibition of c-Jun phosphorylation. Again, EMSA could also be employed to detect if AP-1 binding of DNA is down-regulated. Finally,

immunofluorescence studies or co-immunoprecipitations would allow examination of the interaction between the GR and NF κ B or AP-1 to be investigated.

Mediators of GC phagocytosis

Serum components are implicated in the phagocytosis of apoptotic cells, both as bridging molecules such as thrombospondin and MFG-E8 and in opsonisation of the apoptotic cell (Hart et al., 2004). The experiments presented here demonstrate that lack of serum during the differentiation of both control and Dex- treated MDM Φ strongly inhibits phagocytic ability without inducing apoptosis in the MDM Φ . In these experiments the neutrophils were cultured overnight to induce apoptosis in the presence of serum, which should allow time for them to become opsonised. This may also mean that there are enough serum components during the phagocyte-apoptotic cell interaction to provide bridging components. This could be addressed by carrying out experiments to add serum back during the phagocytosis assay, with a failure to restore phagocytic ability confirming this idea. Instead, my data suggests that serum components are required during the differentiation process for a phagocytic MDM Φ since phagocytic ability was rescued by addition of serum to the cultures. Further identification of the component(s) in the serum responsible for the phagocytic phenotype could be achieved by biochemical fractionation of serum.

Roles for protein kinase C (PKC) during phagocytosis of apoptotic cells as well as Fc-mediated phagocytosis have been described (Hu et al., 2002; May and Machesky, 2001). The results presented in this chapter show that activation of PKC by PMA increases the phagocytic ability of control MDM Φ above that of the inert 4- α PMA. A small increase in phagocytic ability (lower than that induced by PKC activation) was also observed following treatment of the control MDM Φ with the PKC inhibitor, RO 31-8220, which suggests that PKC activation is a determinant of phagocytic ability in these cells. However, inhibition by RO 31-8220 failed to inhibit internalisation of apoptotic neutrophils. My results contradict studies such as those

by Hu et al., which demonstrated an inhibition in internalisation but not binding of apoptotic thymocytes by murine tissue macrophages (Hu et al., 2002). However, contradictory results have been found for the involvement of PKC in Fc-mediated phagocytosis (Allen and Aderem, 1996; May and Machesky, 2001). Inhibition of PKC can prevent focal adhesion formation in fibroblasts in association with a dispersal in adhesion components such as talin and actin (Woods and Couchman, 1992). PMA activation of PKC may regulate adhesion formation (Woods and Couchman, 1992) and studies also suggest that PKC activation may increase integrin-mediated adhesion (Gladwin et al., 1990). In my studies, control MDM Φ , activated by PMA, became more spread and adherent (data not shown) suggestive of altered adhesion regulation.

Dex-treated MDM Φ lack podosome adhesions concomitant with a loss in phosphorylation and recruitment of key adhesion components (Giles et al., 2001) and coupled with the PKC data presented here may suggest that Dex causes a block on the PKC pathway. Dex-treated MDM Φ phagocytosis was unaffected by the activation or the inhibition of PKC, and it is therefore likely that Dex augments phagocytosis via a PKC-independent mechanism. In light of this interesting result I would investigate the roles of other signalling pathways implicated in internalisation of apoptotic cells such as PI3kinases and tyrosine phosphorylation (Leverrier and Ridley, 2001; May and Machesky, 2001; Stephens et al., 2002; Todt et al., 2004). One method employed by Greenburg and colleagues, investigating tyrosine phosphorylation events during Fc-mediated phagocytosis, was to examine tyrosine phosphorylation by immunoblotting and immunoprecipitation lysates from Fc-receptor stimulated macrophages (Greenberg et al., 1994).

CHAPTER 4: MDM Φ BINDING AND INTERNALISATION OF APOPTOTIC NEUTROPHILS

Introduction

The process of apoptotic cell phagocytosis begins with the recognition of the apoptotic cell by the macrophage, most probably by receptors directed against 'eat me' signals, such as PS, or carbohydrate modifications on the surface of the apoptotic cell. A selection of the described receptors (see chapter 1) have been implicated in human MDM Φ recognition of apoptotic cells. Recognition of apoptotic B cells (Flora and Gregory, 1994) requires CD14 (Devitt et al., 1998) whilst the vitronectin receptor/CD36/thrombospondin complex (Savill et al., 1990) is thought to be the major receptor for apoptotic neutrophils, with minor roles for scavenger and lectin receptors (Fadok et al., 1998b).

The identification of multiple, apparently redundant receptors can perhaps be explained by specific roles for the different receptors. Some receptor recognition mechanisms may be specific to particular tissues, for example the phenotype of SRA deficiency is particularly evident during peritoneal inflammation but not in the thymus (Platt et al., 1996). Secondly, receptors may be species-specific or recognise changes to specific apoptotic cell types (Savill and Fadok, 2000). CD14, for example, is chiefly implicated in recognition of lymphocytes (Devitt et al., 1998) and lectin-like receptors in mouse macrophage recognition of thymocytes (Duvall et al., 1985). The 'tether and tickle' hypothesis put forward by Henson and colleagues (Henson et al., 2001) suggested that redundancy can be explained by a two-step recognition mechanism. They proposed that apoptotic cell removal required 5 separate events (see Figure 4.1): cell surface changes and ligand expression on the apoptotic cell; macrophage receptor recognition of these ligands; instigation of macrophage signalling events, triggered by the 'tethering' receptor or by a separate 'uptake' receptor; engulfment and finally digestion of the apoptotic cell within the

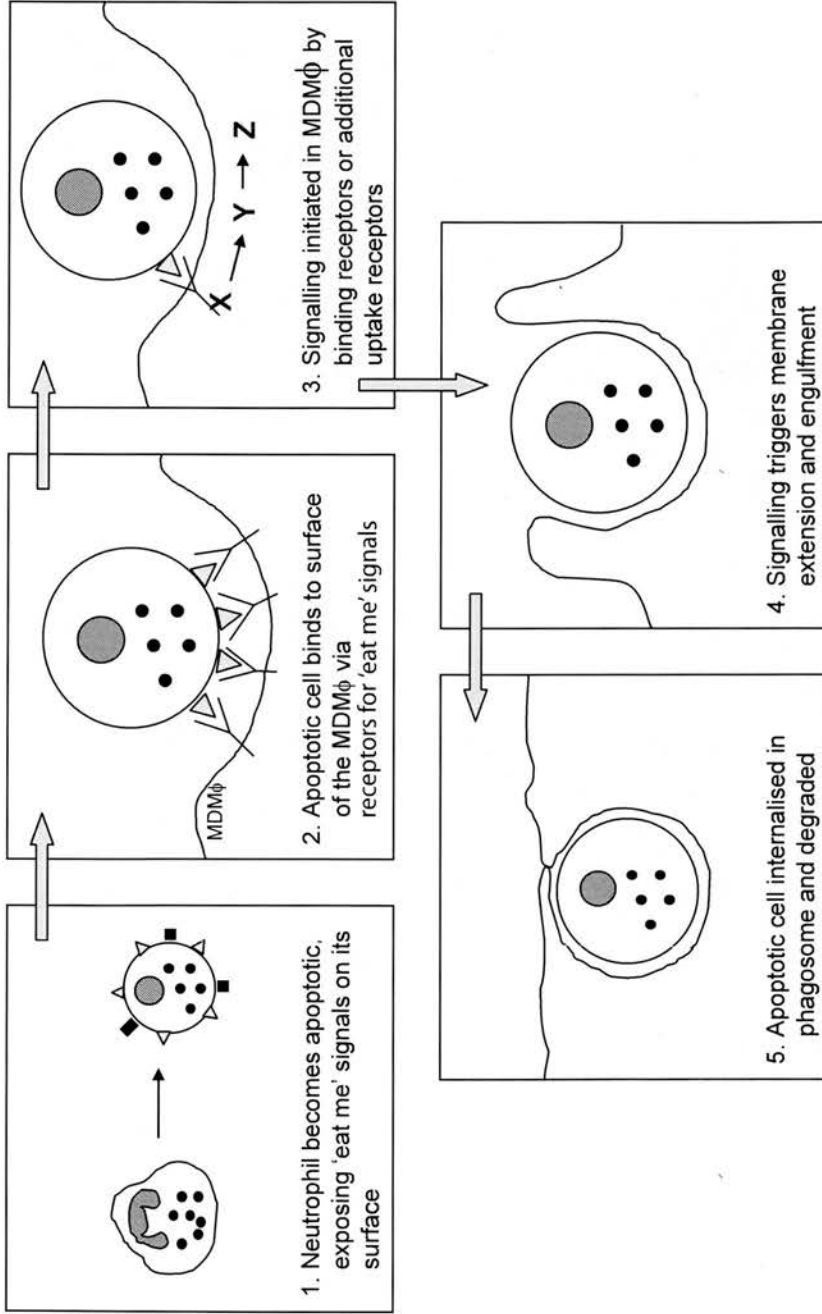


Figure 4.1 Proposed mechanism of apoptotic cell recognition and engulfment
Adapted from text of Henson, P. M. et al 2001

phagosome. The initial tethering of the apoptotic cell may therefore be independent from the initiation of signalling events required for engulfment, which potentially utilises a second set of receptors, engaged after the apoptotic cell is bound (Henson et al., 2001). This two-step recognition model could explain the large number of potential phagocytic receptors, with some functioning as ‘tethering’ receptors and others as ‘uptake’ receptors.

Erythrocytes modified to express defined ligands or antibodies against known phagocytic receptors on MDM Φ were used to examine individual receptors roles in binding and uptake (Hoffmann et al., 2001). They demonstrated a ‘tethering’ role for many of the previously described phagocytic receptors, including CD36, $\alpha v\beta 3$, CD14 and CD68. SRA induced low but equal levels of engulfment and tethering. However, PS did not mediate tethering, although PS-PSR interaction was required for uptake of the erythrocytes, suggesting that it provided the ‘tickle’ or signalling required for actin polymerisation and engulfment. However, this data appears at odds with previous demonstrations that PS was not involved in the phagocytic cell uptake by unstimulated MDM Φ (Fadok et al., 1992b; Savill et al., 1990), raising the possibility that PS expression on the surface of erythrocytes is recognised differently to PS on the surface of an apoptotic cell. Nevertheless, the proposed model is attractive because it makes signalling via low avidity PS-binding feasible and confers specificity on the process of recognition by having multiple binding receptors followed by a separate signalling receptor. Furthermore, the ‘tether and tickle’ model negates the potential problem causing by recognition and signalling following binding to transiently expressed PS on non-apoptotic cell surfaces (Somersan and Bhardwaj, 2001).

However, the idea of a two-step recognition process poses many questions. PS is potentially recognised by multiple receptors which include CD36, the OxLDL and the PS-receptor (for which the data is equivocal), and many molecules have been proposed to bind PS, including annexins and MFG-E8. Since PS can potentially be recognised by both the proposed ‘binding’ and ‘signalling’ receptors, the model requires that the receptor determines whether signalling is induced. The current

suggestion that multiple ligand-receptor interactions serve to bind the apoptotic cell to the surface of the macrophage, whilst only one receptor is capable of triggering signalling seems lacking, particularly since many of the putative 'binding' receptors have been shown to initiate signal transduction within the cells, for example, integrins (Akakura et al., 2004; Albert et al., 2000), CD36 (Trezza et al., 1990) and the receptor tyrosine kinase MerTK (Todt et al., 2004). For the proposed model to hold true, it seems likely that further signalling receptors remain to be defined. Firstly, PS recognition is not involved in human MDM Φ engulfment of apoptotic cells (Fadok et al., 1992b), and secondly, as both necrotic and apoptotic cells express PS on their surfaces, it is unclear how can signalling via the same PS interaction can mediate both pro and anti-inflammatory cytokine responses from the phagocyte?

In conclusion, verification of the two-step recognition model is required together with clearer definition of the roles of phagocytic receptors. Experiments examining the roles of putative phagocytic receptors have used apoptotic cell binding or phagocytosis, or both as read out, and examination of the literature allows some refinement as to whether receptors are 'binding/tethering' receptors or 'signalling/tickling' receptors. CD14 lacks intracellular signalling domains, thus it is reasonable to suggest that the role for this receptor would be involved in tethering of cells whilst SHPS-1 has been recently proposed as tethering receptor for the ligand CD47 on apoptotic thymocytes (Tada et al., 2003). Studies by Duvall and colleagues which suggested the presence of a lectin-like receptor on the surface of the macrophage, which recognised carbohydrate molecules on the apoptotic cell, examined binding not internalisation (Duvall et al., 1985). Whilst this does not exclude a dual role for a lectin-like receptor it certainly implicates it as a binding receptor.

In this chapter I will present data investigating the stages and receptors involved in MDM Φ receptor-mediated internalisation of apoptotic neutrophils. Furthermore, experiments will be shown which examined the effect of Dex-treatment on MDM Φ binding and internalisation of apoptotic neutrophils.

Results

Neuraminidase treatment augments MDM Φ phagocytosis of apoptotic neutrophils

In order to investigate the nature of changes to the MDM Φ surface induced by Dex-treatment which would be conducive to phagocytosis of apoptotic cells, 5 day MDM Φ were incubated with the sialidase enzyme neuraminidase prior to a phagocytosis assay. Neuraminidase cleaves sialic acid from glycoproteins exposed on the surface of MDM Φ . The proportion of control MDM Φ (differentiated in the presence of 10% autologous serum) capable of phagocytosis of apoptotic neutrophils was increased to levels similar to that of Dex-treated MDM Φ following incubation with neuraminidase (Figure 4.2). However, neuraminidase treatment of Dex-treated MDM Φ had no effect on phagocytic ability. These results demonstrate that cleavage of sialic acid from the surface of the control MDM Φ increases the percentage of control MDM Φ that phagocytose, converting non-phagocytic cells into phagocytic cells, whereas neuraminidase treatment has no effect on Dex-treated MDM Φ phagocytosis.

Dex treatment of MDM Φ augments the binding step of phagocytosis

Neuraminidase augmentation of control MDM Φ phagocytic capacity for apoptotic neutrophils, led me to test the hypothesis that Dex induces changes to the surface of the macrophage, making it a more capable phagocyte. Phagocytosis is a multi-step process requiring initial binding of the apoptotic cell to the surface of the macrophage, followed by internalisation. I then investigated whether surface changes induced by Dex altered the initial binding stage.

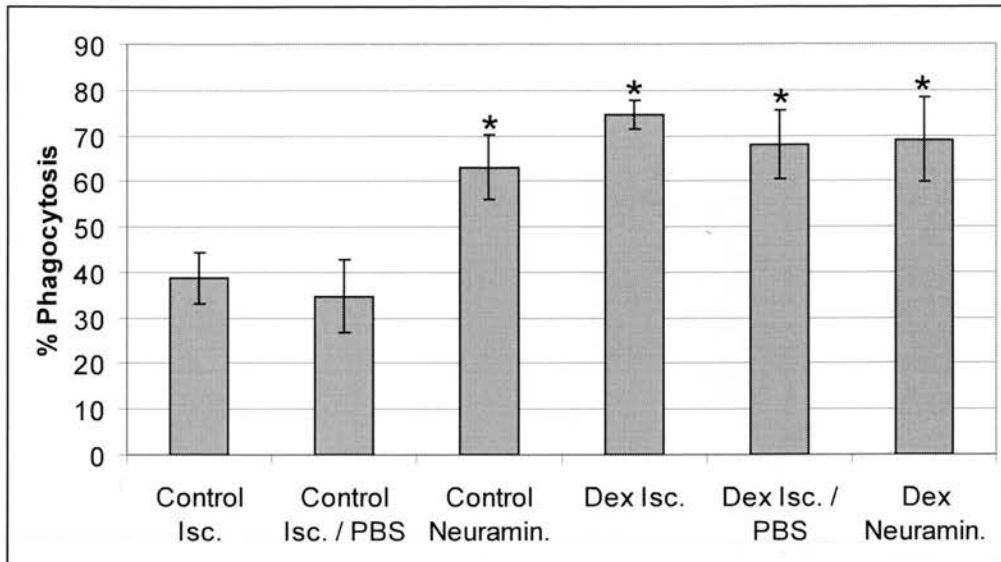


Figure 4.2 Neuraminidase augments Control MDM Φ phagocytosis of apoptotic cells

5 day MDM Φ were pre-treated with 0.1U/ml of neuraminidase (diluted in PBS) for 20 minutes at 37°C. The media was removed and fluorescent labelled 'aged' apoptotic neutrophils added. To control for any effect of changing the media, other MDM Φ had the media replaced with PBS. Following 1 hours incubation, the phagocytic ability of the different macrophage populations was assessed by flow cytometry.

Transformed data were analysed by Tukey-Kramer repeated ANOVA. * $p < 0.05$ vs. Control Isc and Control Isc/PBS (n=3).

By overlaying apoptotic neutrophils onto adherent monolayers of MDM Φ at 4° C, apoptotic neutrophils bound to MDM Φ but were not internalised. Low level binding of ‘fresh’ neutrophils (typically comprising less than 1% apoptotic cells) by both untreated and Dex-treated 5 day MDM Φ was observed (Figure 4.3). TNF/gliotoxin-treated neutrophils, which typically comprised 85-100% apoptotic cells, bound to untreated 5 day MDM Φ poorly. However Dex-treated MDM Φ showed an augmentation both in the percentage of the MDM Φ binding neutrophils (Figure 4.3) and in the number of apoptotic cells bound to each MDM Φ (Binding Index \pm SEM: control 5.9 ± 2.6 ; Dex 80.7 ± 22.2 ; n=8). These initial experiments show low percentage binding, but subsequent optimisation of the binding conditions demonstrated higher percentage binding for both control and Dex-treated MDM Φ , with the significant up-regulation in apoptotic neutrophil binding being maintained following Dex-treatment (see Figures 4.6 and 4.7).

Figure 4.4 shows scanning electron micrographs (SEM) of untreated and Dex-treated 5 day MDM Φ bound to apoptotic neutrophils. (A) and (B) show untreated MDM Φ binding one or two apoptotic cells, whereas the Dex-treated MDM Φ were observed to bind numerous apoptotic cells (C and D). Interestingly, apoptotic cells could be seen binding to both the cell body and the membrane extensions from the Dex-treated MDM Φ .

Binding and internalisation are two separate events distinguishable by requirement for cations

Many receptors have been implicated in the phagocytosis of apoptotic neutrophils by human macrophages and previous studies have failed to identify a receptor or receptors which were responsible for the augmentation of phagocytosis induced by Dex treatment (Giles et al., 2001). A selection of the cell surface molecules implicated in phagocytosis have been described as cation-dependent or – independent, and by examining phagocytosis in the presence or absence of extracellular divalent cations the receptor(s) involved in Dex-mediated phagocytosis

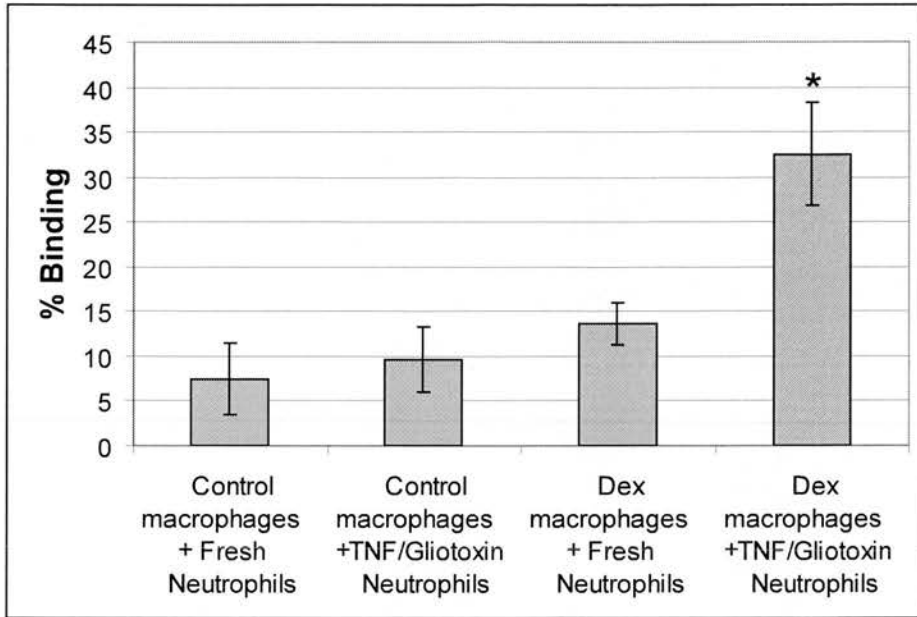
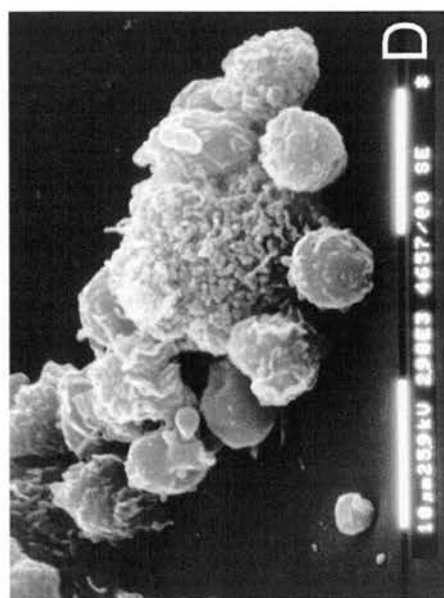
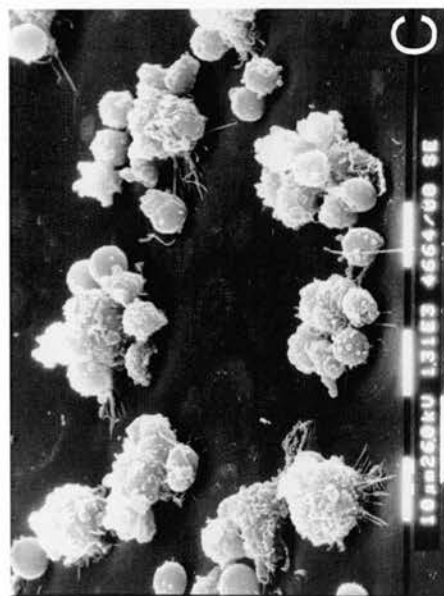
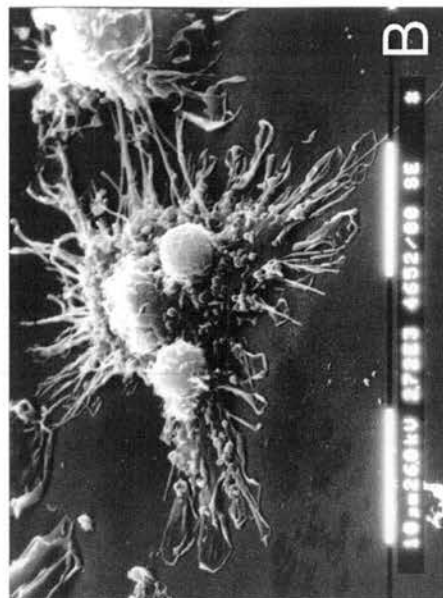
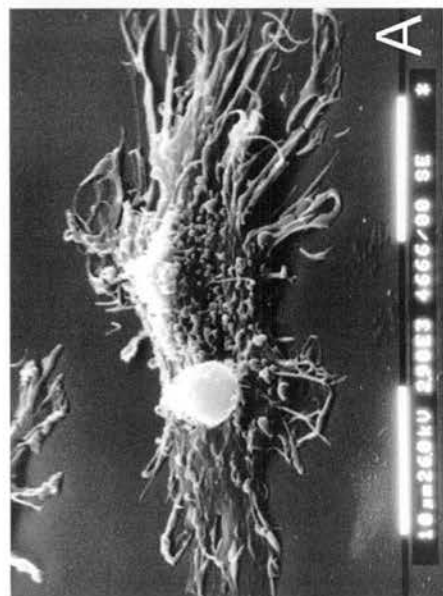


Figure 4.3 Dex augments MDM Φ binding of apoptotic neutrophils

5 day MDM Φ were overlaid with 'fresh' or TNF/glio-treated apoptotic neutrophils and incubated at 4°C for 1 hour. 'Fresh' neutrophils typically comprised < 1% apoptotic cells, TNG/glio treatment resulted in 85-100% apoptosis. Following washes to remove unbound apoptotic neutrophils, the percentage of MDM Φ with one or more neutrophil bound were counted. A minimum of 500 MDM Φ were counted per well, with each condition being carried out in duplicate and the mean value calculated. Data shown are the mean percentage binding for three separate donors \pm SEM. Transformed data were analysed by Tukey Kramer repeated ANOVA. * $p < 0.05$ vs. Control MDM Φ + fresh neutrophils (n=3).

Figure 4.4 SEM images of apoptotic neutrophils bound to 5 day Control and Dex MDMΦ

Untreated or Dex-treated 5 day MDMΦ were overlaid with apoptotic neutrophils and incubated at 4°C for 1 hour. Following washing to remove unbound neutrophils, the cells were fixed and dehydrated by washing through graded acetones and coated with gold/palladium. Cells were viewed and imaged using a Philips 505 Scanning Electron Microscope. Representative images from three separate donors are shown. control MDMΦ shown (A and B) are representative of the highly spread cells with multiple membrane extensions. The majority of control MDMΦ has no apoptotic neutrophils bound. Typically, only one or two apoptotic neutrophils were bound to the control MDMΦ which had bound the apoptotic neutrophils. Dex-treated MDMΦ (C and D) are small, round and highly ruffled cells. They bound to high numbers of apoptotic neutrophils, with apoptotic cells also seen attached to the membrane extensions. 1 white section of the scale bar is equal to 10μM



may be elucidated. Incubation of Iscove's DMEM medium with Chelex resin removes the divalent cations as demonstrated in Figure 4.5(A). Binding of antibody 24 to CD11/18 on neutrophils requires the presence of divalent cations, particularly Mg^{2+} and Mn^{2+} (Dransfield et al., 1992). To test the effective removal of divalent cations from the Iscove's DMEM media, antibody binding was examined in the presence of Iscove's DMEM alone, Iscove's DMEM with added divalent cations, the cation chelator EDTA and Iscove's DMEM treated with Chelex resin. CD11/18 fluorescence was detected in Iscove's DMEM containing divalent cations, and binding increased with the addition of extra $MnCl_2$. No binding was detected in the presence of EDTA or Chelex treated Iscove's DMEM demonstrating the successful removal of the divalent cations from the media.

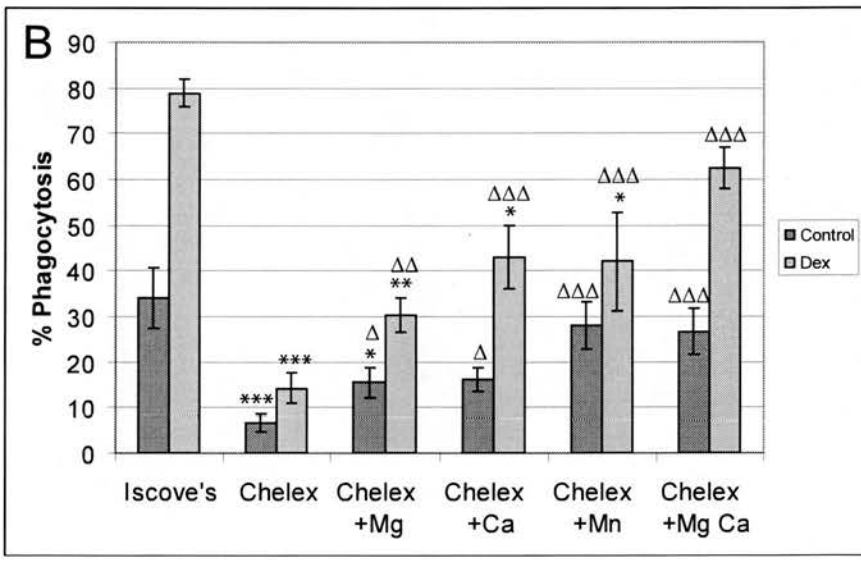
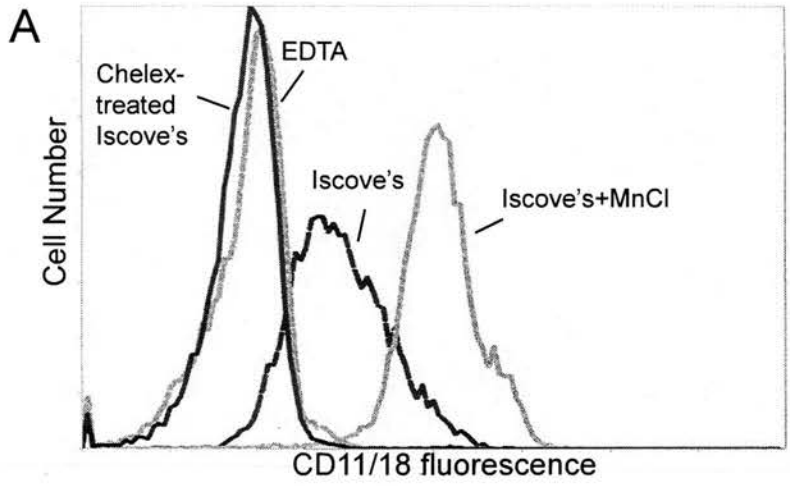
Figure 4.5(B) shows that MDM Φ phagocytosis of apoptotic neutrophils required the presence of extracellular divalent cations. A significant inhibition of both control and Dex phagocytosis was observed in the absence of divalent cations, with the re-addition of both Ca^{2+} and Mg^{2+} to the Chelex-treated Iscove's restoring phagocytosis. Interestingly, re-addition of Mn^{2+} restores control but fails to fully restore Dex-treated MDM Φ phagocytosis. However, the binding of apoptotic neutrophils to untreated or Dex-treated MDM Φ was cation-independent (Figure 4.6). The binding assay measured the number apoptotic cells tethered to the surface of the macrophage whereas the phagocytosis assay measured the percentage of internalised apoptotic cells. The binding stage was shown in Figure 4.6 to be cation independent, whilst the subsequent internalisation was cation dependent (Figure 4.5). This data demonstrates that the initial binding and subsequent receptor-triggered internalisation are readily distinguishable steps, requiring two or more distinct receptors.

Figure 4.5 Phagocytosis is a cation-dependent process

(A) Binding of cd11/18 to neutrophils requires the presence of divalent cations and was used to confirm the removal of divalent cations from Chelex treated Iscove's. Binding was carried out in the presence of Iscove's DMEM alone, Iscove's DMEM + MnCl, Chelex treated Iscove's DMEM or EDTA before analysis by flow cytometry. Binding of cd11/18 was not detected when the assay was carried out in Chelex treated Iscove's or EDTA.

(B) Control and Dex treated 5 day MDM Φ were used in a 1 hour phagocytosis assay with fluorescent overnight aged neutrophils. Following overnight culture the neutrophils were washed and then re-suspended in Iscove's, Chelex treated Iscove's (no divalent cations), or Chelex treated Iscove's with Mg, Ca, Mn or Mg+Ca added back.

Transformed data were analysed by Tukey repeated ANOVA. * p<0.05, ** p<0.01, *** p<0.001 vs. Iscove's column. Δ p<0.05, $\Delta\Delta$ p<0.01, $\Delta\Delta\Delta$ p<0.001 vs. Chelex column, (n=3).



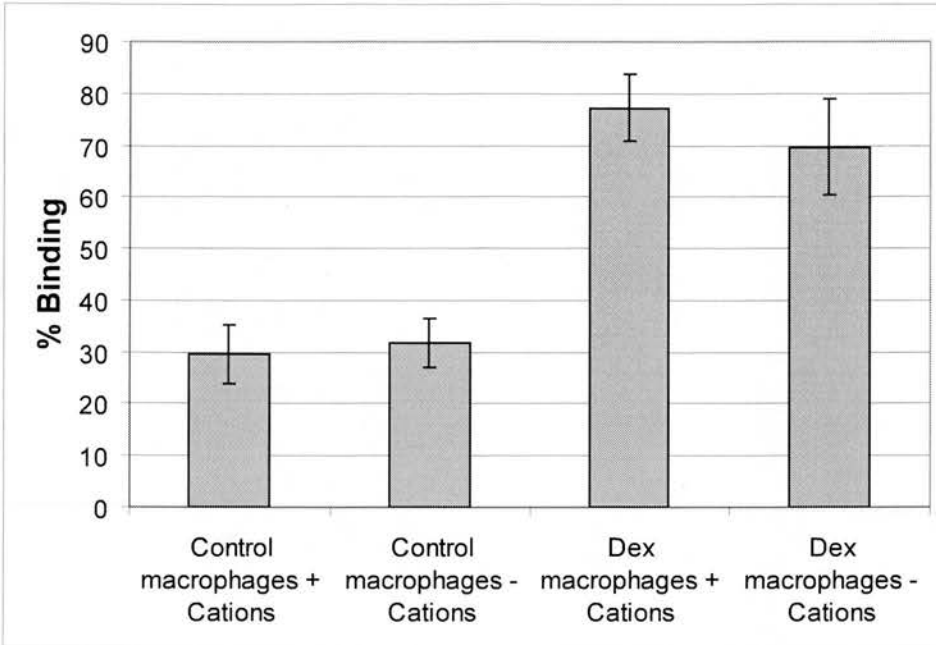


Figure 4.6 Binding of apoptotic neutrophils to MDM Φ is cation independent

5 day control or Dex-treated MDM Φ were overlaid with apoptotic neutrophils at 4°C for 1 hour in Iscove's DMEM (divalent cations) or Chelex-treated Iscove's DMEM (no divalent cations). Following washes to remove unbound apoptotic neutrophils, the percentage of MDM Φ with one or more neutrophil bound were counted. A minimum of 500 MDM Φ were counted per well, with each condition being carried out in duplicate and the mean value calculated. Data shown are the mean percentage binding for three separate donors \pm SEM. The binding of apoptotic neutrophils to both control and Dex-treated MDM Φ was not dependent on the presence of divalent cations.

Inhibition of known receptors does not inhibit binding of apoptotic cells

Having demonstrated that the binding of the apoptotic neutrophil to the MDM Φ is cation-independent, I sought to investigate if any of the known cation-independent receptors for apoptotic cells were involved in the binding. It is important to note that all reagents used here were tested to be functional in other systems used within the CIR (Dr. A. Devitt and Dr. S. Brown, personal communication) and were observed to bind to MDM Φ in indirect immunofluorescence assays (data not shown). A blocking antibody against CD14 (61D3) along with a control antibody (non-blocking) against CD14 (63D3) were used to pre-treat 5 day MDM Φ prior to use in a binding assay. However, blocking CD14 did not inhibit MDM Φ binding of apoptotic neutrophils (Figure 4.7A).

Although there are no suitable reagents to investigate the role of SRA in binding of apoptotic neutrophils to human MDM Φ , I sought to use murine BMDM Φ to test for the role of SRA. Bone marrow was extracted from SRA^{-/-} or WT background-matched mice, and macrophages differentiated for 7 days in the presence or absence of Dex prior to use in a binding assay with human apoptotic neutrophils. An increase in binding of apoptotic neutrophils was observed in the Dex-treated MDM Φ compared to untreated MDM Φ , demonstrating that GCs also augment mouse BMDM Φ phagocytosis of apoptotic cells *ex vivo*. However, untreated or Dex-treated BMDM Φ lacking SRA, showed no defect in their ability to bind to apoptotic neutrophils (Figure 4.7B).

Homophilic ligation of CD31 on apoptotic neutrophils and CD31 on MDM Φ promotes binding whilst CD31-CD31 binding between viable leukocytes and MDM Φ promotes detachment, preventing ingestion of non-apoptotic cells (Brown et al., 2002). It was therefore of interest to determine if inhibition of CD31 on the untreated and Dex-treated MDM Φ blocked binding of apoptotic neutrophils. Pre-treatment of control and Dex-treated MDM Φ with an IgG2a control antibody or Hec7.2 CD31 blocking antibody (Brown et al., 2002) followed by binding of apoptotic neutrophils showed no inhibition in control or Dex-treated MDM Φ binding. Binding of apoptotic neutrophils, following MDM Φ incubation with isotype

Figure 4.7 Inhibition of known receptors does not inhibit binding of apoptotic cells

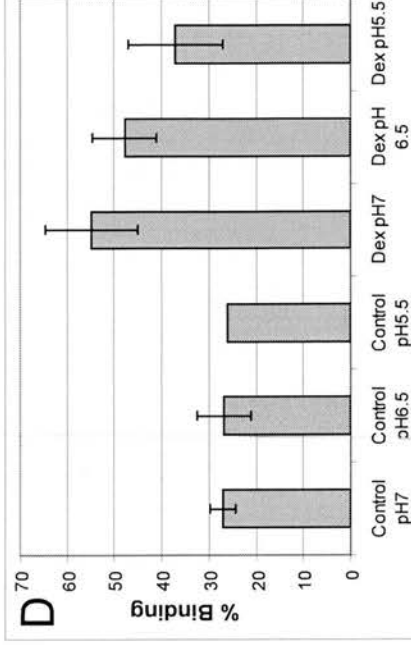
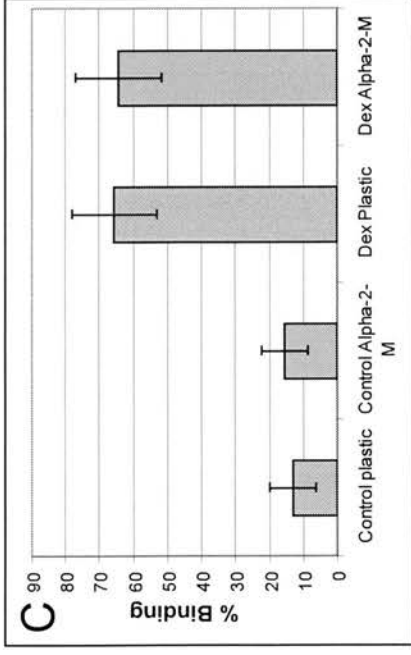
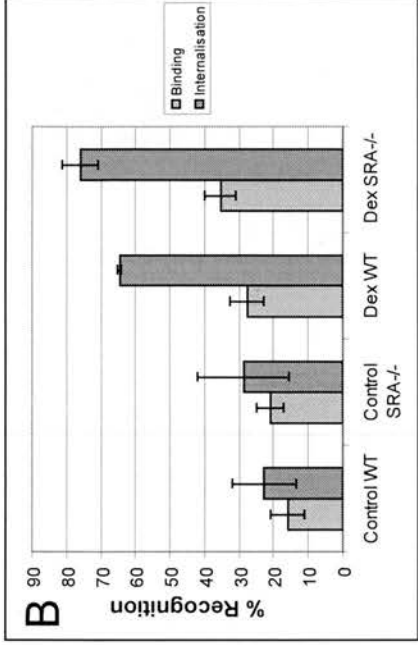
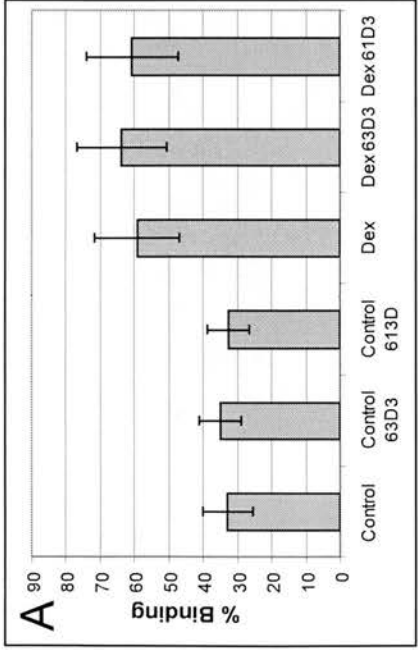
(A) 5 day control and Dex-treated MDM Φ were pre-treated with a blocking antibody against CD14 (61D3) and a non-blocking, control antibody against CD14 (63D3). The MDM Φ were then overlaid with apoptotic neutrophils at 4°C for 1 hour, then washed to remove unbound apoptotic neutrophils. The percentage of MDM Φ with one or more neutrophil bound were counted. A minimum of 500 MDM Φ were counted per well, with each condition being carried out in duplicate and the mean value calculated. Data shown are the mean percentage binding for three separate donors \pm SEM. Blocking CD14 on the MDM Φ did not inhibit either control or Dex-treated MDM Φ binding of apoptotic cells.

(B) Bone marrow was extracted from age and sex matched SRA^{-/-} mice and background control mice (WT). BMDM Φ were differentiated over 7 days with 125nM Dex added on day 2. Phenotyping the 7 day untreated and Dex-treated SRA^{-/-} and WT BMDM Φ , demonstrated that the SRA^{-/-}BMDM Φ did not express SRA on their cell surface (see Materials and Methods). 7 day BMDM Φ were overlaid with human apoptotic neutrophils in a 4°C binding assay as described above or in a 37°C phagocytosis assay. Data shown are the mean percentage binding for three separate experiments \pm SEM. Loss of SRA did not affect Control or Dex-treated MDM Φ binding of apoptotic neutrophils

(C) 5 day control or Dex-treated MDM Φ were plated onto virgin tissue culture plastic, or onto α -2-macroglobulin coated plastic. α -2-macroglobulin binds to and sequesters CD91 from the surface of the MDM Φ . The MDM Φ were then used in a binding assay as described above. Data shown are the mean percentage binding for three separate donors \pm SEM. Blocking CD91 on the MDM Φ did not inhibit control or Dex-treated MDM Φ binding of apoptotic cells

(D) 5 day control and Dex-treated MDM Φ were used in binding assays as described above but with alteration to the pH of the media used in the assay. Lowering the pH had no effect on the control MDM Φ binding of apoptotic neutrophils. Lowering the

pH attenuated Dex-treated MDM Φ augmentation of apoptotic cell binding. Data shown are the mean percentage binding for three separate experiments \pm SEM



matched IgG2a was assumed to be 100%, and in three separate experiments binding to control MDM Φ treated with Hec7.2 was 112%, 160% and 87% whilst binding to Dex-treated MDM Φ treated with Hec7.2 was 85%, 118% and 93%.

A role has been described for CD91 in phagocytosis of apoptotic cells. To investigate whether this receptor is an apoptotic cell binding receptor, tissue culture plates were either left virgin, or coated with a solution of alpha-2-macroglobulin as described in a previous study (Ogden et al., 2001). Alpha-2-macroglobulin is a ligand for CD91, and so by plating 5 day MDM Φ onto the ligand CD91 expressed on the MDM Φ should be sequestered to the basolateral surface of the cell where it binds the alpha-2-macroglobulin and therefore will not be available on the surface of the macrophage. No inhibition of binding ability was observed by plating untreated or Dex-treated MDM Φ onto alpha-2-macroglobulin (Figure 4.7C). Together these data suggest that none of the previously identified receptors are involved in untreated or Dex-treated MDM Φ binding of apoptotic neutrophils.

One of the mechanisms by which neuraminidase treatment caused an increase in control MDM Φ phagocytosis may have been that cleavage of sialic acid altered the surface charge of the macrophage, reducing electrostatic repulsion between cell types, and thereby increasing the binding of apoptotic cells. Indeed previous studies have suggesting a link between surface charge and recognition of apoptotic cells (Savill et al., 1989a), therefore I sought to further investigate this possibility. By altering the pH of the media used for the binding assay, a role for surface charge may be established. Interestingly, lowering the pH had no effect on the binding of apoptotic cells by untreated MDM Φ , but it did lower the percentage of Dex-treated MDM Φ binding apoptotic cells (Figure 4.7D).

Role of $\alpha v \beta 3$ integrin in the internalisation of apoptotic cells

The demonstration that internalisation of apoptotic neutrophils was a cation-dependent process, coupled with studies showing human MDM Φ phagocytosis of apoptotic cells primarily involves the cation-sensitive $\alpha v \beta 3$ integrin, led to a re-

examination of the role of this integrin as an internalisation receptor for Dex-treated MDM Φ . 5 day untreated and Dex-treated MDM Φ were incubated with blocking antibodies against αv and $\alpha v\beta 3$ prior to phagocytosis of apoptotic neutrophils. Despite effective binding of the antibodies to the MDM Φ (see Figure 5.4), there was no inhibition of phagocytosis (Figure 4.8 A). Figure 4.8(B-E) shows the immunofluorescence staining of 5 day Dex-treated MDM Φ for the active $\beta 3$ integrin, using LIBS1 antibody. LIBS1 staining was punctuate and often polarised in MDM Φ regardless of whether or not they were binding apoptotic neutrophils (Figure 4.8B). However, the binding or internalisation of apoptotic neutrophils was associated with LIBS1 staining in all examined cells e.g. in image C and also D and E, where staining is shown on membranes extending towards the apoptotic neutrophils.

The extent of phagocytosis of apoptotic neutrophils is dependent on the method by which apoptosis is induced

Neutrophil apoptosis was induced by two different methods. The first was by overnight culture of neutrophils in Iscove's DMEM + 10% AS, resulting in a population of neutrophils containing 45-60% morphologically-defined apoptotic cells (Figure 4.9A). 2 hour culture of neutrophils in Iscove's DMEM + 10% AS, with the addition of TNF- α and gliotoxin, induced apoptosis in 85-100% of the cells (Figure 4.9B). Intriguingly, these two populations of apoptotic cells were phagocytosed to different extents by macrophages from the same donor. A small reduction in phagocytosis of the TNF/gliotoxin-treated neutrophils by untreated MDM Φ was seen compared to 'aged' neutrophils. However, despite Dex-treated MDM Φ demonstrating augmented phagocytosis of 'aged' neutrophils compared to untreated MDM Φ , Dex-treated MDM Φ did not demonstrate an augmented phagocytosis of the TNF/gliotoxin-treated neutrophils. This finding is interesting since the TNF/gliotoxin treated neutrophil population contained approximately twice as many apoptotic

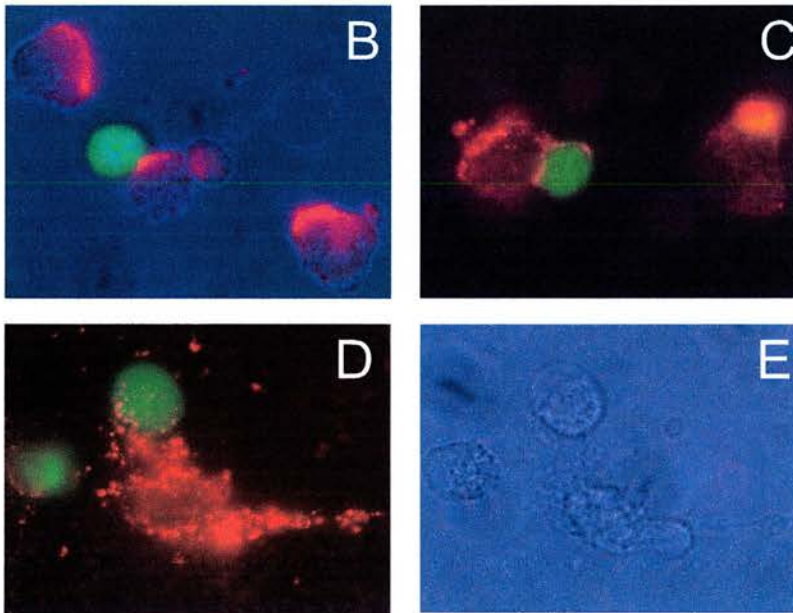
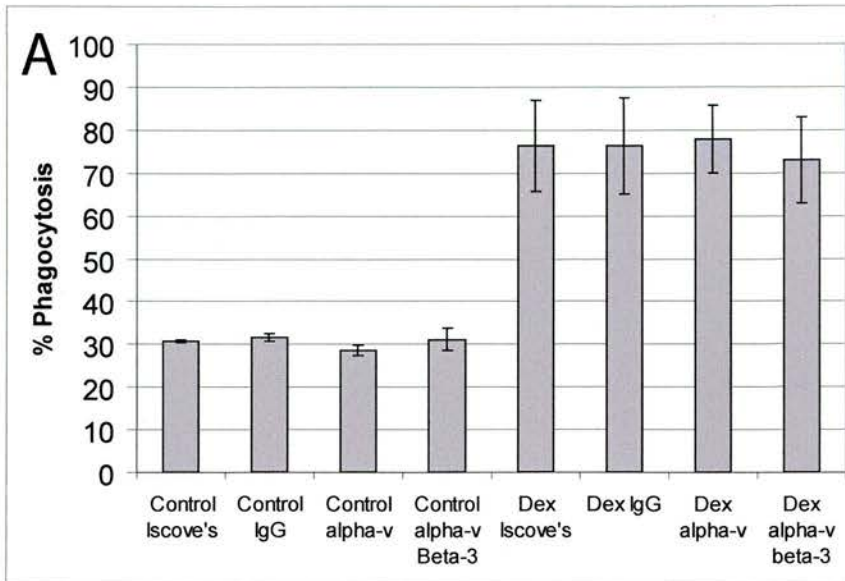


Figure 4.8 Control and Dex MDM Φ phagocytosis of apoptotic neutrophils is not inhibited by blocking antibodies against αv or αv - $\beta 3$.

(A) 5 day MDM Φ were pre-incubated with isotype control or blocking antibodies against αv or $\alpha v\beta 3$ before being used in a 1 hour phagocytosis assay with overnight aged neutrophils. Data shown is the mean percentage phagocytosis \pm SEM (n=2). (B)-(E) show 5 day Dex-MDM Φ which were phagocytosing labelled apoptotic neutrophils (green), before being fixed and labelled with an antibody against active $\beta 3$ integrins (red). (B) is a composite image of immunofluorescence and phase contrast, (C) is an immunofluorescence image and (E) is the phase contrast image for (D), showing membrane extending towards the apoptotic cell. Active $\beta 3$ integrins appeared to be associated with binding of the apoptotic neutrophils.

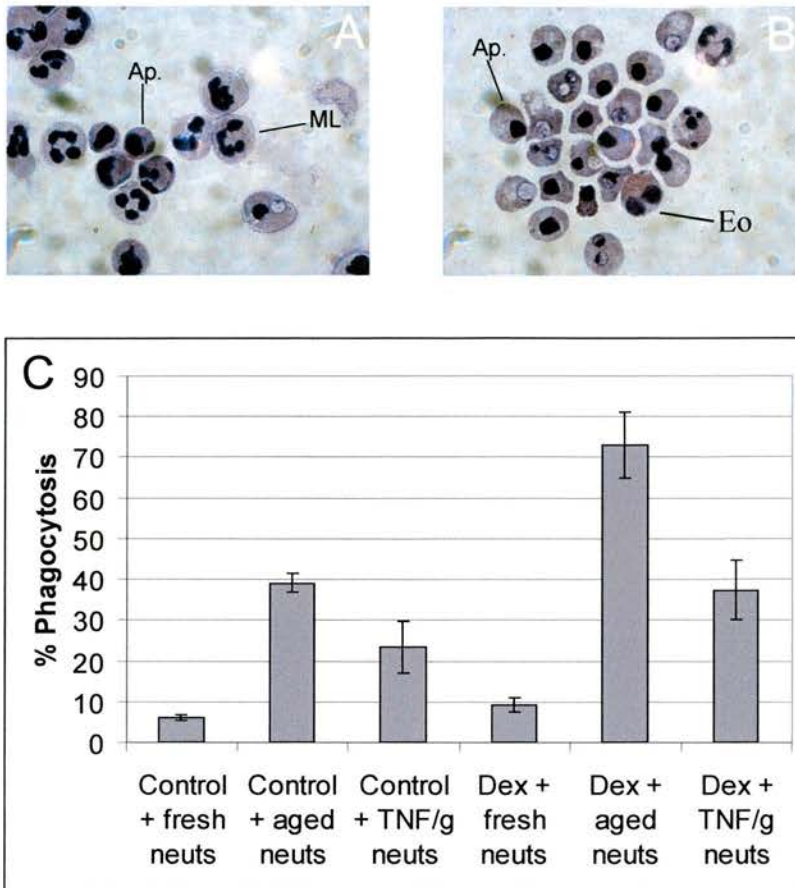


Figure 4.9 Phagocytosis of different apoptotic neutrophil populations

(A) is an H&E stained cytospin of neutrophils cultured overnight in Iscove's + 10% AS. Apoptotic neutrophils (Ap), defined morphologically, have a condensed nucleus, whilst the non-apoptotic cells have multi-lobed nuclei (ML). The neutrophil population typically contains 45-60% apoptotic cells defined by morphology.

(B) is an H&E stained cytospin of neutrophils cultured TNF- α + Gliotoxin for 2 hours. Apoptotic cells contain condensed nuclei (Ap.) and these typically comprise 85-100% of the population as defined by morphology. An apoptotic eosinophil (eo) is also seen in this image.

(C) Control and Dex MDM Φ were cultured for 5 days, before phagocytosing either fresh neutrophils (<1% apoptosis), overnight aged neutrophils as described in (A) or TNF/glio neutrophils as described in (C). Neutrophils were FL-1 labelled and the data were analysed by flow cytometry. Data shown are the mean percentage phagocytosis \pm SEM, (n=3).

cells as the overnight 'aged' population.

These data indicated that TNF/gliotoxin treatment potentially induced a differential surface phenotype to that of overnight aged neutrophils, leading to altered recognition by the Dex-treated MDM Φ . I therefore compared the cell surface phenotype of the two neutrophil populations (Figure 4.10A). No obvious changes in expression were observed between the two populations in any of the surface antigens examined. However, examination of apoptosis-induced surface expression of phosphatidylserine (PS) by annexin V staining showed a difference in expression between the two populations (Figure 4.10 B and C). The overnight 'aged' neutrophils had an annexin V positive population which correlated with the morphologically apoptotic population and an annexin V negative, non-apoptotic population. By contrast, the TNF/gliotoxin-treated neutrophils had a lower median fluorescence than the annexin V positive 'aged' neutrophils (49.4 and 5.7 median fluorescence intensities respectively). Confirmation of this result was seen by examination of annexinV immunofluorescence staining by microscopy. Bright cell surface annexin V staining of the 'aged' neutrophils was associated with condensed nuclei (Figure 4.10D) whilst a lower level of staining was detected on the apoptotic TNF/gliotoxin-treated neutrophils (Figure 4.10E).

Dex-treated MDM Φ internalisation of apoptotic neutrophils can be inhibited by annexinV

The previous experiments led me to consider the following hypothesis: Down-regulation of PS exposure on the surface of the TNF/glio neutrophils results in reduced Dex-treated MDM Φ phagocytosis of these cells. To test this hypothesis neutrophils were aged overnight as previously described, resulting in a high level of PS exposure on the outer membrane. These PS^{high} apoptotic neutrophils were incubated with annexin V which bound to the surface expressed PS, prior to the phagocytosis assay. Figure 4.11 shows that pre-incubation of the neutrophils with annexin-V attenuated Dex-treated MDM Φ phagocytosis. However, annexin V binding did not alter untreated macrophage phagocytosis.

Figure 4.10 Surface phenotyping of 'aged' and TNF/gliotoxin treated neutrophils

Overnight 'aged' and 2 hour TNF/Gliotoxin treated neutrophils were phenotyped for the expression of surface antigens (A) or for expression of PS (B-E)

(A) shows the average mean fluorescence expression for surface antigens from three experiments \pm SEM.

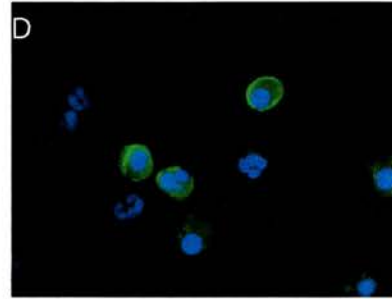
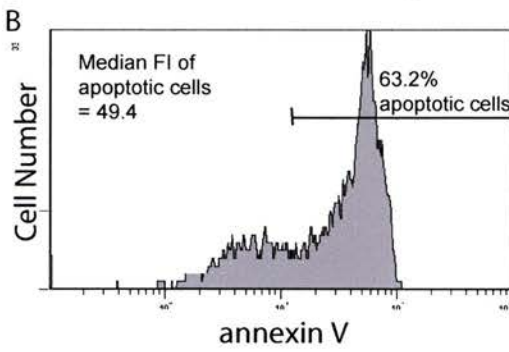
(B and C) show representative annexin V profiles from 3 experiments. Annexin V binds to PS on the surface of the neutrophils.

The 'aged' neutrophils are a mix of apoptotic and non-apoptotic cells (typically 45-65% apoptotic) whilst TNF/Gliotoxin induces between 85-100% apoptosis by 2 hours. The apoptotic cells are marked on the flow profiles. Comparison of the median fluorescence intensity for the apoptotic aged and apoptotic TNF/Gliotoxin neutrophils (49.5 and 5.7 respectively) demonstrates a down regulation in annexin V binding on the TNF/Gliotoxin cells. This was confirmed by immunofluorescence co-staining of the neutrophils for PS by annexin V (green) and the nucleus with Hoest (blue).

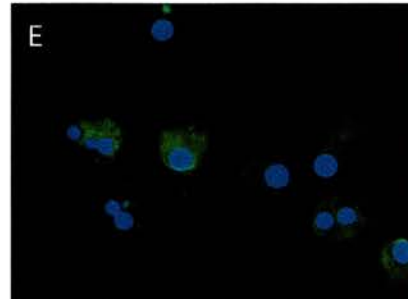
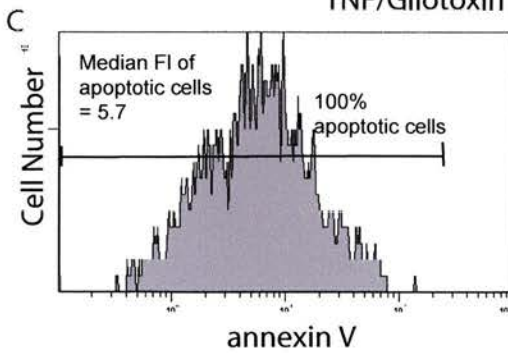
A

Surface Antigen	Mean Fluorescence: overnight aged neutrophils \pm SEM	Mean Fluorescence: TNF α /glio neutrophils \pm SEM
CD11a	4.1 \pm 1.7	2.5 \pm 0.4
CD11b	11.8 \pm 1.1	18.5 \pm 2.5
CD16	4.5 \pm 0.9	6.3 \pm 3.6
CD18	7.8 \pm 5.7	6.1 \pm 4.7
CD29	0.5 \pm 0.5	0.2 \pm 0.1
CD31	4.4 \pm 0.6	2.8 \pm 0.1
CD32	9.5 \pm 2.2	5.0 \pm 0.2
CD43	20.2 \pm 11.7	9.0 \pm 4.3
CD45	10.9 \pm 2.2	10.6 \pm 0.3
CD62L	0.2 \pm 0.1	1.4 \pm 1.3
CD66	9.6 \pm 3.9	13.3 \pm 2.1

'Aged' Neutrophils



TNF/Gliotoxin Neutrophils



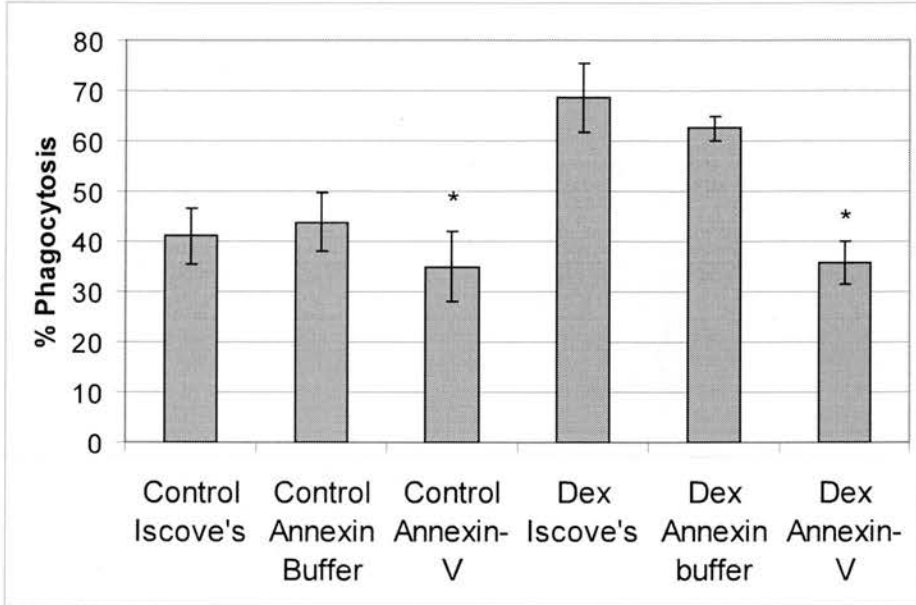


Figure 4.11 Blocking neutrophil PS expression attenuates Dex-treated MDM Φ phagocytosis

Overnight 'aged' neutrophils were spun down and resuspended in Iscove's, Annexin V buffer (HBSS + 5mM CaCl) or Annexin V buffer plus Annexin V and incubated on ice for 20 minutes. The neutrophils were pelleted and resuspended in Iscove's DMEM. Phagocytosis of the 'aged' neutrophils by Control and Dex-treated 5 day MDM Φ was examined by flow cytometry. Data shown were the mean % phagocytosis of three separate experiments \pm SEM. Transformed data were analysed by Tukey-Kramer repeated ANOVA. * $p < 0.05$ is vs. Dex Iscove's (n=3).

One interpretation of these results is that the Dex-induced augmentation (above that of untreated MDM Φ phagocytosis) may be attributed to a PS-dependent recognition mechanism.

Discussion

Dex-treated MDM Φ have an increased ability to bind apoptotic neutrophils

Viable cells, including MDM Φ , are thought to carry a net negative surface charge (Papadimitriou, 1982), resulting in an electrostatic repulsion between two cells. Induction of apoptosis in thymocytes has been associated with a decrease in the surface charge density (Miyazawa et al., 1981; Morris et al., 1984) which may therefore reduce the electrostatic repulsion between the apoptotic cell and the MDM Φ . Interestingly, a charge-sensitive mechanism has been demonstrated for the uptake of apoptotic neutrophils (Savill et al., 1989a). Exposure of the untreated-MDM Φ (but not Dex-treated MDM Φ) to neuraminidase augmented phagocytosis of apoptotic cells. Neuraminidase cleavage of sialic acid from glycoproteins on the surface of the MDM Φ may cause the unmasking or inactivation of surface antigens or may act to alter the net surface charge of the MDM Φ , potentially reducing electrostatic repulsion between the apoptotic neutrophil and MDM Φ . The change that was induced on the surface of the untreated MDM Φ by neuraminidase treatment, which led to augmentation of phagocytic ability, was not observed with Dex-treated MDM Φ . One possible explanation for this observation is that the surface of the Dex-treated MDM Φ already has changes similar to those induced by neuraminidase, and this could be examined by the use of fluorescently labelled sialic acid-binding lectins to examine sialic acid expression on the surface of the control and Dex-treated MDM Φ . Dex-treated MDM Φ may exhibit an altered charge, when compared to the untreated MDM Φ , with reduced repulsive forces between the phagocyte and apoptotic neutrophil. As a consequence the Dex-treated MDM Φ may bind apoptotic neutrophils more successfully or readily. A Dex-induced MDM Φ surface charge alteration may be tested by examining the binding of latex beads, carrying different charges, to control or Dex-treated MDM Φ , or by microelectrophoretic mobility, which allows the charge on the surface of a single cell to be measured.

One possibility raised by the neuraminidase data was that Dex-treated MDM Φ were more capable of binding apoptotic cells. In order to examine this, I developed a binding assay. By allowing the interaction of apoptotic cells and MDM Φ at 4°C, binding between the two cells was permitted but internalisation was not (presumably because signalling and subsequent actin polymerisation were inhibited). Binding studies clearly demonstrated that Dex-treatment induced changes to the MDM Φ which facilitated binding of apoptotic cells. Whilst the increased phagocytosis of apoptotic cells by Dex-treated MDM Φ has been suggested to be a consequence of increased Rac activity and altered cytoskeletal dynamics (Giles et al., 2001), my data clearly indicate that the increased phagocytosis also reflects increased binding of apoptotic neutrophils to the MDM Φ surface.

Binding and internalisation are two clearly distinguishable events in apoptotic cell uptake

Further experiments demonstrated that the process of binding was cation independent, whilst internalisation of the apoptotic neutrophils required extracellular divalent cations. This data demonstrates for the first time that there are two clearly distinguishable steps in the recognition and uptake of an apoptotic neutrophil by MDM Φ . Furthermore, because only internalisation requires divalent cations, it is also apparent that these two steps utilise different receptors.

Investigation of the involvement of possible receptors for the binding step yielded largely negative data, failing to demonstrate a role for receptors previously suggested to be involved in apoptotic cell uptake. A role for the integrin $\alpha\beta3$ 'vitronectin receptor'/CD36/ thrombospondin complex was ruled out, as ligand binding to the $\alpha\beta3$ is dependent on divalent cations. Interestingly, Hoffman and colleagues presented data to the contrary, suggesting a role for this complex in binding but not internalisation (Hoffmann et al., 2001). However, the data presented in Hoffman's study used erythrocytes coated with antibody against $\alpha\beta3$ or with the ligands thrombospondin or vitronectin. Whilst their data shows much greater binding than engulfment when these erythrocytes were presented to MDM Φ , some engulfment

was observed. For example, an average of two erythrocytes with vitronectin on the surface bound to one MDM Φ , whilst only one was engulfed. An average of 7.5 erythrocytes with anti-CD36 and anti- $\alpha v\beta 3$ antibodies on the surface bound to one MDM Φ , with only one being engulfed per MDM Φ . There are several possible interpretations of these data. One explanation is that when only a single ligand or receptor is involved in uptake (as in the erythrocyte (Ebab) system), the receptors are able to perform dual roles in the binding and internalisation process. Secondly, Hoffman's data does show higher average binding compared to engulfment, but this antibody-based system discounts the role of the receptor, whereas engulfment of apoptotic cells may be critically dependent on either the receptor affinity or the receptor distribution.

Based upon published data CD14 is a candidate binding receptor with no reported requirement for cations in its recognition of apoptotic cells. However, a blocking antibody against CD14 failed to inhibit binding in my system, potentially excluding a role for CD14. SRA^{-/-} BMDM Φ , demonstrated to express no SRA on their cell surface (see Materials and Methods), were slightly more effective at binding to apoptotic cells than BMDM Φ which expressed SRA on their cell surface.

Furthermore, SRA^{-/-} BMDM Φ showed no inhibition in internalisation of apoptotic neutrophils either. Although these studies used mouse macrophages as phagocytes and human apoptotic neutrophils as target cells, the fact that relatively high levels of phagocytosis were observed suggests that cross-species recognition is not a problem. This data effectively rules out a role for the SRA receptor in uptake of apoptotic neutrophils and in Dex-augmentation of apoptotic cell clearance. I also examined CD91 recognition of apoptotic cells. No inhibition in binding of apoptotic neutrophils was observed following MDM Φ binding to alpha-2-macroglobulin, suggesting this receptor is not involved in the binding step. However, it is possible that not all of the CD91 on the macrophage surface was sequestered, and therefore some interaction with apoptotic cells may have occurred through this receptor.

Indirect immunofluorescence staining for CD91, would reveal the extent to which MDM Φ binding to alpha-2-macroglobulin sequestered CD91 to the basolateral surface of the cell. Finally a role for CD31 was investigated. Binding of a viable cell via a homophilic CD31 interaction at 37°C and under low shear has been shown to

induce signalling which leads to dissociation of the cell (Brown et al., 2002). My binding assay was conducted at 4°C in the absence of shear, and therefore would have only allowed examination of CD31 as a binding receptor, and not as a receptor conveying a dissociation signal. However, a role for CD31 in MDMΦ binding of apoptotic neutrophils was not observed. Interestingly, when the binding studies were performed at 4°C with subsequent warming to 37°C, and examination by real-time video microscopy, many of the bound apoptotic cells were observed to detach and consequently were not always engulfed (data not shown).

Phagocytosis of apoptotic cells has been associated with alterations in surface charge. Savill and colleagues identified a recognition mechanism that was inhibitable by amino sugars and basic amino acids neutralising the anion charge on the apoptotic cell surface (Savill et al., 1989a). Apoptotic cell engulfment was inhibited by a reduction in pH, again probably by neutralising the anionic charge on the neutrophil. Negatively charged ligands such as PS and OxLDL have been identified on the apoptotic cell surface. I therefore investigated the effect of pH on the binding of apoptotic cells to MDMΦ. Interestingly, lowering the pH to levels that has been previously demonstrated to inhibit engulfment had no effect on untreated MDMΦ binding. Anionic changes (such as exposure of PS) to the surface of the apoptotic cell may therefore not be involved in this step of recognition. In contrast, lowering the pH reduced Dex-treated MDMΦ binding to apoptotic neutrophils. Whilst this data may suggest that Dex treatment augments binding through altered properties of the MDMΦ surface, this result must be interpreted cautiously. Dex-treatment reduces the adherence of MDMΦ to tissue culture plastic, and it was noted that lowering the pH caused a percentage of the MDMΦ to 'lift' from the plate. It is possible, therefore, that selective loss of 'high-binding' Dex-treated MDMΦ was responsible for this result. This could be tested by either, the recovery and microscopic examination of the 'lifted' cells, or by developing a binding assay which was carried out in suspension.

Internalisation receptor

It has been proposed that this second stage of receptor engagement signals for the engulfment of the apoptotic cell (Hoffmann et al., 2001). My data provides the first demonstration that the MDM Φ phagocytosis of apoptotic neutrophils is governed by two or more receptors, acting in a sequential manner to first tether and then internalise the cell. The requirement for divalent cations in the internalisation step, suggested a role for the integrin $\alpha\beta3$ 'vitronectin receptor'/CD36/ thrombospondin complex. Using antibodies which blocked either αv and $\alpha v\beta3$ function I failed to demonstrate a role for this complex in either untreated or Dex-treated phagocytosis of apoptotic neutrophils, in direct contrast with other studies which have successfully demonstrated this (Fadok et al., 1992b; Savill et al., 1990). However, immunofluorescence studies using LIBS1, an antibody specific for the ligand occupied conformation of $\alpha v\beta3$, did show apparent co-localisation of active $\beta3$ to the MDM Φ membrane adjacent to partially engulfed apoptotic neutrophils. My data did not confirm or refute a role for the vitronectin receptor complex as an internalisation receptor. It would be important to confirm whether this receptor complex is involved in the internalisation step. One possibility would be the use of the RGDS peptide ligand, which binds to multiple integrins, including $\alpha v\beta3$. Ligation of the $\alpha v\beta5$ integrin on 293T cells evokes signalling via CrkII-DOCK180-Rac1 during phagocytosis of apoptotic cells (Albert et al., 2000), whilst retinal epithelium cell phagocytosis requires both prior activation of $\alpha v\beta5$ and actin polymerisation to bind particles (Finnemann and Rodriguez-Boulan, 1999), demonstrating that integrin receptor complexes are involved in phagocyte signalling, and that the integrin $\alpha v\beta3$ 'vitronectin receptor' could potentially signal in a similar way. However, active $\beta3$ was localised throughout the phagocytosing MDM Φ , and as phagocytosis requires the delivery of new membrane to the site of particle internalisation (Aderem, 2002), it is possible that the $\beta3$ localises there as a bystander.

I developed the binding assay using neutrophils treated with TNF/gliotoxin, a method which induced apoptosis in 85-100% of the cells, with very little evidence of necrosis. In binding assays no difference in the binding of TNF/Gliotoxin neutrophils

compared to 'aged' neutrophils was observed (data not shown). However, a clear difference was observed when these two apoptotic populations were used in phagocytosis assays. The observed Dex-treated MDM Φ augmentation of phagocytosis of 'aged' neutrophils was attenuated when TNF/gliotoxin-treated neutrophils were used as targets. The untreated MDM Φ showed only a small down-regulation in phagocytosis of the TNF-gliotoxin neutrophils compared to the overnight 'aged' neutrophils. Comparison of the surfaces of the two apoptotic cell populations revealed few molecular changes except for the extent of PS expression. The 'aged' neutrophils, which had morphological characteristics of apoptosis, expressed high levels of PS on their surface. However, the TNF/gliotoxin-treated neutrophil population, whilst showing morphological characteristics of apoptosis, had a broad range of PS expression, with a greatly reduced overall expression when compared with the apoptotic 'aged' neutrophils.

In order to examine whether this change in PS expression was responsible for the altered phagocytosis of these cells by the Dex-treated MDM Φ , PS^{high} 'aged' neutrophils were labelled with annexin V, which has previously been shown to block PS-mediated recognition (Callahan et al., 2003). Annexin V binding of PS on the surface of an apoptotic cell attenuated Dex-augmented phagocytosis. My data suggests that the augmentation in phagocytic capacity provided by Dex-treatment is mediated via recognition of PS. Because pre-incubation of neutrophils with annexin V did not completely inhibit phagocytosis it may be that Dex-treatment induces the PS-PSR recognition mechanism in addition to another, as yet unknown, receptor-ligand recognition system utilised by the untreated MDM Φ . This induced switch would not be unprecedented. β -glucan treatment of human MDM Φ has been shown to switch the main recognition mechanism from $\alpha v \beta 3$ to PS (Fadok et al., 1992b). It would be of great interest to obtain the antibody to the proposed PS-receptor and to profile the surface of untreated and Dex-treated MDM Φ to examine if there is an up-regulation in expression following Dex-treatment. It has been reported, although evidence was not shown, that PS recognition by phagocytes is cation independent (Fadok et al., 1998b). However, my data suggests a role for PS during the cation dependent internalisation process and not the cation independent binding stage.

Several receptors, including CD36, have been reported to potentially recognise PS and perhaps the requirement for divalent cations in PS recognition may alter depending on the receptor.

A suggestion by Pradhan was that the $\alpha v\beta 3$ 'vitronectin' receptor complex uptake was associated with cells in the later stage of apoptosis (Pradhan et al., 1997). However, PS expression is an early marker on the apoptotic cell (Martin et al., 1995), raising the possibility that Dex-treated MDM Φ may recognise a different subset of apoptotic neutrophils to the control MDM Φ . I therefore carried out some preliminary experiments to attempt to address whether Dex-treated MDM Φ recognise different subsets of apoptotic cells to control MDM Φ . This was investigated by fluorescently labelling aged neutrophils for CRP (C-reactive protein), a marker of late apoptosis (Dr. S.P. Hart, unpublished) whilst also staining all neutrophils with a cell tracker dye, and then examining what proportion of total control or Dex-treated MDM Φ phagocytosis were FITC-CRP labelled neutrophils. The results showed that $35 \pm 14\%$ SD of control MDM Φ phagocytosis were FITC-CRP labelled neutrophils, whereas $22 \pm 3\%$ SD of the Dex-treated MDM Φ phagocytosis were FITC-CRP labelled neutrophils, suggesting that control MDM Φ were more efficient at phagocytosing late apoptotic cells than the Dex-treated MDM Φ . These interesting preliminary findings would clearly require further testing, but may provide insight into reported differences in molecular pathways implicated in the phagocytic process.

Summary

I propose the following model based upon the results presented in this chapter: (Figure 4.12). Untreated-MDM Φ binding of apoptotic cells occurs in a cation-independent manner. For internalisation of the bound apoptotic cell, a second receptor which requires divalent cations to function, also binds the apoptotic cell. The second 'internalisation' receptor may be pre-expressed by the MDM Φ , or may be up-regulated as a consequence of apoptotic cell binding to the first receptor.

BINDING

SIGNALLING

Cation-independent

Cation-dependent

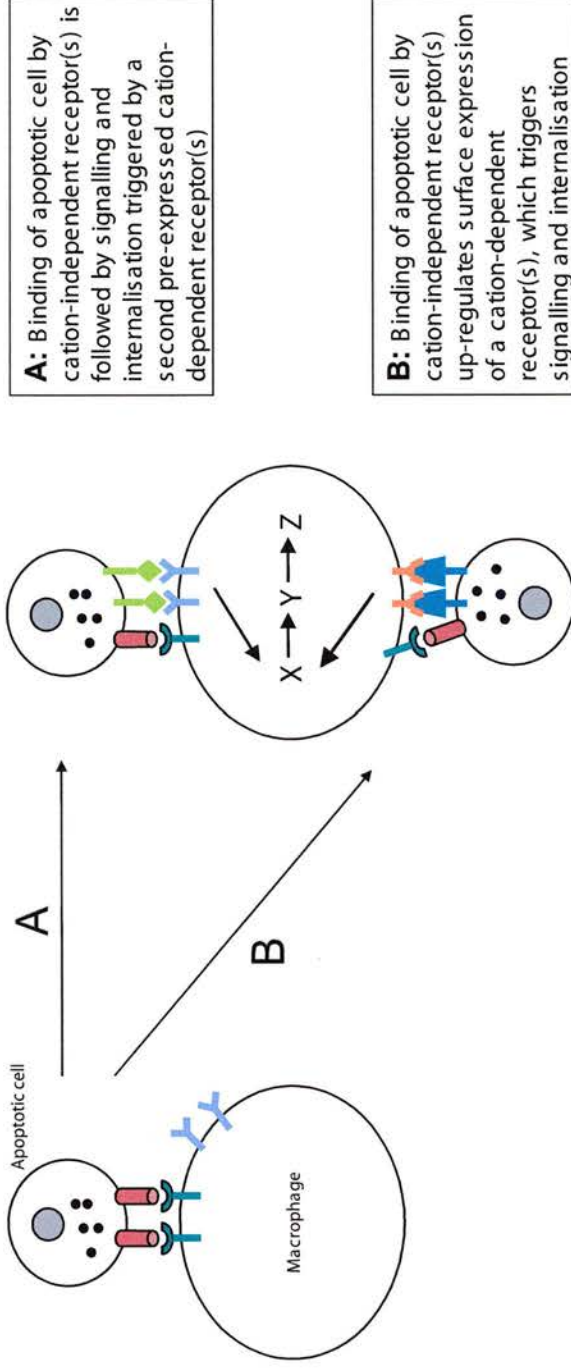


Figure 4.12 2-step binding and signalling model for apoptotic cell recognition and phagocytosis

The apoptotic neutrophil binds to the MDM Φ via a yet unknown receptor in a cation-independent fashion. This is followed by engagement of a second receptor(s), in a cation dependent manner. The second receptor could be pre-expressed by the MDM Φ , or upregulated following binding of the apoptotic cell. The second receptor, is likely to be required for signalling which triggers internalisation of the apoptotic cell.

The ligand binding of the internalisation receptor(s) triggers signalling events required for engulfment.

I have demonstrated that Dex-treatment up-regulates binding of apoptotic neutrophils. No receptor has been identified, but pH experiments may suggest that Dex-treatment results in changes to the cell surface which alters the charge on the MDM Φ , favouring binding of apoptotic neutrophils. Dex also induces important changes to the internalisation receptor, possibly recruiting a PS recognition mechanism not used by untreated MDM Φ . I hypothesise that the up-regulation of this second stage is essential for the increased signalling required for the augmentation in internalisation apparent following Dex-treatment. My results (presented in chapter 5) coupled with those of previous studies (Giles et al., 2001) show that Dex-treatment critically up regulates Rac GTPase activation. Seemingly, Dex-treatment enhances the binding and signalling receptor stages, as well as inducing changes to the cytoskeleton conducive to internalisation.

CHAPTER 5: ADHESION AND MIGRATION

Introduction

Podosomes

Podosomes are specialised adhesion complexes which were originally identified in Rous sarcoma virus-transformed fibroblasts (Tarone et al., 1985), and are now known to be restricted to monocyte-derived cells such as macrophages, dendritic cells and osteoclasts (Marchisio et al., 1987). Podosomes consist of a central core which is enriched for F-actin. Actin regulators such as Cdc42H, WASp and the Arp 2/3 complex, alongside intermediate filaments and their associated proteins (Babb et al., 1997) are also located in the core region. Surrounding the core is a ring of actin- and integrin-associated proteins which include: α -actinin, paxillin, talin, vinculin, the scaffolding protein and Src substrate, F-actin and kinases such as Pyk2 (Abram et al., 2003).

Podosomes are structurally and functionally related to adhesions found in other cells such as focal adhesions (for a recent review on different adhesion types see (Kaverina et al., 2002)). Both contain the proteins α -actinin, vinculin and talin and many of the podosomal ring proteins are also conserved in focal adhesions. Since ring proteins serve as complexes linking the cytoskeleton to the core, this suggests a conservation in structural linkage of adhesions to the cytoskeleton (Linder and Aepfelbacher, 2003; Pfaff and Jurdic, 2001). However, F-actin and the actin-linker protein fimbrin are unique to podosomes. A further disparity is the requirement for de novo synthesis for focal adhesion but not for podosome formation (Tarone et al., 1985). Podosomes are involved in the adhesion and migration of myeloid cells and have also been shown to play a role in forming the sealing zone in osteoclasts (Vaananen and Horton, 1995), and metalloproteinases in the core of podosomes are involved in local extracellular matrix (ECM) degradation (Sato et al., 1997) (Chen et

al., 1984). Focal adhesions too are important in adhesion and migration, whilst also sensing elasticity and force via connection to stress fibres (Wehrle-Haller and Imhof, 2002).

The focal adhesion links the cytoskeleton to ECM proteins via integrins. The integrin family are heterodimer receptors comprising an alpha and a beta subunit (Miranti and Brugge, 2002), which bind to ECM proteins mediating cellular adhesion and signalling. $\beta 1$ integrin subunits have been shown to localise to the core, and $\beta 2$ and $\beta 3$ to the ring structure, as well as being found adjacent to the podosome (Johansson et al., 1994). During adhesion, Pyk2 and paxillin bind the tail of the $\beta 3$ subunit of the $\alpha \beta 3$ integrin in osteoclasts (Pfaff and Jurdic, 2001) (McHugh et al., 2000) or to $\beta 2$ integrins in macrophages (Duong and Rodan, 2000). In summary, podosomes provide a link between the ECM and the actin cytoskeleton, acting as scaffolding for the assembly of signalling complexes which are required for actin-controlled processes such as migration.

Regulation of podosomes formation, growth and disassembly

Podosomes are highly dynamic structures, with a half life of between 2 and 12 minutes (Linder and Aepfelbacher, 2003). They are found in polarised and migrating cells, where their formation and turnover at the leading edge and dissociation at the cell rear are critical for migration. Evans and colleagues studied podosome regulation in the lamellipodia of macrophages. Fluorescent-actin and -fimbrin were used to show that podosomes form de novo in a microtubule-independent manner at the leading edge of the cell. The lifetime of short-lived podosomes can be extended by fusion with another podosome or by fission of a podosome into daughter podosomes. A further mechanism observed was the formation of a large indistinct structure, they termed a podosome cluster precursor (PCP), from a single podosome which then forms a cluster of daughter podosomes. These microtubule-dependent processes provide a mechanism for the generation of podosomes at an extending leading edge,

since the branching and forming of daughter podosomes always occurs towards the leading edge of the cell (Evans et al., 2003).

Studies into the regulation of focal adhesions and podosomes have implicated a number of different pathways in their formation and turnover (see Figure 5.1). The GTPases Rac and Cdc42 mediate the formation of small focal complexes in fibroblasts (Hotchin and Hall, 1995; Nobes and Hall, 1995), whereas Rho and Cdc42 are involved in the formation of osteoclast and human Hela cell podosomes respectively (Dutartre et al., 1996; Zhang et al., 1995). Studies in the CSF-1-dependent macrophage cell line, Bac.1, also demonstrated the requirement for Rac and Cdc42 in the formation and distribution of podosomes, with Cdc42 signalling upstream of Rac in this pathway (Allen et al., 1997). Work in primary human macrophages, demonstrated that Cdc42 co-localised and bound to WASp in the core of podosomes where they mediated the disassembly and formation of podosomes. This was demonstrated both by the injection of the c-terminal of WASp, leading to sequential loss of WASp, actin and vinculin from podosomes and by studies demonstrating that macrophages from WAS patients, whose cells contain no WASp, have no podosomes. WASp is known to bind the Arp2/3 complex and this may result in a concentration of Arp2/3 complex at the site of adhesion, where it mediates actin assembly (Linder et al., 1999). Linder and colleagues further demonstrated that microtubules, linked to WASPs via the Cdc42 interacting protein CIP4, are essential for podosome formation, although loss of podosomes does not affect microtubular structure (Linder et al., 2000). In addition, microtubules have also been shown to cross-talk with focal adhesions (Krylyshkina et al., 2003).

Kinases such as FAK, Pyk2 and Src also play an important role in the regulation of adhesions. FAK was shown to be important in adhesion disassembly in a Rho and Rac independent manner by the increased size and numbers of peripheral focal adhesions in FAK^{-/-} mice fibroblasts. Webb and colleagues demonstrated that the FAK-Src signalling complex phosphorylates the adaptor proteins, paxillin and p130cas, and postulated that this could alter binding of other adhesion components and hence regulate adhesion formation and disassembly. The targeting of Src to

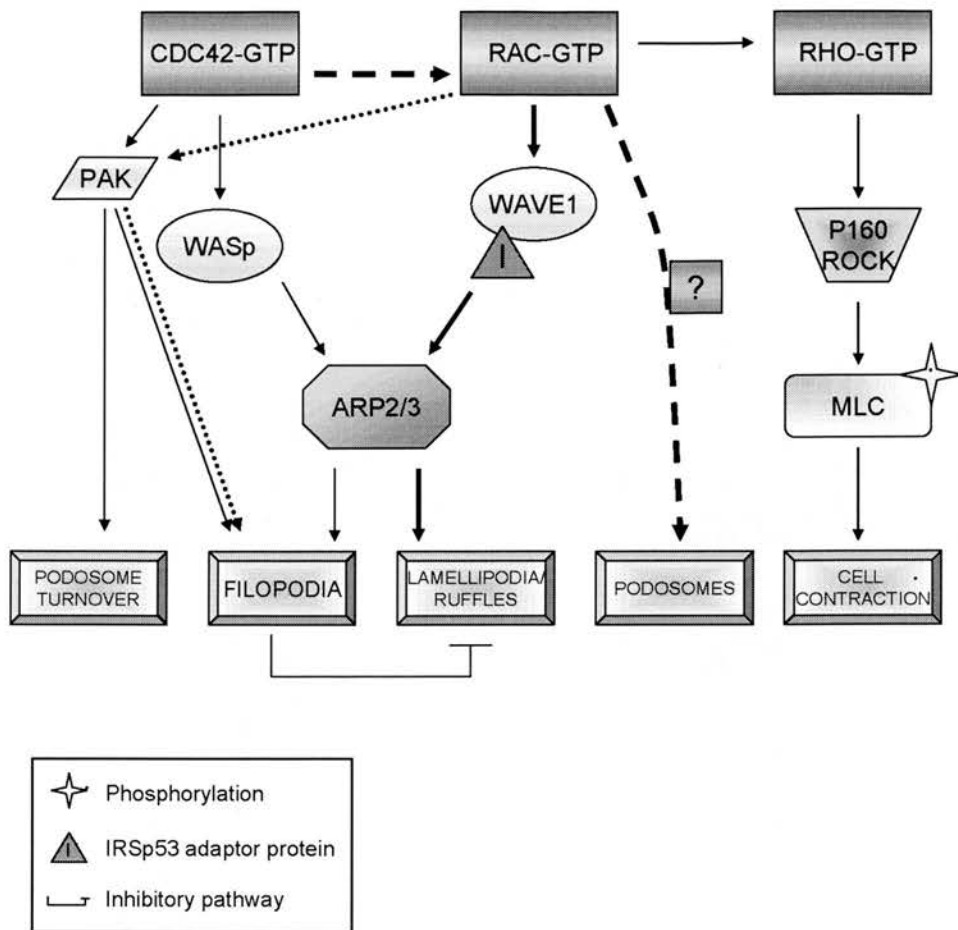


Figure 5.1 Schematic diagram of potential pathways involved in macrophage adhesion and migration

specific sites is carried out by members of the Rho GTPase family. For example, Rho directs localisation to focal adhesions whilst Rac1 and Cdc42 target localisation to focal complexes associated with lamellipodia and filopodia respectively, providing a mechanism for directing adhesion turnover at specific regions in a cell (reviewed in (Frame et al., 2002)). A further mechanism regulated by the FAK-Src complex was demonstrated using inhibitor studies in fibroblasts. The activation of the MAP kinase signalling cascade by the FAK-Src complex resulted in activation of ERK, which in turn activates myosin light chain kinase (MLCK). MLCK activates acto-myosin based contractility which may sever linkages and result in the disassembly of adhesions required for migration (Webb et al., 2004). Interestingly, studies of the IC-21 macrophage cell line showed that Pyk2, which may functionally substitute for FAK (Duong et al., 1998), became phosphorylated following adhesion to fibronectin, and that the phosphorylation was dependent on α M β 2 integrin (Duong and Rodan, 2000). In a manner similar to FAK-src, p21-Activated Kinase (PAK) has a role in cell adhesion and contraction via MLC phosphorylation in endothelial cell migration (Kiosses et al., 1999).

Migration is essential for macrophage function

The migration of monocytes into infected tissues is necessary for the clearance of bacteria and apoptotic neutrophils during an inflammatory response (Chae et al., 2002; Nakano et al., 1994). Monocytes leave the bone-marrow as non-differentiated cells and circulate in the blood for 1-3 days before migrating from blood vessel lumens into the inflamed tissue (Nichols et al., 1971). Monocytes initially form transient adhesions via selectins to the endothelial cells which results in the 'rolling' of the monocytes along the blood vessel wall. Following stimulation by chemokines such as CCR2, the monocytes become firmly adhered or 'tethered' to the endothelial cells via β 1 and β 2 integrins, e.g. LFA-1 and VLA-4, expressed on their surfaces. The polarised monocytes then migrate through tight junctions between the endothelial cells into the inflamed tissues where they differentiate into monocyte-

derived macrophages. In order to phagocytose target cells, be they bacteria or apoptotic neutrophils, MDM Φ migrate towards them following chemoattractant gradients, such as FMLP released by bacterial cells. It was also recently shown by Lauber and colleagues that apoptotic cells release the phospholipid, lysophosphatidylcholine (LPC), in a caspase-3 dependent manner which resulted in monocyte migration towards them (Lauber et al., 2003).

For full resolution of inflammation the recruited macrophages themselves need to be removed from the inflammatory site. Any persistence of aged macrophages may result in the leakage of their histotoxic contents into the tissue. Following the discovery that macrophages can be induced to undergo apoptosis (Albina et al., 1993; Mangan and Wahl, 1991), it was proposed that macrophages underwent apoptosis and were phagocytosed in situ (Savill et al., 2002). However, recent examination of the fate of the inflammatory macrophages by Bellingan and colleagues illustrated that the macrophages emigrate to the draining lymph nodes (Bellingan et al., 1996) in a tightly controlled manner mediated by the integrins VLA-4 and VLA-5 (Bellingan et al., 2002a).

Migration

Cellular migration is a highly organised multi-step process which is fundamental to many functions including embryogenesis and development, immune responses and wound healing (Lauffenburger and Horwitz, 1996).

Initially a cell becomes polarised in response to a stimulus, e.g. a cheomattractant such as FMLP. Lamellipodia and filopodia form at the leading edge and a uropod (retracting tail) at the rear of the cell. The lamellipodium is a thin sheet of cytoplasm which extends from the front of the cells supported by an actin meshwork. Filopodia are short protrusions extending out of the lamellipodia which are composed of actin bundles (Figure 5.2C). The extension of the lamellipodia is stabilised by the formation of adhesions in this region (Figure 5.2B), which is followed by actin-myosin based contraction of the cell body. Completion of migration requires the

disassembly of adhesions in the uropod and recycling of the adhesion components to the leading edge of the cell. Thus, adhesion and migration are intimately linked, with the disruption of adhesion-associated proteins disrupting migration (Kiosses et al., 1999) (Jones et al., 2002).

Migration is driven by actin polymerisation, with members of the Rho family GTPases central to the regulation of this process (Allen et al., 1997; Ridley, 2001). They act as molecular switches which cycle from an active GTP form to an inactive GDP form. A cell binding to chemoattractant via uniformly distributed receptors, interprets the gradient via the production of lipid products e.g. PIP₃, in an asymmetrical manner, correlating to the chemoattractant gradient. PIP₃ can generate GEFs which activate the Rho GTPases in a localised manner within the cell (Weiner, 2002). Rho GTPases have been implicated in the nucleation and polymerisation of actin during lamellipodia and filopodial formation in macrophages (Small et al., 2002). The growth of filopodia in macrophages requires the activation of Cdc42 which, via N-WASP, activate the ARP2/3 complex. The ARP 2/3 complex promotes the directed nucleation and polymerisation of actin filaments as branches from existing filaments (Figure 5.1).

Experiments using constitutively active and dominant negative Rac demonstrated a role for this protein in adhesion, ruffling and lamellipodia growth (Allen et al., 1997). Rac drives activation, via the adapter protein IRSp53 (Miki et al., 2000), of a second member of the WASp protein family, Wave1, which like WASp binds to the ARP2/3 complex. Recent studies in BMDMΦ from RAC1^{-/-} mice demonstrated that the Rac-1 isoform is not involved in macrophage migration (Wells et al., 2003), thereby implicating Rac-2 in the regulation of this process.

Additionally, Rho activated p160 Rho-kinase (ROCK) phosphorylates MLC causing cell body contraction (Ridley and Hall, 1992) (Leung et al., 1996). Thus the coordinated responses of Rho-GTPases orchestrate central aspects of cellular migration. In addition, other signalling pathways such as the MAPkinase signalling cascade, PI(3)Kinase pathway (Vanhaesebroeck et al., 1999) and calcium concentrations have also been associated with migration ability of leukocytes. For example, ERK1/2 becomes activated and is required for human peripheral blood

monocyte migration following stimulation by the monocyte chemoattractant proteins (MCPs) in a potentially PIP₃ Kinase-dependent manner (Srinivasan et al., 2003; Wain et al., 2002).

Migration plays a fundamental role in the function of a macrophage; from the initial migration of a monocyte into the inflammatory tissue, to migration of the differentiated macrophage towards apoptotic target cells and emigration of the spent macrophage to the draining lymph nodes. Consequently a breakdown in any of these processes would result in the failure in the resolution of inflammation. Therefore, the experiments presented in this chapter aim to investigate the effect of Dex on macrophage adhesion and migration, by studying both the process of migration and the underlying signalling pathways.

Results

Macrophages have distinct adhesions which are important in migration

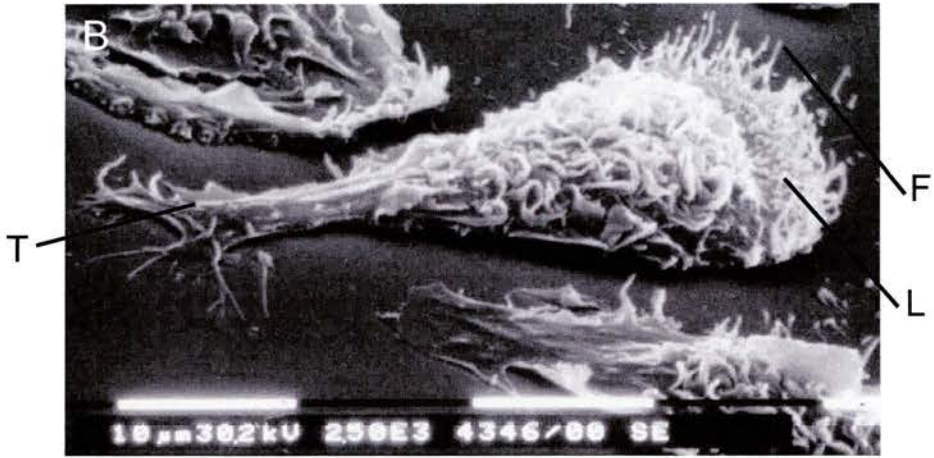
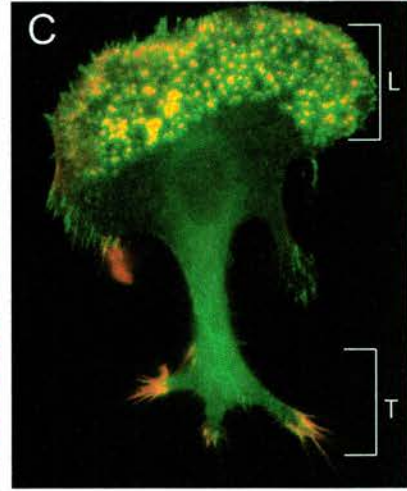
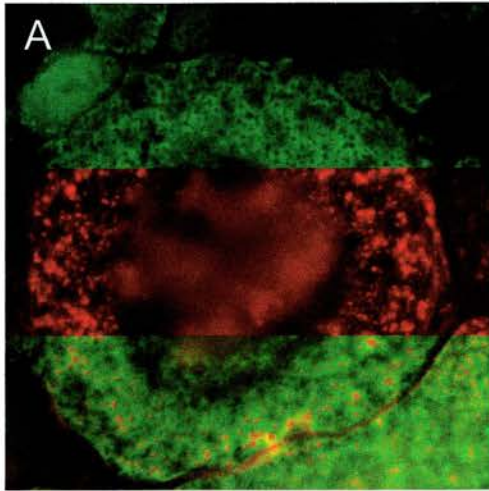
Cellular adhesions are important for the migration of a macrophage, stabilising the extending lamellipodia, allowing for communication via integrins with the underlying substratum and their disassembly being a requirement for the detachment of the retracting tail. In view of this critical link, I investigated the association between podosomes and the migratory morphology of 5 day monocyte-derived macrophages (MDM Φ), allowing the 'normal' process of macrophage migration to be described. Thus, peripheral blood monocytes were cultured on glass cover slips for 5 days in 10% AS, whereafter they were fixed and then either processed for immunofluorescence staining, or dehydrated and prepared for scanning electron microscopy by coating with gold particles. The MDM Φ seen in Figure 5.2A is a 'giant' multinucleated MDM Φ , with typical cortical podosome staining. The podosomes, as discussed in the introduction, are composed of a core of proteins which include F-actin, surrounded by a ring of proteins including paxillin. The top third of the figure shows the MDM Φ stained for the protein paxillin, which defines a ring-like structure with a central core that is negative for paxillin. The middle third of the image shows the punctate F-actin staining in the core region. The bottom third shows an overlay of the top two images with the core of F-actin surrounded by a ring of paxillin staining, typical of 'podosome-like' structures. Figure 5.2B shows a scanning electron micrograph (SEM) of migrating 5 day MDM Φ . The macrophage is polarised with extending lamellipodia (L) and filopodia (F) at the leading edge, and a retracting tail (T) at the back behind the highly ruffled cell body. Figure 5.2C shows a 5 day migrating MDM Φ which has been stained for the podosomal proteins, paxillin and actin. In this example, the MDM Φ was observed to have a similar morphology to that depicted in Figure 5.2B, exhibiting polarisation with a leading edge at the top of the cell.

Figure 5.2 Podosome staining and images of migrating macrophages

(A) shows a 5 day MDM Φ stained for podosomes. The top third shows the staining for the ring protein, paxillin (green), the middle third for the core protein F-actin and the bottom third is an overlay of the two. The image was captured using a Zeiss Immunofluorescence microscope x63 lens.

(B) is a scanning electron microscopy image of a migrating 5 day MDM Φ . The lamellipodia (L) and filopodia (F) seen at the leading edge are areas of active actin polymerisation, with the retracting edge (T) at the rear of the cell.

(C) shows a migrating 5 day MDM Φ stained for paxillin (green) and F-actin (red) to label the podosomes. The podosomes were present at the leading edge, where they stabilised the extending lamellipodia (L). The image was captured using a Zeiss immunofluorescence microscope using x63 lens.



The podosomes are present through the leading edge, where they are known to be required for stabilisation of the extending lamellipodia (L). The filopodia, which extend from the front of the cell, also contained paxillin and actin, although the proteins are not organised into podosomes in these areas.

An organised microtubule network is absent from Dex-treated MDM Φ

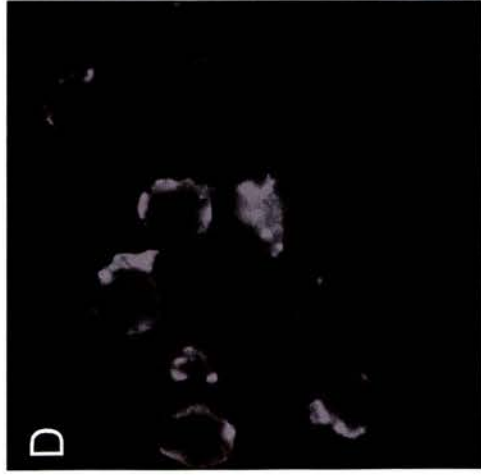
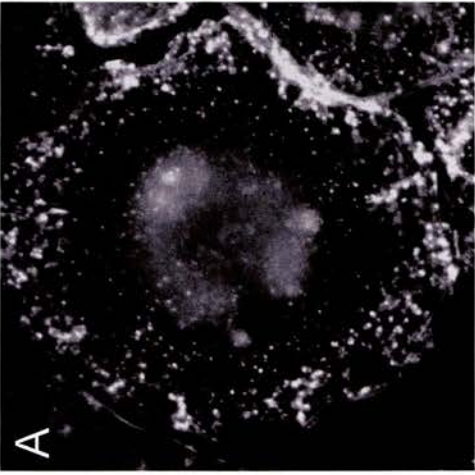
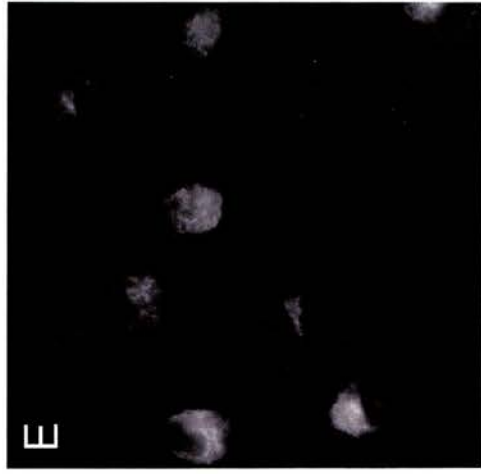
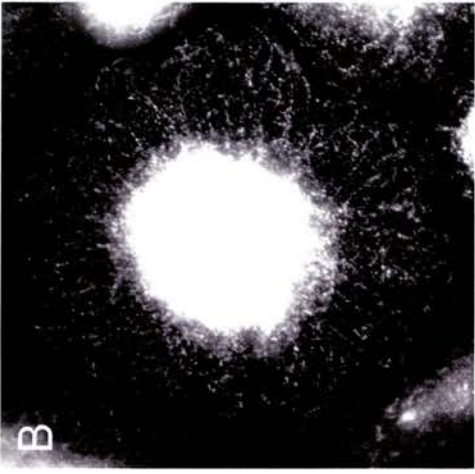
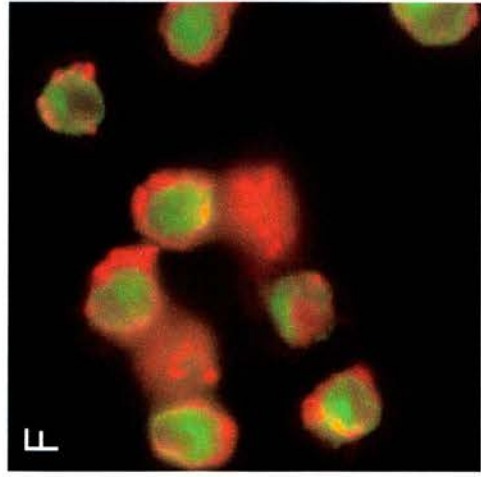
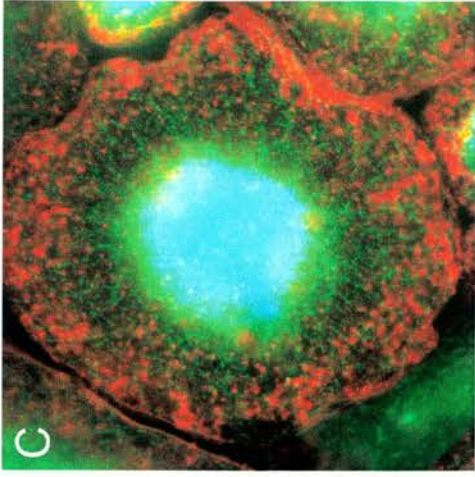
The observation that the Dex-treated MDM Φ lacked podosomes (Giles et al., 2001)(Figure 6.7), together with the study by Linder and colleagues which demonstrated that microtubules are necessary for the formation of podosomes (Linder et al., 2000) led me to investigate the microtubule network in the untreated (control) and Dex-treated MDM Φ by staining for the microtubule subunit, β -tubulin, in conjunction with F-actin staining of the podosomes. Figure 5.3A shows a control MDM Φ labelled for the podosomal marker protein F-actin and Figure 5.3B shows the β -tubulin labelling for the same cell. The β -tubulin was seen to radiate out from the centre of the cell in an organised network of fine 'threads'. Figure 5.3C shows the overlay of Figure 5.3A and B, where the podosomes were present either along the length of, or at the termination of the microtubules.

Figure 5.3D shows a 5 day Dex-treated MDM Φ labelled for F-actin, which was present in the ruffles at the edge of the cells only with no punctate staining of podosomes. Figure 5.3E shows the β -tubulin staining of the microtubules. The organised structural microtubule network observed in the control MDM Φ was not apparent in the Dex-treated MDM Φ . The β -tubulin appeared as a diffuse staining with occasional short strands of microtubules, which did not appear to link up with other microtubules into an organised network. The absence of a highly organised microtubule network accounts for the lack of podosomal structures associated with the microtubules.

Figure 5.3 Microtubules are associated with podosomes in Control but not Dex-treated macrophages

(A) and (D) are F-actin stained 5 day untreated (control) and Dex-treated MDM Φ respectively, confirming the previous observation that control but not Dex-treated MDM Φ have podosomes. (B) and (E) are β -tubulin stained microtubules in control and Dex cells respectively. The control macrophages had an organised network of microtubules, whereas the Dex-treated MDM Φ microtubules were short and unorganised. (C) and (F) are images for the control and Dex-treated MDM Φ respectively; with the F-actin staining in red and β -tubulin in green. The podosomes in the control cells were present either along the length or at the termination of the microtubules. The Dex-treated MDM Φ have no obvious connection between the microtubules and the F-actin staining.

Images were captured using a Zeiss Immunofluorescence microscope using x100 lens.



Dex-treated MDM Φ exhibit decreased surface expression of integrins

Integrins are important in the relaying of signals between the ECM and the cell via the podosomes, as well as playing an important role in migration, and I therefore wished to profile surface integrins to examine any differences in expression between the control and Dex-treated MDM Φ .

5 day MDM Φ grown in 10% AS \pm 125nM Dex were gently removed from the tissue culture plates and labelled for the surface expression of a panel of different integrins, before analysis by flow cytometry.

The alpha subunits α_L (CD11a), α_M (CD11b), and α_X (CD11c), and β_2 subunit (CD18) which together form LFA-1, Mac-1 and p150,95 respectively were examined. α_4 (CD49d) and α_5 (CD49e) along with the β_1 subunit (CD29), which pair to form VLA-4 and VLA-5, were also investigated alongside α_V (CD51) and $\alpha_V\beta_3$ (CD51/CD61) (Figure 5.4A).

α_L and α_M were expressed at high levels on the surface of the control MDM Φ , and were dramatically down-regulated on the Dex-treated MDM Φ surface, whilst α_X was expressed at similar levels on the control and Dex-treated MDM Φ . Down-regulation of α_L and α_M is likely to contribute to the reduced expression of β_2 on MDM Φ following Dex-treatment. Examination of α_4 and α_5 showed that they were both expressed on the surface of the resting control or Dex-treated MDM Φ at fairly similar levels. β_1 levels were also unchanged following Dex treatment, although staining levels were low and given that α_5 showed high expression this may suggest that the β_1 antibody was not working well. Nevertheless, the failure of Dex-treatment to alter β_1 , α_4 or α_5 expression levels suggests that VLA-4 and VLA-5 levels were consistent between control and Dex-treated MDM Φ . Phenotyping α_V and $\alpha_V\beta_3$ integrins demonstrated a decrease in expression following Dex treatment. The significant down regulation of α_L on Dex-treated MDM Φ observed by flow cytometry was confirmed by immunofluorescence microscopy using the WAC70 clone (Figure 5.4B). The control MDM Φ had specific α_L staining around the cell periphery along with some diffuse intracellular staining. However, contrary to the flow cytometry results no peripheral surface staining was detected on the Dex-treated

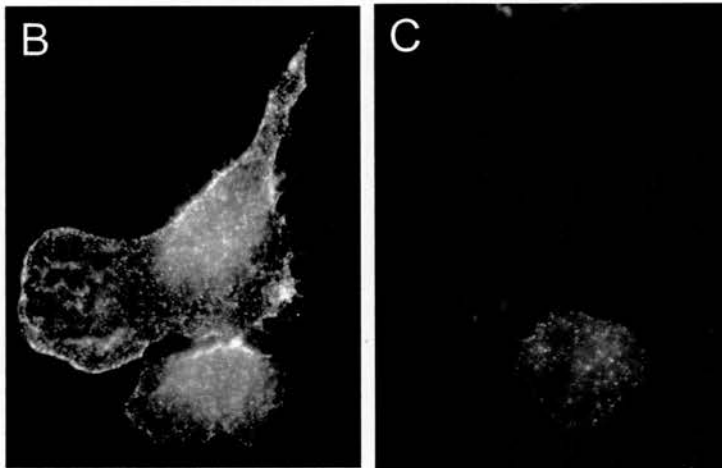
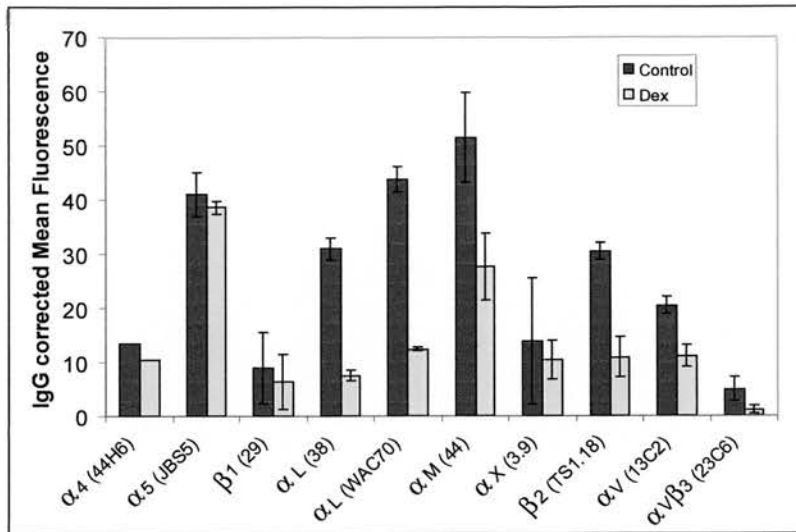


Figure 5.4 Integrin surface staining of 5 day MDMΦ

(A) In order to examine changes in integrins following Dex treatment, 5 day control and Dex MDMΦ were stained for the following integrins and analysed by flow cytometry: the alpha subunits α L, α M and α X and beta subunit, β 2, of LFA-1, MAC-1 and LeuCamc respectively. The cells were also stained for the alpha subunits α 4 and α 5 and β 1 which form VLA-4 and VLA-5, along with α V β 3. Data shown are the mean fluorescence \pm SEM, (n=3).

To confirm the significant down-regulation of α L in Dex-treated MDMΦ, control (B) and Dex-treated (C) MDMΦ were stained for α L using WAC70 clone. Images were captured by Zeiss Immunofluorescence microscope using a x100 objective lens.

MDM Φ by immunofluorescence, suggesting that the flow cytometry is a more sensitive technique for detecting low levels of antigen.

Establishment of a migration assay

In order to examine the process of migration, I sought to establish an assay which would allow the movement of the different populations to be studied. Although transwell assays (Boyden chamber) have been used in the laboratory, I decided that the best option would be to use time lapse video microscopy as it would provide insight into MDM Φ migratory behaviour. The MDM Φ monolayers were 'wounded' using a pipette tip and then imaged over a period of several hours as cells migrated into the 'wound'.

Preliminary observations suggested that the MDM Φ at the edge of a wound became polarised and migrated, presumably in response to loss of cell-cell adhesion. In order to establish key assay parameters such as the length of time the MDM Φ took to migrate, initial experiments were carried out in which cells were wounded in a tissue culture plate and then returned to the tissue culture incubator before examination by light microscopy. These initial trials established that approximately 9 hours was required for the control MDM Φ to completely 'fill' the wound.

I also investigated the possibility of looking at chemotactic migratory responses of MDM Φ . However, this was not easily studied by time-lapse microscopy as the stage was moved following 'capture' of each image, resulting in agitation of the media in the flask. Thus chemoattractant added to one end of the flask would rapidly become distributed throughout the media, making assessment of directed migration difficult. The possibility of adding chemoattractant into an agarose plug was also investigated but owing to the small flask neck this was only plausible in a tissue culture dish without the ability to image via time lapse microscopy. Initial experiments used highly migratory monocytes as a test system. Agarose was carefully pipetted onto gelatine-coated glass slides to form a uniform layer; then, using a metal punch, three linear and equidistant holes were made in the agarose. Iscove's DMEM was added to the top well, monocytes to the middle well and a chemoattractant diluted in Iscove's

DMEM to the bottom well. The monocytes adhered to the tissue culture plate at the bottom of the well and could then migrate towards the stimulus (Nelson et al., 1975). However, the small volumes of media, coupled with the length of time needed for the assay often meant that the wells dried out. A decision was taken to concentrate on the time-lapse wounding method as it provided temporal information relating to the migratory process.

Dex-treated MDM Φ have an altered migratory potential

The data in Figure 5.5 shows the rate of migration and number of control MDM Φ in the wound, with the wound filled by the end of the assay. This can be seen in an example video in Figure 5.6 Video 1. The control MDM Φ became polarised, formed large lamellipodia and migrated slowly in the direction of the newly formed lamellipodia (Figure 5.6 Video 3).

The migration of Dex-treated MDM Φ was different to that observed for the control MDM Φ . The Dex-treated MDM Φ were observed to display an initial 'burst' in migratory activity (Figure 5.5 and Figure 5.6 Video 2), migrating much faster than the control MDM Φ (For donor 1 MDM Φ migratory rates during the 1st hour of migration were: control = 9 cells/hr, Dex = 54 cells/hr). Careful analysis of the time-lapse microscopy images revealed that the Dex-treated MDM Φ also responded in a very distinctive manner (Figure 5.6 Video 4), whereby they, apparently randomly, rapidly extended and retracted multiple membrane protrusions, often as long as the cell itself. Occasionally lamellipodia, similar to those observed in control MDM Φ were formed with a small uropod at the rear of the cell, and a short period of directional migration occurred (Figure 5.6 Video 4). Another difference between Dex-treated and control MDM Φ was that Dex-treated MDM Φ failed to sustain migratory behaviour following the 'burst' described above.

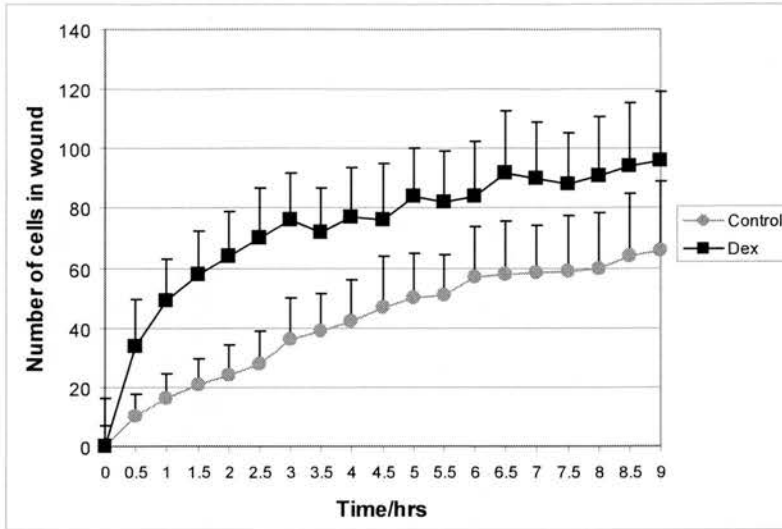


Figure 5.5 Dex treated macrophages have an altered migratory potential

5 day MDM Φ were cultured in microscopy flasks and, prior to the assay, a wound was scored down the middle of the monolayer. The media was changed to remove any unadhered cells and the migration captured by time-lapse microscopy. An image was taken every 5 minutes for 9 hours. Data were analysed as described in Materials and Methods.

The figure shows that Dex MDM Φ show an initial burst in migratory potential which has slowed down by around 3 hours. The control MDM Φ showed a slow and steady rate of migration until the wound was filled. Data are shown for mean of 3 experiments + SEM

Figure 5.6 Video microscopy showing the migration of 5 day control and Dex-treated MDM Φ

Monolayers of 5 day untreated (control) and Dex-treated MDM Φ were wounded and then washed to remove the non-adherent cells. The migration of the MDM Φ back into the wound was imaged by phase contrast microscopy, with one image captured every five minutes.

Video 1: control MDM Φ migration over a 20 hour period

Video 2: Dex-treated MDM Φ migration over a 20 hour period

Video 3: Clip of control MDM Φ migration demonstrating formation of lamellipodia and migratory phenotype.

Video 4: Clip of Dex-treated MDM Φ migration, showing the altered migratory phenotype following Dex treatment, * marks the lamellipodia.

Representative videos shown for each condition from 5 experiments, each from a separate donor.

However, they continued to rapidly extend and retract processes, suggesting that cytoskeletal regulation was not compromised.

Owing to a difference in size between the control MDM Φ and Dex-treated MDM Φ the 'wound' required a larger number of Dex-treated MDM Φ in it for it to be filled. Thus, despite the increased number of Dex-treated MDM Φ in the wound compared to control MDM Φ shown in the graph in Figure 5.6A, the Dex 'wound' is less full than the control 'wound'.

Images of migrating control and Dex-treated MDM Φ , taken from captured images during phase contrast time-lapse microscopy are shown in Figure 5.7. The control MDM Φ form lamellipodia at the leading edge which differed in area, perhaps reflecting the stage of lamellipodia formation (Figure 5.7 A-D), and in some cases the lamellipodia surrounded the entire cell (Figure 5.7 E). The lamellipodia and uropods did also form in some Dex-treated MDM Φ (Figure 5.7F and G), although the filopodial-like extended membrane was a more common morphology (Figure 5.7 H-J).

F-actin is found predominantly at the leading edge of Dex-treated MDM Φ

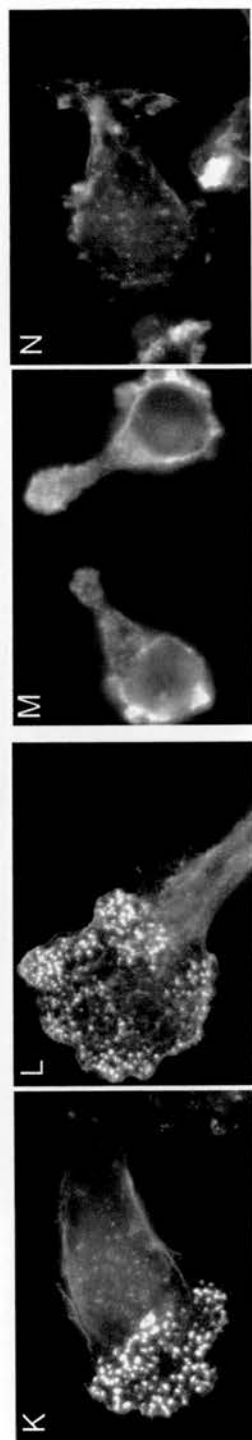
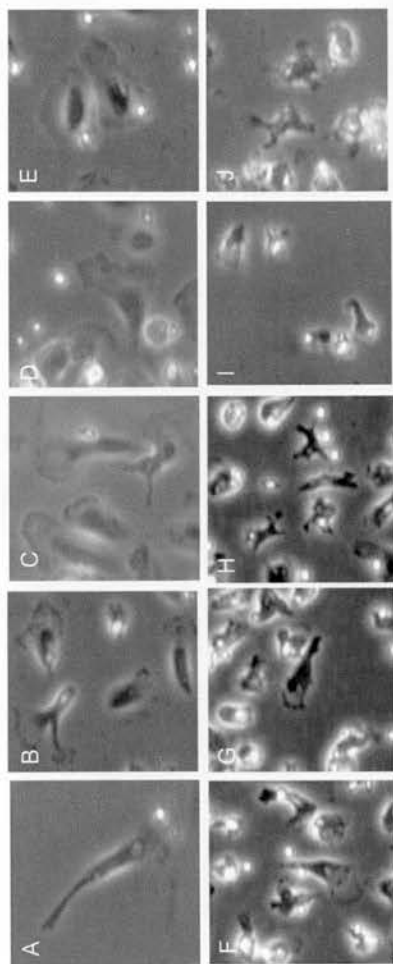
Following the observation that, despite a lack of podosomes, the Dex-treated MDM Φ are capable of migrating and forming temporary lamellipodia, it was of interest to examine the F-actin distribution and podosome formation during MDM Φ migration. 5 day MDM Φ , grown on glass cover slips, were wounded using a pipette tip to induce migration. The cells were returned to the incubator for 2 hours and then fixed and labelled for F-actin. The control MDM Φ formed a polarised morphology with the F-actin present predominantly in podosomes at the leading edge of the cell, as observed previously (Figure 5.7K and L). The Dex-treated MDM Φ had a distinct F-actin distribution, with the majority of staining observed at the leading edge in ruffles. Small punctate regions of F-actin were apparent in the uropod and cell body and occasionally in the region behind the leading edge (Figure 5.7N). These adhesions did not appear in as large a number as the control MDM Φ podosomes, nor

Figure 5.7 The cellular morphology and F-actin distribution in migrating MDM Φ

(A-J) 5 day Dex-treated MDM Φ were imaged during a wounding assay by time-lapse microscopy. Images are representative of control and Dex-treated MDM Φ during migration from 5 experiments.

Images (A-E) show the lamellipodia and retracting tail in the polarised control MDM Φ , required for migration. Images (F-J) show the morphologies adopted by the Dex-treated MDM Φ during the migration assay. (F-G) show Dex-treated MDM Φ with lamellipodia and uropods, whilst (H-J) show the membrane extensions which were extended and retracted from the Dex-treated MDM Φ between periods of migration.

(K-N) 5 day MDM Φ , differentiated in the presence of 10% autologous serum \pm 125nM Dex were fixed and stained for F-actin. (K-L) are control MDM Φ , demonstrating that the lamellipodia are stabilised by the formation of podosomes. (M-N) are Dex-treated MDM Φ which contain F-actin predominantly in ruffles at the leading edge of the cell, as well as in adhesions throughout the cell body. Images were captured using a Zeiss Immunofluorescence microscope using a x100 lens.



did they stain as brightly (Figure 5.7M and N). This data provides evidence that although Dex-treated MDM Φ form lamellipodia which are stabilised by small adhesions, they do not form podosomes-like adhesion structures during migration.

Dex-treated MDM Φ have increased activation of Rac-GTPase

The Rho-GTPase, Rac has been implicated in the actin polymerisation during adhesion formation, ruffling and cellular migration, and so I examined the levels of total and active Rac within different macrophage populations, to investigate if any changes in this important Rho family GTPase could explain the observed alterations in Dex macrophages.

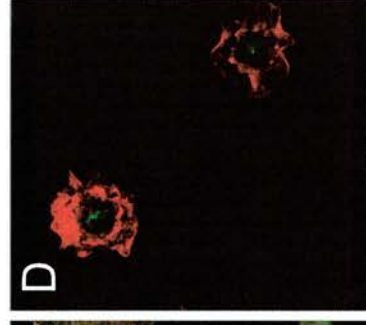
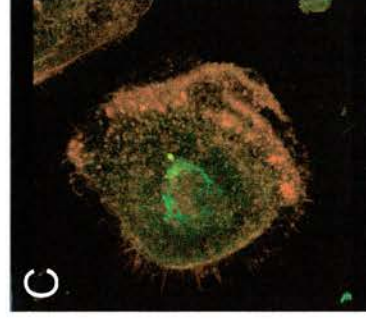
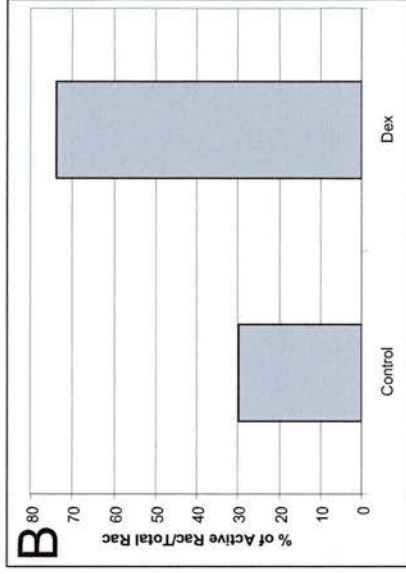
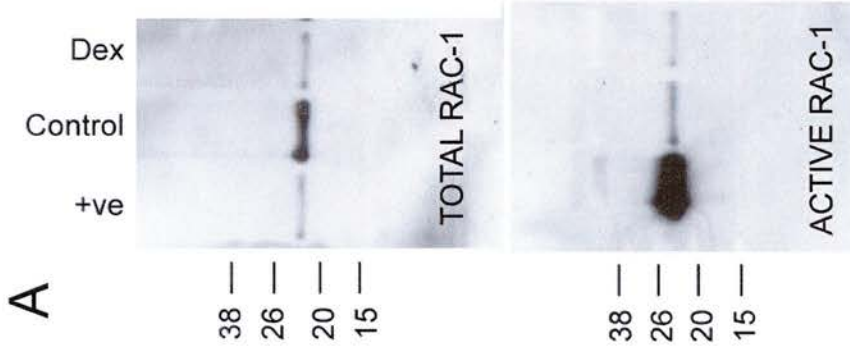
5 day MDM Φ , which had been cultured in 10% AS alone \pm Dex were used in a Rac pulldown assay as described in Chapter 2. Lysates were equalised for protein concentration before the assay. Figure 5.8 shows a western blot which had been probed for total and active Rac-1 in the macrophages. The levels of total Rac-1 showed some differences between the samples, with Dex-treated MDM Φ containing lower levels of total Rac-1 protein than the control MDM Φ . Calculation of the relative levels of the total Rac-1 compared with active Rac-1 (Figure 5.8B) suggested that the relative levels of GTP Rac-1 were higher in the Dex-treated MDM Φ than the control MDM Φ . However, levels of active Rac-1 could well be higher in the control MDM Φ owing to a higher total amount of Rac-1 in these cells. It was also important to consider the localisation of Rac within the MDM Φ , because this would impact on the proteins Rac-1 could bind and therefore on downstream function. To this end immunofluorescence imaging of macrophages for total Rac-1 was carried out in control and Dex-treated MDM Φ (Figure 5.8C and D). The images shown are maximum projections of confocal stacks where slices are taken through the cells. A maximum projection adds together all positive staining in the cell, and so staining at the basal and apical surface of the cell appears in the same image. Rac-1 (green)

Figure 5.8 Rac-GTPase concentration is altered in Dex-treated MDMΦ

(A) and (B) Total Rac protein levels were compared between 5 day MDMΦ differentiated in 10% AS ± 125nM Dex. The total Rac levels were lower in the Dex-treated MDMΦ when compared to the control MDMΦ. 30% of the control MDMΦ Rac was activated, but this rose to 75% in the Dex-treated MDMΦ.

(C) and (D) shows maximum projection images (shows all staining through all sections of cells in one image) of control and Dex-treated MDMΦ respectively. The MDMΦ were stained for F-actin (red) and Rac-1 (green). In both the control and Dex-treated MDMΦ the Rac stained an area on the very top of the cells, and was also seen in a diffuse pattern throughout the control MDMΦ. It did not associate with F-actin rich ruffles in the Dex-treated MDMΦ.

C = x63 lens with 1.5x zoom; D = x63 lens with 2x zoom



localised to the apical surface of both the control and Dex-treated MDM Φ , as well as being observed diffusely throughout the rest of the cell. Macrophages were co-stained with F-actin (red), which was seen throughout the control MDM Φ and specifically in podosomes on the basal surface. Interestingly, the F-actin mainly localised to the ruffles on the apical surface of the Dex-treated MDM Φ , but the Rac-1 and F-actin did not co-localise.

Macrophage re-adhesion alters MAP Kinase phosphorylation

During a wounding assay only a small number of cells are migrating at any point and hence any protein changes associated with migration may have been lost amongst the non-migrating cellular protein levels. In order to investigate changes in protein modification associated with migration, a method was developed which allowed the responding population of cells to be examined. Macrophages were 'lifted' from the tissue culture plastic and allowed to re-adhere over an hour. During re-adhesion, the macrophages re-form podosomes, which may be akin to podosome formation during migration.

The MAP Kinase family member ERK1/2 has been shown to be involved in podosome regulation and chemotaxis (Jimenez-Sainz et al., 2003; Riboldi et al., 2003; Wain et al., 2002), and I therefore investigated ERK1/2 protein levels and phosphorylation in adherent and re-adhered MDM Φ . The two MDM Φ populations, control and Dex-treated, were equalised for protein concentration before examination by western blotting. ERK comprises a p42 and a p44 subunit which are resolved under reduced conditions on a polyacrylamide gel. Levels of total ERK 1/2 were consistent between all 4 samples (Figure 5.9). The adherent control MDM Φ contained high levels of phosphorylated ERK1/2 which did not alter upon re-adhesion. The Dex-treated MDM Φ , which has been adhered to the tissue culture plastic for 5 days prior to detection, showed low levels of ERK phosphorylation, but this dramatically increased upon re-adhesion, particularly in p42.

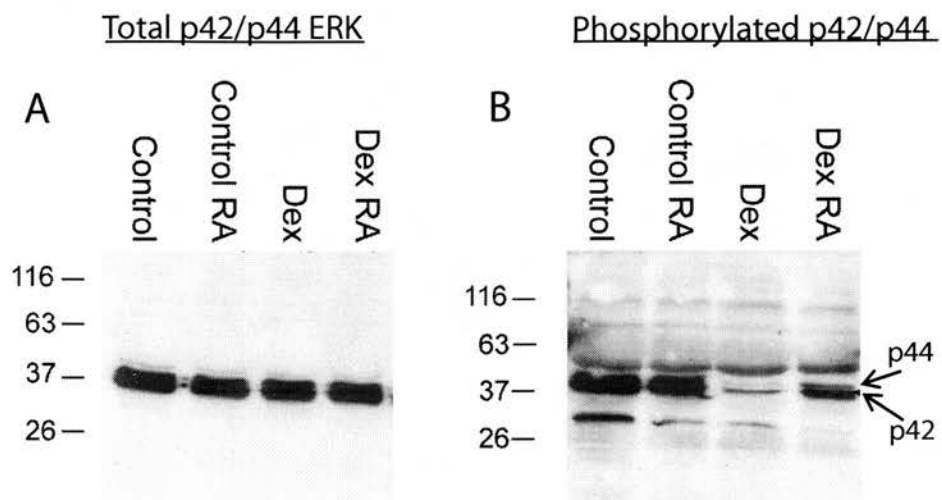


Figure 5.9 MDMΦ readhesion alters the phosphorylation of ERK1/2

5 day MDMΦ were either left adhered to, or lifted and re-adhered (RA) to, tissue culture plastic over one hour. Cell lysates were made and protein concentrations of all samples were equalised. Lysates were run reduced and blotted for total ERK1/2 (A) and P-ERK1/2 (B). Representative blot of 2 shown.

Role of MAP Kinase in the initial burst in Dex-treated MDM Φ migration

Migration assays were carried out in the presence of the MEK Kinase inhibitor, PD98059, for three different macrophage donors. Two of the three donor's Dex-treated MDM Φ showed no inhibition following PD treatment. However, analysis of the third donor's Dex-treated MDM Φ resulted in a loss of migration (Figure 5.10). Intriguingly, these preliminary results suggest that other factors may be important in determining whether MAP kinase has a role in Dex-treated MDM Φ migratory response.

Re-adhesion does not affect phagocytic ability

The process of adhesion, migration and phagocytosis are closely linked, due to a conservation of mechanisms between these different functions (Leverrier and Ridley, 2001; May and Machesky, 2001). Since they all require actin polymerisation and share many of the same proteins and pathways it was of interest to examine the phagocytic ability of the re-adhered macrophages.

5 day MDM Φ were differentiated in 10% AS \pm 125nM Dex \pm 10ng/ml IL-4 or 10ng/ml IFN- γ , differentiating to form phenotypically distinct MDM Φ (see chapter 6). The MDM Φ were either left attached to the tissue culture plate or lifted and allowed to re-adhere for 1 hour at 37°C 5% CO₂, a time point at which podosomes are clearly visible in control MDM Φ and ERK phosphorylation showed clear differences in re-adhered Dex-treated MDM Φ . The MDM Φ were then used in a 1 hour phagocytosis assay and the extent of phagocytosis examined by flow cytometry. Figure 5.11 demonstrates that no significant change in phagocytic ability was observed between the MDM Φ which had been left adhered or those re-adhered within any of the 6 MDM Φ populations. This data suggests that the proteins required for podosome formation are not limiting phagocytic ability.

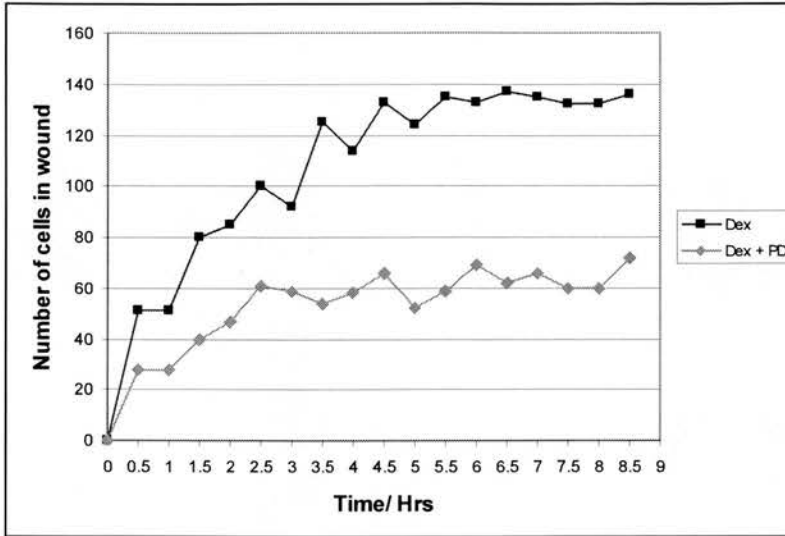


Figure 5.10 Effect of ERK1/2 inhibitor on Dex-treated MDM Φ migration

Dex MDM Φ were pre-treated with the ERK1/2 inhibitor PD98059 (PD), prior to the wounding assay. From the three donors analysed, only one shown above demonstrated an inhibition following PD treatment.

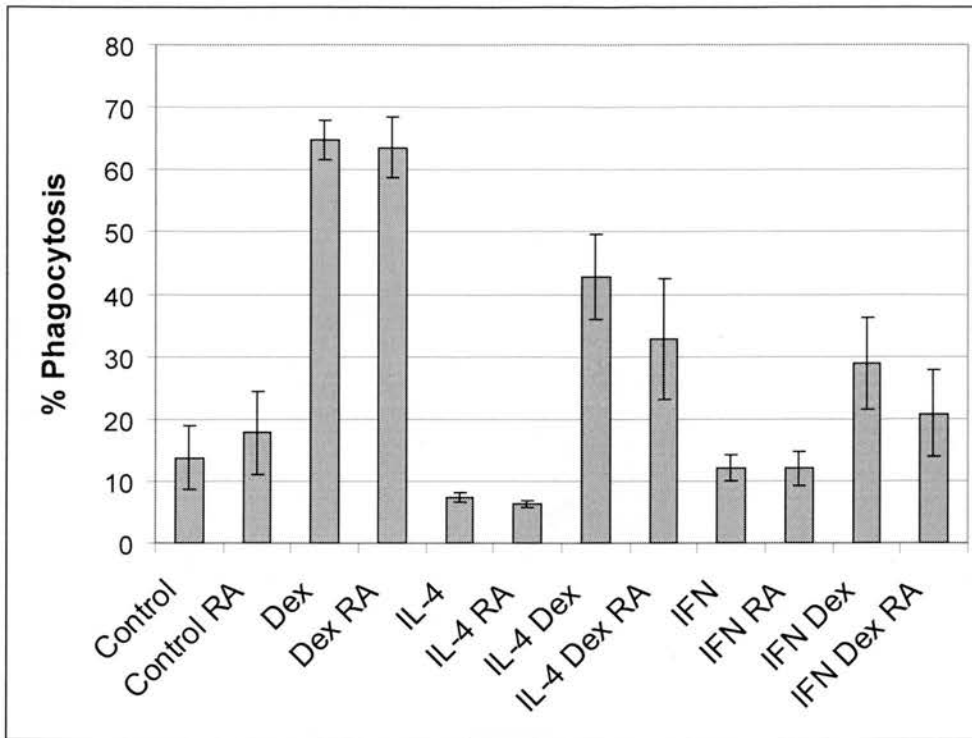


Figure 5.11 Readhesion of macrophages has no effect on phagocytic ability

5 day MDM Φ were lifted and then allowed to readhere to tissue culture plastic for 1 hour. Following a 1 hour phagocytosis assay, the internalisation of fluorescently labelled apoptotic neutrophils was examined by flow cytometry. Readhesion caused no significant alteration in any of the MDM Φ population's phagocytic ability.

Data shown are mean % phagocytosis \pm S.E. Tukey Kramer repeated ANOVA on the transformed data showed, (n=3) no significant difference was observed between any of the adhered vs re-adhered pairs.

Discussion

Loss of adhesions is associated with altered migratory phenotype

Examination of MDM Φ migration by video microscopy revealed that Dex-treatment resulted in an altered migratory ability when compared to untreated (control) MDM Φ . The migratory morphology exhibited by the 5 day control MDM Φ involved formation of lamellipodial membrane extensions that appeared to determine the direction of subsequent migration, and a retracting tail at the rear of the cell. In all cells examined, migration was preceded by the adoption of this morphology and any subsequent change in direction required the formation of lamellipodia in that direction. The Dex-treated MDM Φ exhibited an initial period of directed migration during which they adopted a distinct migratory morphology. However, lamellipodia formed were less extensive and subsequent directional migration was short-lived, with the studies examining the F-actin staining of the migrating MDM Φ revealing a distinct cytoskeletal architecture when compared with untreated MDM Φ .

It was notable that migrating Dex-treated MDM Φ formed very few large organised adhesions compared to the control MDM Φ . One possibility is that the difference in cytoskeletal organisation may play an important role in the observed migration patterns. Firstly, Linder and colleagues have demonstrated that a microtubule network is required for the formation of podosomes (Linder et al., 2000) and the lack of a highly organised microtubule network in the Dex-treated MDM Φ may therefore have resulted in the failure to stabilise podosomes-like adhesions. In the absence of podosomes behind the leading edge, and presumably with a lack of supporting adhesions, the extending lamellipodia would be expected to be short lived. Secondly, the less intense staining of the adhesions present in the Dex-treated MDM Φ may suggest that they are smaller, although not necessarily weaker, than those in the control MDM Φ . Examination of other known adhesion components, such as pyk2, talin and vinculin would allow the adhesions associated with Dex-treated migrating

MDM Φ to be distinguished from those found behind the leading edge of the untreated migrating MDM Φ . Correlations between the extent of adhesion contacts and migration have been reported, with FAK^{-/-} fibroblasts exhibiting increased numbers of focal adhesions whilst having a reduced migratory rate (Ilic et al., 1995). Further, there is work suggesting that rapid cell migration is driven by small, often undetectable adhesions at the leading edge (Webb et al., 2002). Therefore, the reduced presence of podosomes at the leading edge of the Dex-treated MDM Φ may have allowed for the initial period of rapid migration observed following wounding.

Dex-treatment of MDM Φ alters protein expression or phosphorylation of key cytoskeletal components. The down-regulation of paxillin and pyk2 phosphorylation in 5 day MDM Φ following Dex-treatment and the associated loss of podosome adhesions has been previously described (Giles et al., 2001). Significantly, alterations in expression and phosphorylation of these adhesion components have also been linked with defects in migration. Transfection of Chinese hamster ovary K1 cells, MDA-MD-231 human breast cancer cells, and NBT-II cells with a phosphorylation mutant of paxillin inhibited cell migration and prevented directed migration required for wound closure (Huang et al., 2003). However, in contrast to the Dex-treated MDM Φ , where loss of paxillin phosphorylation correlated with loss of podosome adhesions, transfection of the mutant paxillin in these cells was associated with the formation of large, stable adhesions, postulated to prevent the cells from migrating. Studies examining Pyk2^{-/-} mouse macrophages showed an impaired chemokine-induced migration due to altered cell polarisation and reduced cell contraction (Okigaki et al., 2003). Interestingly, loss of pyk2 in these macrophages was associated with the protrusion of multiple extensions, a phenomenon also observed in the Dex-treated MDM Φ . One possibility, therefore, is that pyk2 phosphorylation is required for orientation of extending membrane in the direction of migration.

In vivo consequences of Dex-treated MDM Φ altered migratory ability

There are a number of reports in the literature demonstrating that both *in vitro* and *in vivo* treatment with GCs causes an inhibition in wound healing (Durmus et al., 2003a; Polat et al., 2002). In a study of diabetes mellitus, wound healing in the presence of GCs was inhibited concurrent with a reduction in infiltrating inflammatory cells, fibroplasia and neovascularisation (Bitar et al., 1999). The observed inhibition of wound healing by GCs could be attributed to its numerous effects on different cells. For example, GCs prevent migration and activation of inflammatory cells (Barnes, 1998), induce apoptosis of some cell types (Amsterdam et al., 2002), as well as altering the cytokine profile (Hubner et al., 1996) and matrix deposition at the wound site (Schacke et al., 2002). The wound-assay results presented in this chapter suggest that the Dex-treated MDM Φ may fail to sustain migration over a larger distance, as they often adopted a phenotype in which they rapidly extended and retracted long processes in multiple directions and failed to migrate. Therefore, Dex-treated MDM Φ 's altered migratory phenotype is consistent with the literature on GC-altered leukocyte migration. A study using a transection-induced bowel anastomoses model showed that wound healing was impaired by Dex treatment and that ICAM-1 expression was down-regulated on the endothelial cells, which would reduce leukocyte migration into the wound site and impair healing. (Polat et al., 2002). Interestingly, the receptor for ICAM-1, $\alpha_L\beta_2$ was down-regulated following Dex-treatment and this may therefore result in reduced monocyte migration from the blood vessel into the inflammatory site.

Dex-treated MDM Φ have altered levels and activation of many key adhesion and migration proteins

In this chapter, the levels of total Rac-1 in the Dex-treated MDM Φ were shown to be down-regulated when compared to control MDM Φ . However, the percentage of activated Rac present relative to the levels of total Rac was higher in the Dex-treated

MDM Φ . The assay was carried out on protein lysates from the different MDM Φ populations which had been equalised to contain the same total protein concentrations. Interestingly, the Dex-treated MDM Φ are much smaller than the control MDM Φ ($<10\mu\text{m}$ compared to $\geq 20\mu\text{m}$ diameter control MDM Φ), and assuming the cells as spherical, the Dex-treated MDM Φ are nearly 8 times smaller in volume than the control MDM Φ , and therefore the levels of total Rac may well be sufficient to fulfil need of Dex-treated MDM Φ . Although it is possible that the reduced total levels of Rac-1 present in the Dex-treated MDM Φ limits certain functions, the presence of a large proportion of Rac-1 in the active form may drive processes such as membrane ruffling. The SEM images seen in Figure 4.4 demonstrate an increased ruffling on the cell surface of the Dex-treated MDM Φ compared to control MDM Φ . In addition to this, the Rac-1 staining in Figure 5.8D shows a high level of Rac at the upper surface of the Dex-treated MDM Φ . The localisation and turnover of Rac are important factors for actin polymerisation required for lamellipodia formation and membrane ruffling. In light of the results demonstrating altered levels of active Rac following Dex-treatment, it would be interesting to compare and contrast Rac in migrating untreated and Dex-treated MDM Φ , perhaps by microinjection and imaging of a fluorescently tagged Rac protein or GFP-tagged PAK in these cells.

Adhesion and the tightly associated process of cellular migration share commonalities in proteins and mechanisms including regulation of actin polymerisation via the Arp2/3 complex. Gene array data presented in chapter one demonstrated an up-regulation in the key signalling proteins, PAK-1 and p160ROCK associated with Dex treatment. PAK-1 is known to bind to and be activated by the activated forms of Rac and Cdc42, with roles in the signalling pathways downstream of these GTPases for filopodial formation and podosome turnover. Studies of endothelial cell migration have demonstrated a potential role for PAK-1 in adhesion formation at the leading edge, and the subsequent contraction and tail detachment (Kiosses et al., 1999). Interestingly, I have also demonstrated an increase in Rac-1 activation following Dex-treatment, and the gene array data predicted a 3-fold up-regulation of Cdc42 and a 2-fold up-regulation in Cdc42 GTPase activating protein.

Signalling via the Rho-GTPase Cdc42 results in actin polymerisation and filopodial extension (Allen et al., 1997), and in view of the result showing that the Dex-treated MDM Φ demonstrate atypical extension and retraction of membrane during the migratory response, it would be important to confirm an up-regulation of Cdc42 protein levels and activation status in the Dex-treated MDM Φ .

In addition, the formation of focal complexes and the maintenance of a spread morphology in the macrophages cell line, Bac1, is dependent on Cdc42 signalling upstream of Rac (Allen et al., 1997). A down-regulation in total levels of Rac protein, such as was seen in the Dex-treated MDM Φ , would therefore be predicted to result in loss of podosomes and rounding up of the cell, both of which were seen in the Dex-treated MDM Φ .

p160ROCK is a Rho associated kinase which is downstream of Rho-GTPase. Over expression and thus over stimulation of this pathway has been shown to cause contraction and rounding up of macrophages (Allen et al., 1997). The observed Dex-treated MDM Φ morphology would be consistent with the predicted outcome of up-regulation of a member of this pathway, with a small and rounded morphology, perhaps due to increased myosin light-chain phosphorylation and contraction of actomyosin. Migration studies using Dex-treated MDM Φ treated with inhibitors of MLC phosphorylation may help to address this question. p160ROCK is also essential for the retraction of the tail of migrating leukocytes, and inhibition results in attachment of the cell to the substratum via the tail, whilst the cell body remains highly motile (Alblas et al., 2001). In accordance with an upregulation in p160ROCK, Dex-treated MDM Φ have less prominent tail retraction when compared to control MDM Φ .

Dex treatment causes the down-regulation of selected integrins on MDMΦ

Phenotyping of the 5 day MDMΦ for the major families of surface integrins revealed that α_L , α_M and α_X were down-regulated on the surface of the Dex-treated MDMΦ when compared to the control MDMΦ. α_4 , α_5 and β_1 , the subunits of VLA-4 and VLA-5, which have been shown to be required for the emigration of macrophages from the inflammatory tissue to the draining lymph nodes (Bellingan et al., 2002b), showed no significant down-regulation following Dex treatment. However, it is possible that the integrins surface expression may alter on MDMΦ following a migratory stimulus. The stimulus for MDMΦ emigration from the inflammatory sites is unknown and therefore it would currently be difficult to test if there were differential changes in expression of integrins by control and Dex-treated MDMΦ under conditions approximating inflammation *in vivo*. It would be interesting to follow up the integrin phenotyping studies presented here with studies investigating adhesion of MDMΦ to the principal integrin ligands, and further to examine MDMΦ across these ligands. Tissue culture plates could be coated with ligands such as ICAM-1 (receptors of which are LFA-1 and Mac-1), fibronectin or motifs of fibronectin (receptors are Mac-1, VLA-4, VLA-5 and $\alpha_v\beta_3$) and collagen (receptor is $\alpha_2\beta_1$). Analysis of control and Dex-treated MDMΦ adhesion to these different ligands may then provide information on whether Dex-mediated down-regulation of integrins would impact on cellular adhesion. Furthermore, given the demonstration that stimulation from both integrin binding to ECM and from Rho GTPase signalling are required for focal adhesion formation in human fibroblasts (Hotchin and Hall, 1995), the down-regulation of integrins following Dex-treatment may be important in explaining the adhesion morphology of these MDMΦ.

In the present analysis, I compared the integrin profiles of 5 day untreated and Dex-treated MDMΦ. However, it would also be interesting to examine how rapidly Dex alters the surface receptor patterns. Any rapid alterations in receptor expression or activity might influence monocyte migration across endothelium into the

inflammatory tissue. Further, the down-regulation of the $\beta 2$ and $\alpha v\beta 3$ integrins may reflect the change in podosomes seen between the control and Dex-treated MDM Φ . Certainly, the lack of podosomes and thus the concentration of integrins at sites of adhesion where they signal from the ECM to the cell, suggests the Dex-treated MDM Φ may have an impaired ability to interpret environmental signals.

MDM Φ migration is not dependent on MAP Kinase activation

A model experimental system was developed to investigate assembly of adhesion/signalling complexes approximating those in MDM Φ . One of the major differences between untreated and Dex-treated MDM Φ was the presence of podosomes. However, continuous turnover of these structures in adherent cells makes analysis of the mechanisms underlying control of their assembly difficult. I found that detachment of MDM Φ from tissue culture plates followed by their re-adherence, resulted in the formation of podosomes (data not shown). Thus, using this system for detachment-reattachment, it might be possible to examine signal transduction pathways biochemically in a synchronous population of cells. Western blotting for the Map kinase ERK1/2 revealed that re-adhesion caused increased phosphorylation (particularly of ERK2) in Dex-treated MDM Φ . No change in phagocytic ability of any of the MDM Φ populations examined was detected following readhesion, indicating that increased ERK1/2 phosphorylation is not a critical determinant of phagocytic ability. Whilst the interpretation of data from the use of pharmacological inhibitors is difficult due to non-specific effects, I sought to use the MAP Kinase inhibitor to investigate the role of p42/44 MAPK in the control of adhesion. Unfortunately the results for these preliminary experiments were equivocal, with a complete inhibition in migration in the case of one donor but in two other experiments, migration appeared unaffected by the PD compound, raising the possibility that the MAP kinases do not play a critical role in human macrophage migration. A role for MAP kinases, and particularly for ERK1/2 have been previously investigated during monocyte chemotaxis. The chemoattractants, MCP (monocyte chemoattractant proteins) 1-4 were shown to rapidly activate ERK2 in

monocytes and incubation with the MAP Kinase inhibitor UO126, abrogated monocyte chemotaxis (Wain et al., 2002). Studies using the specific MAP kinase inhibitor PD98059 have reported contradictory results. Yen and colleagues demonstrated an inhibition in monocyte MCP-1 chemotaxis following incubation with PD (Yen et al., 1997) but Fine and colleagues reported no defect in monocyte chemotaxis to a variety of chemoattractants (Fine et al., 2001). In light of these studies it may be prudent to use a range of inhibitors to clarify the role of MAP Kinases during MDM Φ migration. Interestingly, PD does not block phagocytosis of apoptotic cells (I. Dransfield, unpublished data) supporting the suggestion that MAPK activity may not be critical for phagocytosis either.

Consideration of the assay

The cell wounding assay, which may promote cell migration following the loss of cell-cell adhesions, is a model system for real-time morphological analysis of migration. One possible explanation for the altered migratory behaviour seen between control and Dex-treated MDM Φ following wounding could be due to differential responses to the wound stimulus, perhaps resulting in the release of a signalling factor promoting migration. Untreated MDM Φ may continue to migrate until cell-cell contact is re-established, whereas Dex-treated MDM Φ migration may stop because of cessation of signals required for migration. Following 5 day treatment of peripheral blood monocytes with Dex, expression of ICAM-1 and its receptor $\alpha_L\beta_2$ were down-regulated on the macrophage cell surface. One possibility is that since untreated MDM Φ express both receptor and ligand, they are able to interact with other MDM Φ via these molecules. The down-regulation following Dex treatment may therefore inhibit cell-cell contact. Examination of Dex-treated MDM Φ migration, initiated by other stimuli such as chemoattractants, would answer the question of whether the altered migration presented in these studies was due to differential response to the stimuli or a fundamental alteration in migratory ability. Whilst it was difficult to control the exact width of the wound between experiments, it is also worth noting that the wound size represented a greater number of cell body

lengths for a Dex-treated MDM Φ than for a control MDM Φ . To test the possibility that cell-cell contact provided signals that determined when MDM Φ migration stopped it would be interesting to examine the migration of the MDM Φ when wounds of different sizes were created, using custom built cell-scrapers.

The *in vivo* stimulus for migration can be the establishment of a chemoattractant gradient, and further studies investigating the potential effects of steroids in inflammation should certainly include Boyden chamber-type studies, where the migration of MDM Φ towards different chemokines can be investigated. Unfortunately, experiments to test chemokine guided migration failed to demonstrate polarisation or migration of MDM Φ in response to the chemokine over and above that seen for PBS.

Summary

The process of migration is highly complex, with many pathways integrating both temporally and spatially. Given the protein changes detected in the Dex-treated MDM Φ it is of little surprise that these cells exhibit an altered migratory phenotype. The protein changes, and the potential consequences for signalling pathways and the migratory phenotype in the Dex-treated MDM Φ have been summarised in Figure 5.12. The cells

seem capable of forming a transient lamellipodia, although this appears not to be stabilised by adhesions. The leading edge contains F-actin rich ruffles, not detected in control MDM Φ , and these may play an important role in migration. The small numbers of adhesions present in the migratory Dex-treated MDM Φ may result from a combination of losses of proteins required for, or involved in, podosome formation (p130 Cas, pyk2, paxillin phosphorylation, microtubule network and Rac). Together with the rapid detachment of the retracting tail, most probably as a result of increased p160ROCK, this may aid the initial rapid migration seen in the Dex-treated MDM Φ .

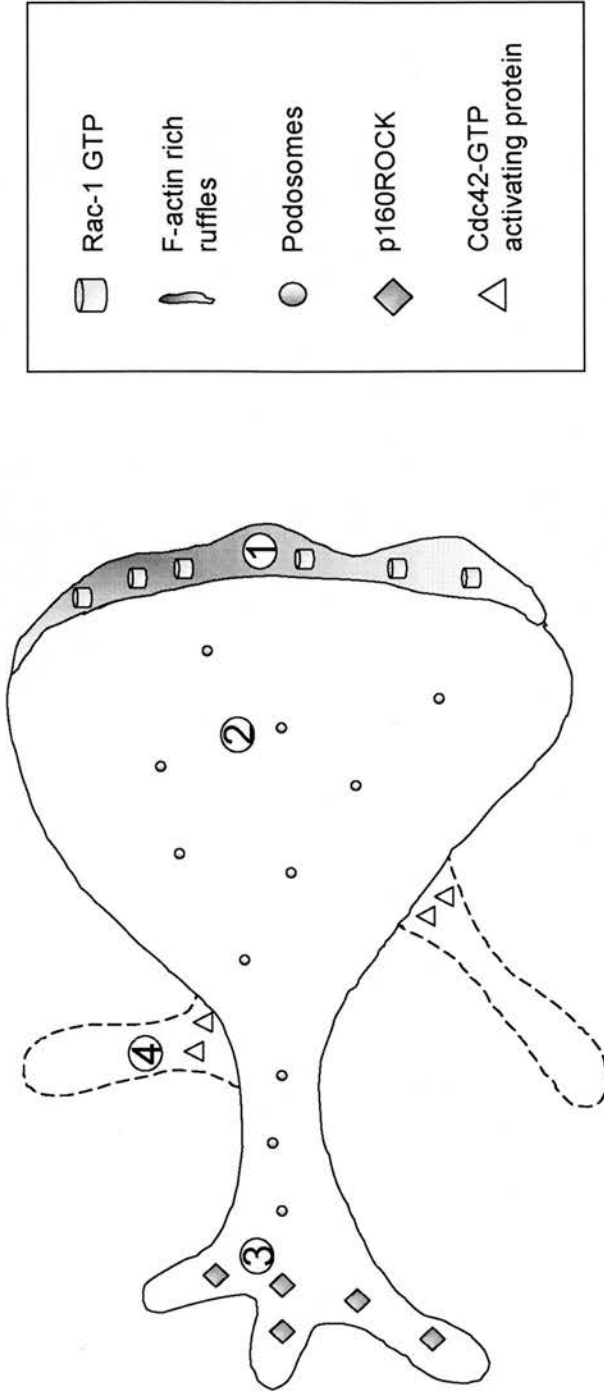


Figure 5.12 Potential signalling pathway changes resulting in alternative migratory phenotype of Dex-treated MDMΦ

1. Increased percentage of active Rac-1 at the leading edge which could cause increased levels of F-actin rich ruffles
2. Reduced number of podosomes, which locate throughout cell, rather than stabilising the extending lamellipodia; result of lack of microtubule network, down-regulation of p130 Cas, pyk2, paxillin phosphorylation and local loss of Rac-1
3. Increased levels of p160ROCK, resulting in shortened uropod and quicker detachment of rear of cell
4. Rapid extension and retraction of membrane extensions due to increased Cdc42 activation

CHAPTER 6: THE EFFECT OF THE CYTOKINE ENVIRONMENT ON GLUCOCORTICOID-TREATED MDM ϕ

Introduction

Inflammation, and its resolution, is controlled by a family of 14-26 kDa polypeptides called cytokines. The process of inflammation requires tight control, from allowing responses that promote inflammation and cellular activation to ridding the tissue of the pathogen, followed by the dampening down and healing phase. Cytokines act as protein signalling molecules, allowing for temporal and functional control of inflammatory cells. For example, the function of macrophages, as they differentiate from infiltrating monocytes, is determined by the cytokine micro-environment within the tissue. The resulting phenotype and function of macrophages exposed to either pro- or anti-inflammatory cytokines is an area of active research, as therapeutic manipulation may offer approaches to treating inflammatory disease (Dries and Perry, 2002). Much like the cytokine-controlled development of the Th1 and Th2 subsets of Th lymphocytes, macrophages too have been subdivided into classically (caM ϕ) and alternatively (aaM ϕ) activated categories owing to contrasting differentiation in the presence of different cytokines.

The development of the caM ϕ phenotype is mediated by pro-inflammatory agents such as LPS and the Th1 cytokines IFN- γ and TNF- α (Mosser, 2003). These cells have defined surface profile and cellular functions. caM ϕ function to phagocytose and subsequently destroy pathogens. To this end it has been shown that caM ϕ and monocytes have upregulated FcR1 and 2, and demonstrate an augmented FcR mediated uptake (Becker and Daniel, 1990). In mouse, the caM ϕ have an enhanced production of nitric oxide (NO) following up-regulation of the enzyme inducible NO Synthase (iNOS) by IFN- γ , enabling increased production of NO from its substrate L-arginine (Hesse et al., 2001; Munder et al., 1998). Together with production and release of O $_2^-$ and nutrient restriction, the ingested pathogen is destroyed.

An up-regulation of all three MHC class II molecules, HLA-DR, HLA-DQ and HLA-DP (Becker and Daniel, 1990; Cao et al., 1989) is induced on the caM Φ cell surface, and, as a result of the increased expression of the MHC class II molecules, caM Φ are more efficient at antigen presentation (Becker, 1985). The caM Φ up-regulates inflammatory cytokine production e.g. TNF- α , IL-12, IL-1 and IL-6 (Cheung et al., 1990; Donnelly et al., 1990), and together these cytokines play their part in the recruitment and polarisation of leukocytes during the inflammatory response.

The effect of IL-4 on the differentiation and maturation of macrophages was originally thought to be a 'deactivation' signal. IL-4 treatment does indeed appear to be antagonistic in its effects on macrophage phenotype and function when compared to IFN- γ -induced caM Φ . IL-4 inhibits the secretion of pro-inflammatory cytokines such as IL-1, TNF- α , and IL-8 from monocytes and macrophages (Donnelly et al., 1990; Hart et al., 1989; te Velde et al., 1988), synergising with glucocorticoids in this effect. In addition, the aaM Φ up-regulate secretion of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (Fenton et al., 1992; Mantovani et al., 2002).

The idea that these cells are not simply deactivated in comparison to IFN- γ macrophages, but differentially activated, was championed by Gordon and colleagues (Gordon, 2003; Stein et al., 1992). They demonstrated that the mannose receptor, an endocytic receptor binding to mannosylated proteins known to be down-regulated by IFN- γ , was strongly induced by IL-4 and to a lesser extent by glucocorticoids. They proposed that the mannose receptor was a marker of an 'alternatively activated' population of cells.

More recently, studies have shown the aaM Φ to have increased expression of a broad repertoire of receptors important for innate immunity, such as the mannose receptor (Stein et al., 1992), the β -glucan receptor and the scavenger receptor 1 (Gratchev et al., 2001b). Despite this apparent capacity for phagocytosis of pathogen, the aaM Φ is ill-equipped to kill ingested pathogens. The production of NO in the IL-4 induced macrophages is reduced in favour of L-ornithine and urea expression (Modolell et al., 1995). IL-4 causes increased expression of the enzyme arginase which competes

for the substrate L-arginine with the NO synthases (Munder et al., 1998; Noel et al., 2004). Since L-ornithine is a key metabolite in polyamine and proline production, required for cellular proliferation and collagen production respectively, the switch in this pathway may be important in inflammatory resolution (Hesse et al., 2001). In addition, and in contrast to caM Φ , these macrophages only express the low-affinity Fc ϵ receptor, with none of the Fc γ receptors found on their cell surface (Becker and Daniel, 1990).

Like caM Φ , the aaM Φ have up-regulated expression of MHC class II molecules, although in the case of aaM Φ this is restricted to a subset of cells and is only seen with the antigens HLA-DR and HLA-DP (Cao et al., 1989; Gerrard et al., 1990). Human MDM Φ cultured in the presence of IL-4 up-regulate the ECM proteins fibronectin and β IG-H3 (a supposed ECM protein due to a secretory domain and integrin recognition site) (Gratchev et al., 2001a).

The literature has expanded the term aaM Φ to include those that have become activated following exposure to the Th2 cytokines IL-4, IL-13 and IL-10, as well as to glucocorticoids and TGF- β (Goerdts and Orfanos, 1999; Stein et al., 1992). Glucocorticoids have also been shown to suppress the production of pro-inflammatory cytokines (Agarwal and Marshall, 2001; Hart et al., 1990), and to induce CD163, the haemoglobin scavenger receptor and receptor for activated endothelium, that is used as a marker of alternative activation (Hogger et al., 1998). In contrast to IL-4, glucocorticoids suppressed expression of ECM proteins such as β IG-H3 and fibronectin (Gratchev et al., 2001a), in agreement with suggestions that glucocorticoids inhibit wound healing (Anstead, 1998; Barba et al., 2000; Durmus et al., 2003b).

Whilst the up-regulation of receptors which have broad specificity for foreign antigen, and the lack of functional ability to breakdown and kill pathogen may be at odds, aaM Φ do express receptors suggesting they are capable of functional antigen presentation. Therefore, they may be important in controlling differentiation of naïve T cells towards a Th2 phenotype, perhaps in contrast to the potential differentiation

of Th1 cells by the caM Φ . Functionally, the aaM Φ are implicated in the later phases of inflammation, in angiogenesis (Kodelja et al., 1997), ECM deposition and wound healing and the down-regulation of inflammation (Gratchev et al., 2001b). This suggestion is supported by the fact that these cells are found during the healing phase of inflammatory reactions (Djemadji-Oudjiel et al., 1996). In addition, however, there is also evidence that aaM Φ are involved in Th2 cytokine-controlled inflammatory diseases such as asthma and parasite infections (Noel et al., 2004). Furthermore, monocytes associated with tumours acquire an aaM Φ profile (Mantovani et al., 2002).

The definition of the caM Φ and the aaM Φ , two macrophage phenotypes that seem suited to roles in early inflammation and the later resolution / healing phases respectively, present two extremes of what must be a spectrum of phenotypes. The current challenge is to begin to understand the effect of combinations of cytokines on macrophage function which would match the complex micro-environment into which the infiltrating monocytes migrate and differentiate.

In this chapter, the role of the cytokine environment on macrophage function will be examined. IFN- γ and IL-4 were chosen as representative Th1 and Th2 cytokines respectively, and studies examining the effects of these cytokines in combination with Dex, upon macrophage adhesion, phagocytosis of apoptotic cells, expression of surface receptors and cytokine production will be presented.

Results

IFN- γ suppresses glucocorticoid augmented phagocytosis of apoptotic neutrophils

The observed *in vitro* augmentation of human MDM Φ phagocytosis by Dex represents a mechanism by which apoptotic neutrophils may be rapidly cleared from the inflammatory site. However, *in vivo*, macrophages would also be subject to the cytokine environment at the inflammatory site, and I wished to examine the effect of this on the phagocytic capacity of MDM Φ . I therefore compared the effects of Dex with the Th1 cytokine IFN- γ and the Th2 cytokine IL-4 in single and combination treatments on MDM Φ phagocytosis of apoptotic neutrophils (Figure 6.1).

In accordance with previous results (Giles et al., 2001; Liu et al., 1999) 5 day treatment with Dex resulted in an augmentation in phagocytosis. Treatment with IL-4 alone inhibited phagocytosis below that observed in the control MDM Φ population, whereas IFN- γ alone had little effect over and above that seen in the control MDM Φ population. Combination treatments of IL-4 and Dex resulted in a population of MDM Φ with a reduction in phagocytic ability when compared to Dex treatment alone. Most strikingly, combination treatment of Dex and IFN- γ resulted in a significant suppression of phagocytosis when compared to Dex treatment alone. These results indicate that the presence of the pro-inflammatory cytokine IFN- γ , during the differentiation process from peripheral blood monocyte to macrophage, suppresses the augmentation of apoptotic cell phagocytosis and hence any potential advantageous effects on the resolution of inflammation induced by Dex treatment.

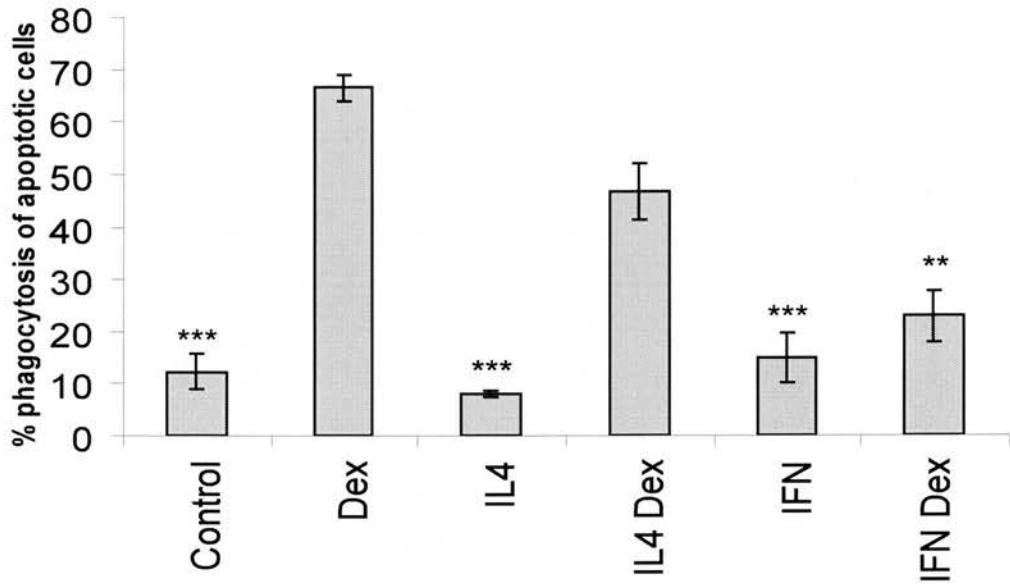


Figure 6.1 The effect of cytokines on MDM Φ phagocytosis of apoptotic neutrophils

Adherent peripheral blood monocytes were cultured for 5 days in the presence of combinations of 125nM Dex, 10ng/ml IL-4 and 10ng/ml IFN- γ . Phagocytic capacity of the MDM Φ was determined in a 1 hour assay and analysed by flow cytometry. Data shown as mean percentage phagocytosis \pm SEM for 5 separate experiments.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Dex-treated MDM Φ , Tukey Kramer repeated ANOVA with post tests performed on transformed data.

Checkerboards of treatments

Having established that co-culture of MDM Φ with either IL-4 and Dex or IFN- γ and Dex failed to augment phagocytosis of apoptotic neutrophils, I was interested in establishing whether the cytokines were capable of inhibiting the phagocytosis either before or after Dex treatment. Peripheral blood monocytes were treated for the first 24 hours in either control conditions, Dex or IL-4. They were then washed and cultured in the second condition for a further 4 days. As shown in Figure 6.2A, IL-4 treatment can be administered within the first 24 hours and then removed, resulting in the inhibition of phagocytic ability. IL-4 could also just be administered in the last 4 days of culture, and again phagocytosis was inhibited. This data suggests that the cells remain susceptible to IL-4 at different points during differentiation with Dex. The IL-4 checkerboard treatments demonstrate a trend showing IL-4 inhibition of Dex-augmented phagocytosis irrespective of whether the MDM Φ were exposed to IL-4 before or after Dex. More experiments are required to verify if this is a significant down-regulation.

Data from the IFN- γ checkerboard (Figure 6.2B) demonstrated a significant increase in phagocytic ability of the cells exposed to Dex for the first 24 hours of culture only when compared with control MDM Φ . In accordance with data presented in Figure 6.1, IFN- γ had no effect on phagocytosis when compared to control MDM Φ and this held true whether the IFN- γ was administered for the first 24 hours only or for the last 4 days only. Interestingly, the inhibition of Dex augmented phagocytosis by IFN- γ observed in Figure 6.1 was also seen when the cells were pre-exposed to IFN- γ before Dex, or vice versa, with 24 hours Dex treatment followed by 4 days of IFN- γ treatment. This data demonstrated that IFN- γ treatment both blocks and reverses Dex augmented phagocytosis in a manner similar to IL-4 treatment.

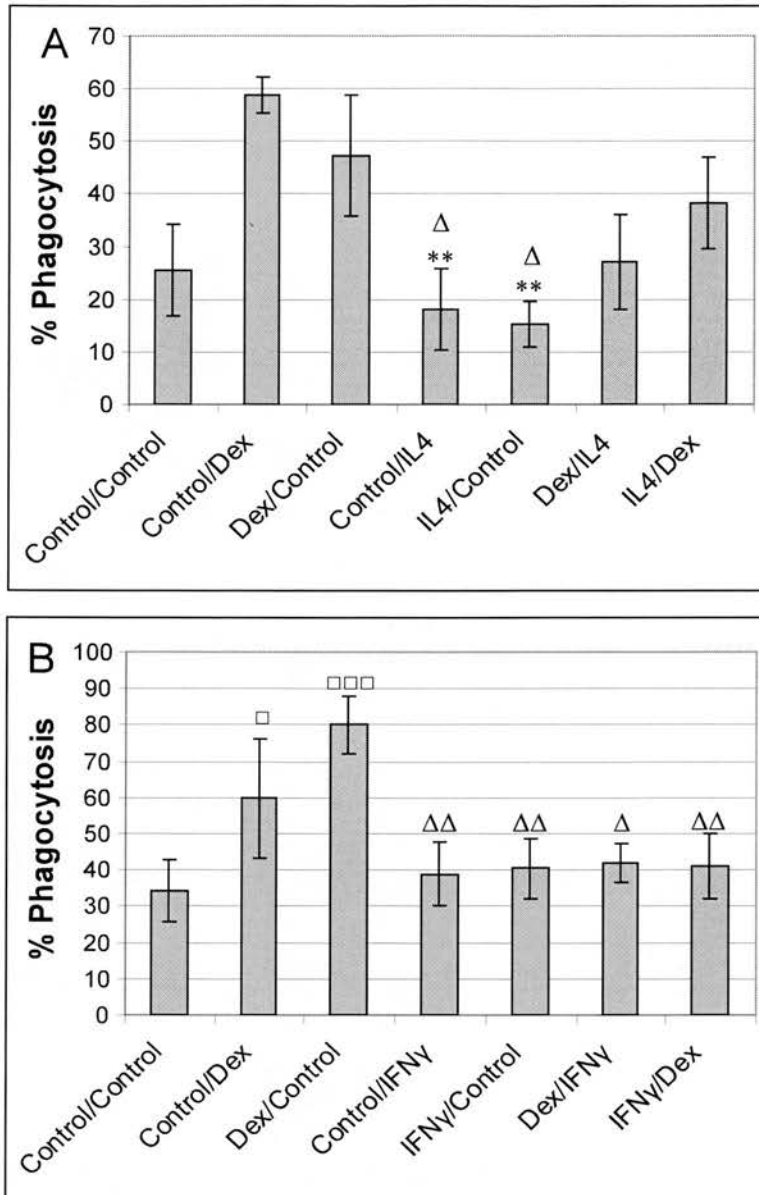


Figure 6.2 Checkerboards of treatments with Dex and IL-4/IFN- γ

Adherent monocytes were cultured for the first 24 hours in Iscove's DMEM containing 10% AS alone, Dex, IL-4 (A) or IFN- γ (B). Cells were washed and then treated for the remaining 4 days in Iscove's DMEM containing 10% AS, Dex, IL-4 (A) or IFN- γ (B) to give a checkerboard of combination treatments (24 hours/4 days). The phagocytic ability of the MDM Φ populations was then determined by flow cytometry. Results are shown as mean percentage of macrophage phagocytosis \pm SEM from 3 separate experiments.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ represented as * vs. Con/Dex; Δ vs. Dex/Con; \square vs. Con/Con, Tukey Kramer repeated ANOVA with post tests performed on transformed data.

Time-course of IFN- γ effect on Dex phagocytosis

The inhibitory effect of IFN- γ upon Dex-augmented phagocytosis was further examined to investigate the temporal nature of the effect of IFN- γ (Figure 6.3). Peripheral blood monocytes were differentiated for 6 days in the presence of Iscove's + 10% AS (control) with the addition of 125nM Dex or 10ng/ml IFN- γ . For the time-course, the macrophages were differentiated in 125nM Dex for all 6 days, with IFN- γ added into the medium on day 1, 3, or 1 or 3 hours prior to the phagocytosis assay on day 6. The data demonstrates that the degree of inhibition observed, following exposure to IFN- γ , was directly related to the length of time of exposure. The addition of IFN- γ for a few hours prior to the phagocytosis assay had no inhibitory effect upon the augmented phagocytosis. Therefore, to observe the full inhibitory potential of IFN- γ on the augmentation of phagocytosis induced by Dex, the MDM Φ required prolonged exposure to the IFN- γ .

Surface phenotype changes induced by IL-4 or IFN- γ are altered by Dex

CD64 is the receptor for Fc γ RI, the high affinity receptor for IgG, and as such functions to clear immune complexes as well as playing a role in antibody-dependent cytotoxicity. CD64 has previously been shown to be up-regulated following treatment with IFN- γ (Becker and Daniel, 1990). The data presented in Figure 6.4 also shows an up-regulation in mean fluorescence of CD64 on MDM Φ that had differentiated in the presence of IFN- γ . IL-4 treatment resulted in the down-regulation of CD64 from the surface of MDM Φ , and this was also seen, albeit to a smaller degree, in the Dex-treated MDM Φ . Combination treatments of IFN- γ and Dex failed to inhibit the IFN- γ up-regulation, demonstrating that Dex was unable to regulate IFN- γ induced CD64 expression.

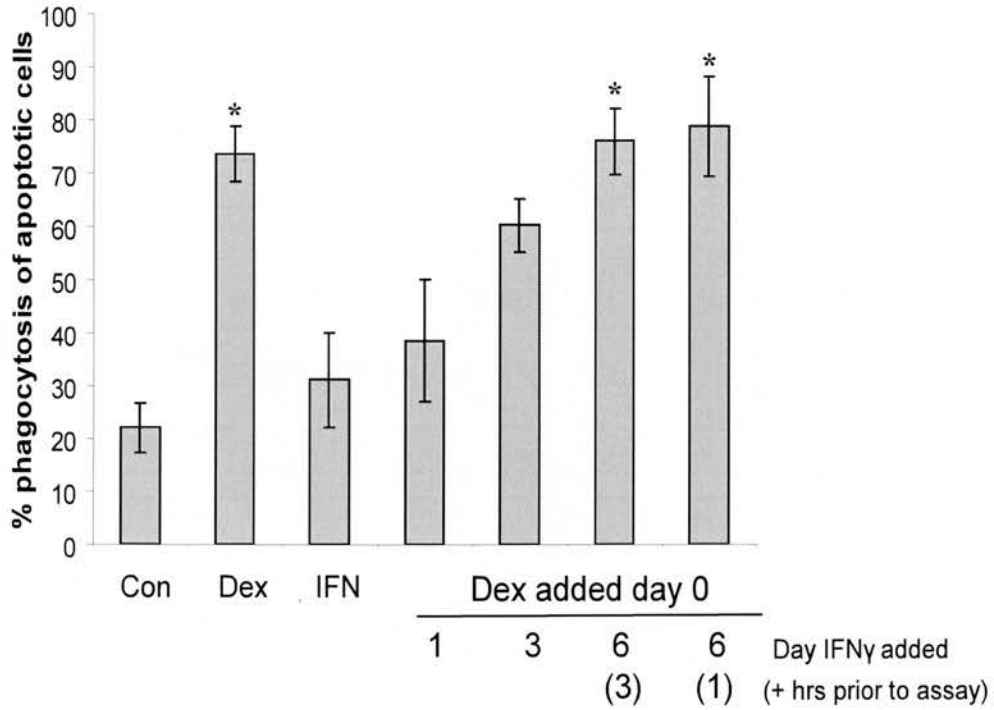


Figure 6.3 A time-course of IFN γ treatment on Dex treated MDM Φ

Adherent monocytes were cultured in Iscove's DMDM containing 10% autologous serum and 125nM Dex, with the subsequent addition of IFN- γ on days 1 or 3, or 1 or 3 hours prior to the phagocytosis assay on day 6.

The phagocytic ability of the MDM Φ populations was then determined by flow cytometry. Results are shown as mean percentage of macrophage phagocytosis \pm SEM from 3 separate experiments. * $p < 0.05$ vs. Con values, Tukey Kramer repeated ANOVA with post tests performed on transformed data.

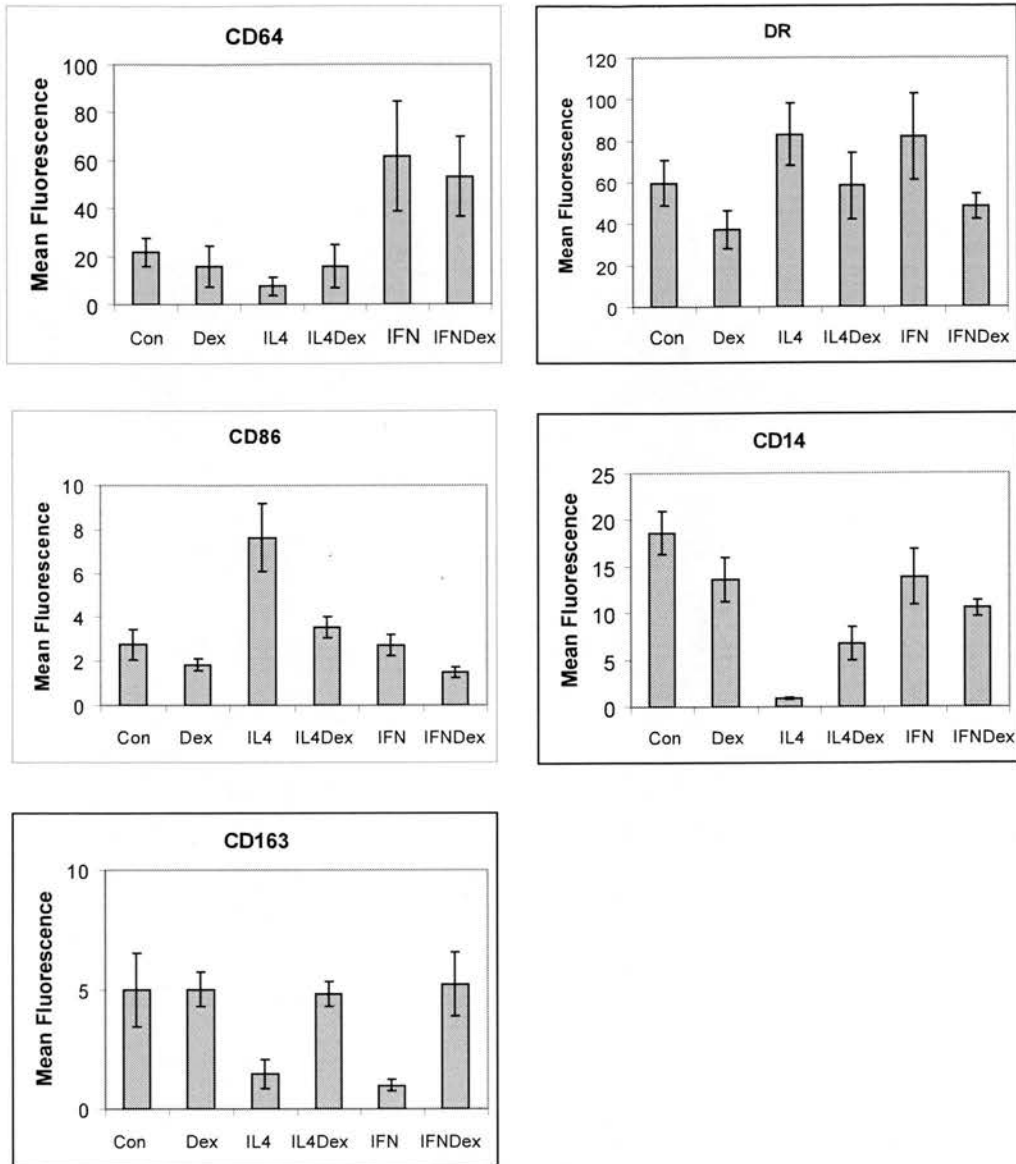


Figure 6.4 Surface phenotyping of 5 day MDM Φ treated with Dex and cytokines

Adherent peripheral blood monocytes were cultured for 5 days in the combinations of 125nM Dex, 10ng/ml IL-4 and 10ng/ml IFN- γ . Surface expression of CD64 (n=3), HLA-DR (n=6), CD86 (n=6), CD14 (n=5) and CD163 (n=6) was then examined by flow cytometry. Data shown are mean fluorescence, corrected for isotype control fluorescence \pm SEM.

The expression of MHC Class II – HLA-DR, a marker of antigen presentation, which has previously been reported to be up-regulated on human monocytes by both IL-4 and IFN- γ (Donnelly et al., 1990) was examined next. Accordingly, HLA-DR up-regulation was observed following treatment with either IL-4 or IFN- γ . Dex treatment, however, led to a down-regulated surface expression of DR, and combination treatments of IL-4 or IFN- γ with Dex resulted in intermediate expression levels, between those seen in Dex cells and IL-4 or IFN- γ .

With respect to control, untreated MDM Φ , expression of the co-stimulatory molecule, CD86, was induced by IL-4 whereas IFN- γ had no effect. Dex treatment resulted in a small down regulation in expression and was dominant over both IL-4 and IFN- γ in combination treatments.

CD14 expression was slightly down-regulated upon Dex treatment, and dramatically so following IL-4 treatment. Combination treatment of IL-4 and Dex resulted in a fluorescence of intermediate levels, providing evidence for Dex being dominant over IL-4. IFN- γ treatment slightly down-regulated CD14 expression, matching the levels observed following Dex-treatment.

Finally, levels of the haemoglobin scavenger receptor CD163, a marker of alternative activation, were examined. The data presented here showed no change in expression in Dex cells compared to control untreated MDM Φ . Previously published results report that Dex up-regulates CD163 on the surface of macrophages (Schaer et al., 2002). Although the low levels of expression suggest that the antibody may not have been binding effectively to the MDM Φ , both IL-4 and IFN- γ down-regulated expression, and the presence of Dex prevented down-regulation in co-incubations of IL-4 or IFN- γ and Dex.

Production of pro-inflammatory cytokines is inhibited in the presence of Dex

Studies have previously demonstrated that IFN- γ treatment of macrophages results in the secretion of pro-inflammatory cytokines, and that IL-4 treatment inhibits this (Donnelly et al., 1990). I set out to investigate the effect of Dex alone or in combination with either IFN- γ or IL-4 on cytokine production (see Figure 6.5). Following 5 day culture the media from MDM Φ treated with 10% AS alone, or in the presence of 125nM Dex \pm 10ng/ml IL-4 or IFN- γ was collected. The media was spun down to remove any debris and stored at -80°C until the assay was run. 6 cytokine levels were recorded for each sample using the BD cytometric bead array kit, as described in the Materials and Methods section. Cytokine standards were used to form a concentration curve for each cytokine.

IL-12p70 levels were low in all samples, with control MDM Φ supernatants containing the highest concentration of all samples tested (12.6pg/ml \pm S.E 10.9). Dex treatment resulted in a reduction in detected concentration (1.4 \pm 1.4) with respect to control supernatants. Treatment with IL-4 or IFN- γ also reduced IL-12 levels (4.2 and 6.4 respectively). Interestingly, combination treatments of IL-4 or IFN- γ with Dex slightly increased IL-12 levels above those seen in the single treatments of the cytokines (6 and 8.9 respectively).

IL-1 levels in control MDM Φ supernatants were relatively high (119 \pm 38) and this increased upon IFN- γ treatment (715.9 \pm 401.5) in accordance with previously published results (Donnelly et al., 1990). IL-1 levels in the Dex-treated MDM Φ samples were below the minimum detection level of the assay. IL-4 treatment significantly reduced the levels of IL-1 detected compared with control MDM Φ , with combination treatments of IL-4 or IFN- γ and Dex also having IL-1 levels below that of the control MDM Φ . The data from these experiments demonstrates a differential effect of IFN- γ and IL-4 on pro-inflammatory cytokine production, consistent with the caM Φ /aaM Φ dogma. These results also demonstrate that Dex reduced pro-inflammatory cytokine production, and that Dex was dominant in reducing the IFN- γ induced IL-1 production.

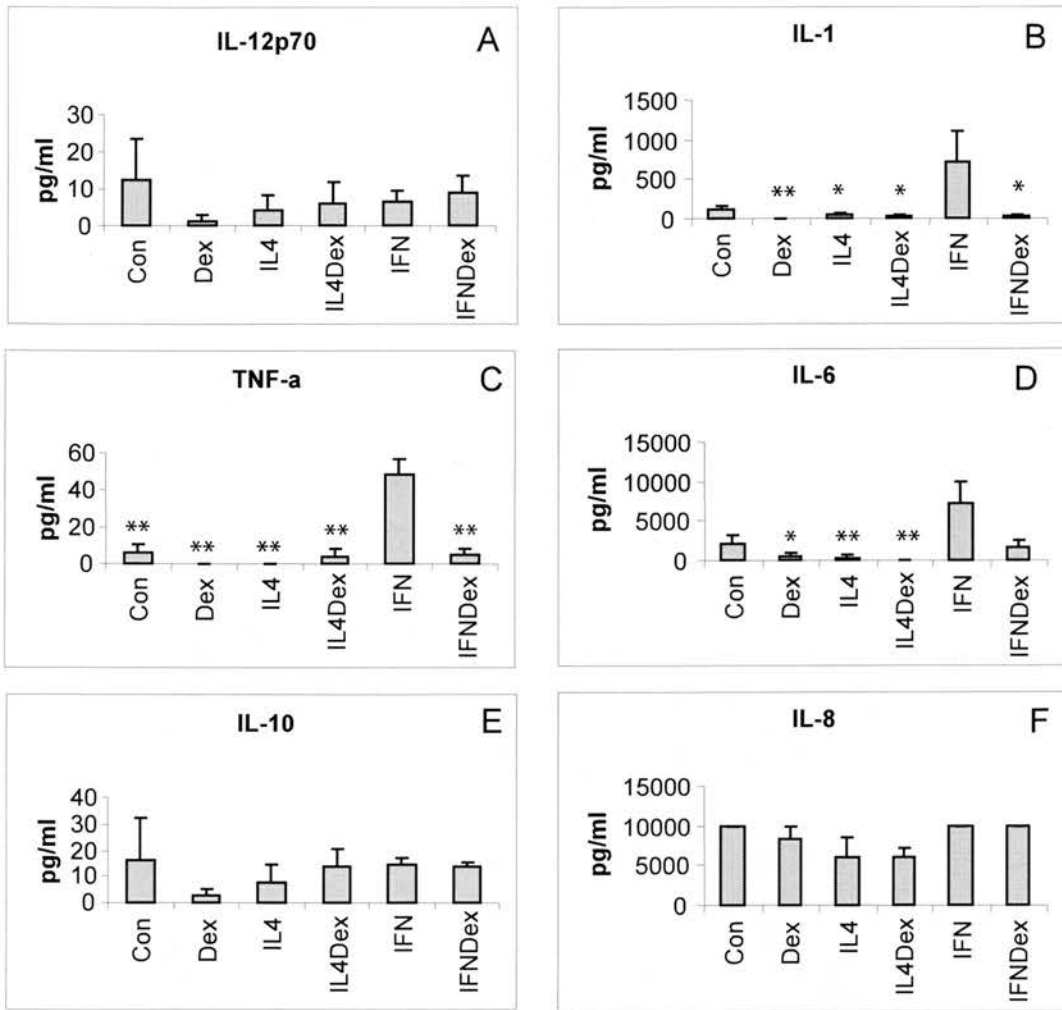


Figure 6.5 Cytokine production by 5 day MDMΦ treated with Dex ± cytokines

Supernatants were collected from the media of 5 day MDMΦ, differentiated from peripheral blood monocytes in combinations of 125nM Dex, 10ng/ml IL4 and 10ng/ml IFN γ . The level of cytokines were detected using the BD Cytokine Bead Array as detailed in the Methods and Materials section. Results shown are the mean concentrations \pm SEM, (n=3).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. IFN- γ MDMΦ, Tukey Kramer repeated ANOVA with post tests performed on transformed data.

TNF- α levels were only up-regulated above control levels in the IFN- γ supernatants (5.4 ± 5.4 in control to 48.7 ± 7.6 in IFN- γ). No TNF- α was detected in either the Dex, IL-4 or IL-4 + Dex treated cell supernatants.

Interestingly, combinatorial treatment of IFN- γ and Dex resulted in a significant reduction in TNF- α levels (5 ± 3), demonstrating the dominant effect of Dex over IFN- γ in the secretion of TNF- α from macrophages.

IL-6 levels were high in the control supernatant, and these were significantly reduced by treatment with Dex (2053.7 ± 1127.6 to 499.1 ± 465). Levels in the IL-4 supernatants were lower than Dex or control, and a synergistic effect was seen when Dex and IL-4 are used in combination, significantly lowering the levels (60.3 ± 41). IFN- γ treatment led to a large increase in IL-6 production (7044 ± 2955) when compared to control macrophages. The combination of Dex and IFN- γ reduced production of the cytokine (1682 ± 816), again demonstrating a dominant effect of Dex over IFN- γ .

IL-10 levels were low in all samples, with no significant changes seen between the different conditions. Whilst, the lowest levels of IL-10 were recorded in the Dex samples. Combination treatment of Dex and IFN- γ did not decrease the concentration below that detected in the IFN- γ only sample.

Of all cytokines analysed, IL-8 levels were detected at the highest concentration. In control, IFN- γ and IFN- γ + Dex samples, the concentration was above the maximum detectable, so although there may well be differences between these samples, in these experiments the assay was unable to differentiate between them.

Dex alone and IL-4 alone resulted in a lower concentration than the control sample, and combination treatments of IL-4 and Dex further lowered this.

In summary, the cytokines present at the highest concentrations were the pro-inflammatory cytokines IL-8, IL-1 and IL-6. In the case of all but IL-8, where the levels were above the upper limit of detection, IFN- γ induced secretion of the pro-inflammatory cytokines TNF- α , IL-6 and IL-1. Combination treatment of Dex and IFN- γ reduced the concentrations of these cytokines, demonstrating a dominant

effect of Dex over IFN- γ . Examination of the cytokines IL-12 and IL-10 showed that the concentration in the IFN- γ + Dex sample was not reduced below that measured for IFN- γ alone, suggesting that the IFN- γ is dominant in these cases.

Morphological change induced by IL-4 and IFN- γ are overridden by Dex

Figure 6.6 shows phase contrast light microscopy images of 5 day MDM Φ differentiated from peripheral blood monocytes. (A) shows an image of control MDM Φ . They formed a heterogeneous population of cells, with a mix of mononucleated smaller cells and multinucleated giant cells. (C) shows an image of the IL-4 population. Clumps of cells, such as those seen in the upper left corner of the picture, were seen throughout the wells of IL-4 differentiated cells. Between the cell clumps, single cells were observed, often with a polarised morphology, such as is seen in IL-4/GMCSF induced-dendritic cell populations. (E) shows MDM Φ differentiated in the presence of IFN- γ for 5 days. IFN- γ induced the formation of multinucleated giant cells, which were seen in high numbers throughout the wells. (B) shows the Dex-treated MDM Φ s. They formed a homogeneous population of small and round cells which were observed by SEM to be more raised than the 'flatter' control MDM Φ . The IL-4 Dex and IFN- γ Dex MDM Φ both shared the same morphology as Dex MDM Φ , and all three populations were weakly adherent to tissue culture plastic.

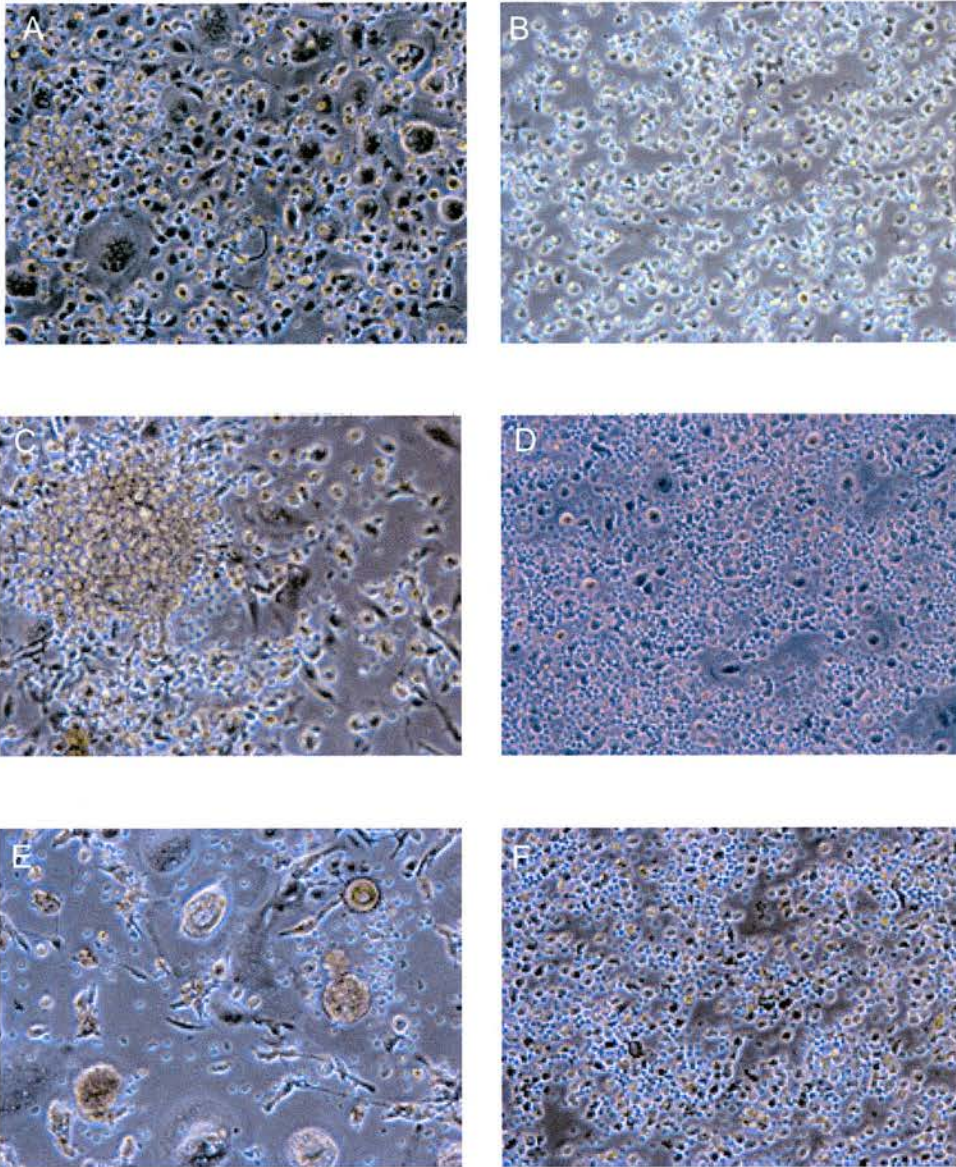


Figure 6.6 Phase contrast images of 5 day MDMΦ cultured in the presence of Dex ± IL-4 or IFN-γ

Adherent peripheral blood monocytes were cultured for 5 days in the 10% AS alone, or with the addition of Dex ± IL-4 or IFN-γ. Representative images are shown, (A) control MDMΦ, (B) Dex MDMΦ, (C) IL-4 MDMΦ, (D) IL-4+Dex MDMΦ, (E) IFN-γ MDMΦ and (F) IFN-γ+Dex MDMΦ. Images were viewed using a x10 objective lens.

Dex is dominant over cytokines in adhesion status

Regions of adhesion found in macrophages are points of contact between the cell and underlying extracellular matrix. The podosomes-like adhesions can be visualised by immunofluorescence staining against F-actin and paxillin. F-actin is present as punctate regions at points of adhesion and are surrounded by several interacting proteins including paxillin and vinculin. In Figure 6.7, the podosomes are seen as red dots of F-actin surrounded by green rings of paxillin.

Control MDM Φ , as seen in Figure 6.7(A), were strongly adherent to tissue culture plastic and formed a large numbers of podosomes. In the multinucleated giant cells which formed part of the heterogeneous population seen in the control MDM Φ , the podosomes were seen as a cortical ring around the edge of the cell.

IL-4 induced a population of MDM Φ which included polarised MDM Φ , as seen in Figure 6.7(C). The IL-4 MDM Φ contained a high number of podosomes and this was reflected in their strong adherence to the tissue culture plates. IFN- γ (Figure 6.7E) induced giant cell formation and, as in the control MDM Φ , the podosomes were seen in a cortical ring around the edge of the cell.

Figure 6.7(B), (D) and (F) represent paxillin and F-actin staining in Dex, IL-4/Dex and IFN- γ /Dex MDM Φ respectively. The MDM Φ had no clear podosomes and the F-actin was present mainly around the edge of the cell or in ruffles. The F-actin was often coincident with paxillin (see regions of orange staining), although the paxillin appeared as a diffuse stain throughout the cells. Dex was dominant over IL-4 and IFN- γ in inhibiting the formation of organised podosomes in the MDM Φ . This was reflected in the poor adherence of these cells to tissue culture plastic.

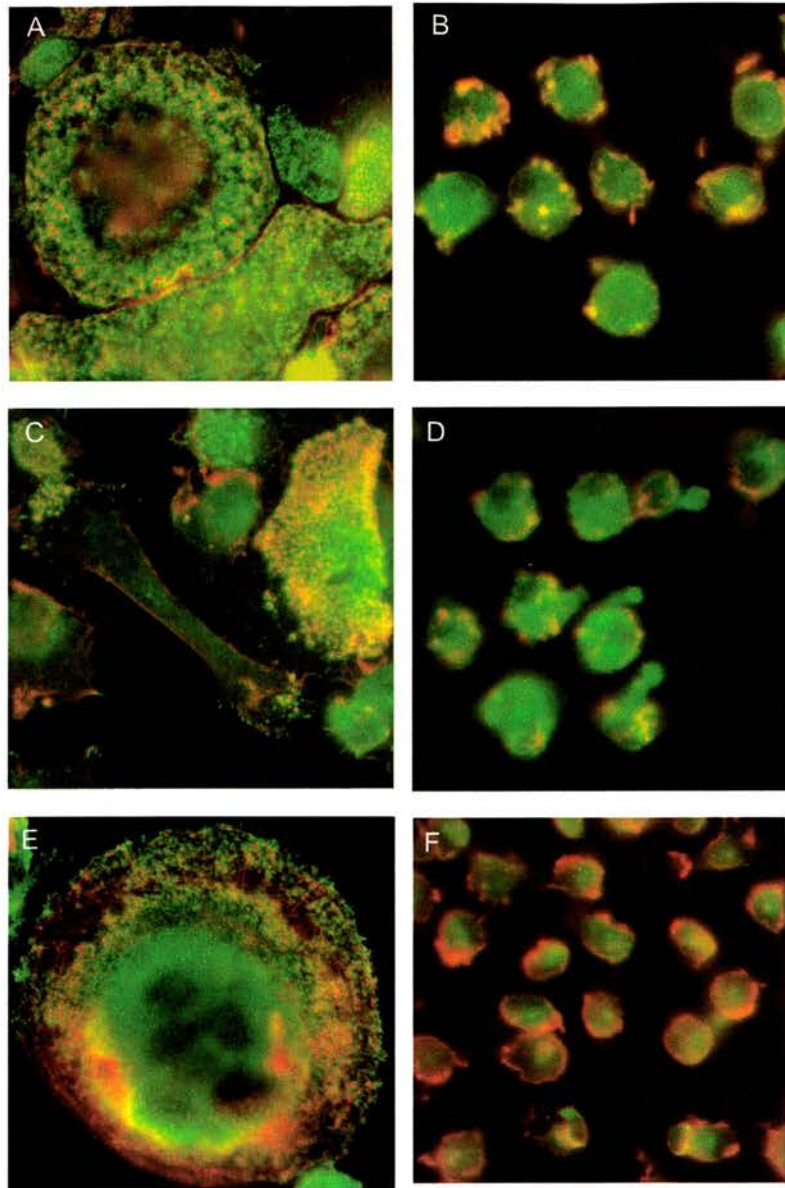


Figure 6.7 Images of podosome adhesions in 5 day MDMΦ

Adherent peripheral blood monocytes were cultured for 5 days in 10 % AS (control) or with the addition of Dex \pm IL-4 and IFN- γ . Podosomes were visualised by staining with F-actin (red) and paxillin (green). Image (A) control, (B) Dex, (C) IL-4, (D) IL-4+Dex, (E) IFN- γ and (F) IFN- γ +Dex MDMΦ. Images were visualised with x63 objective lens. Control, IL-4 and IFN- γ MDMΦ all contain podosomes, seen as punctate actin surrounded by ring of paxillin staining, which are absent in Dex and IL-4/IFN- γ + Dex MDMΦ.

IL-10 augments phagocytosis of apoptotic neutrophils by MDMΦ

Adherent peripheral blood monocytes were cultured in the presence of recombinant human IL-10, 1μM Dex or combinations of the two for 5 days.

The phagocytic ability of the resulting populations of macrophages was assessed by flow cytometry following a 1 hour assay. Figure 6.8 demonstrates that IL-10 significantly augmented the phagocytic ability of the macrophages in a concentration dependent manner. I was unable to fully assess whether the mechanism of IL-10 augmented phagocytosis was the same as that utilised by Dex, because anti-IL-10 receptor antibodies failed to inhibit IL-10 augmented phagocytosis. However, IL-10 was not detected in the supernatants of Dex treated macrophages following phagocytosis of apoptotic neutrophils (data not shown). This suggests that IL-10 is not secreted in response to Dex treatment and therefore does not act in an autocrine or paracrine fashion to stimulate phagocytosis of Dex-treated MDMΦ. Co-culture of the MDMΦ with Dex and IL-10 further augmented the IL-10-induced phagocytosis. It would be expected that we would observe an additive effect upon IL-10-augmented phagocytosis if the mechanism of Dex was independent. However, although the levels of phagocytosis observed following treatment with Dex + IL-10 were higher than with IL-10 alone, the level of phagocytosis was lower than with Dex alone, suggestive of a more complex interplay between these treatments.

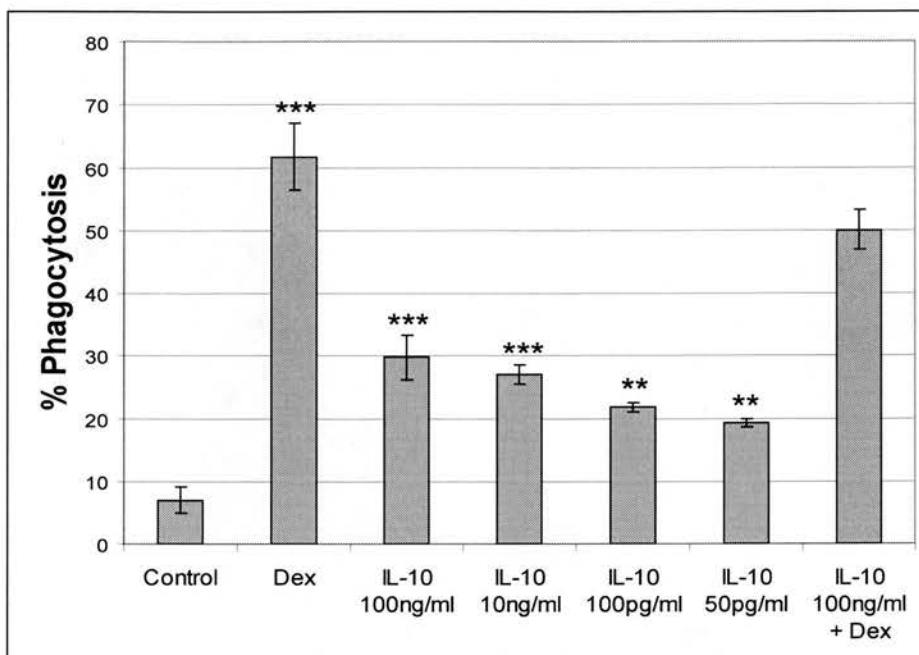


Figure 6.8 IL-10 induced augmentation of MDM Φ phagocytosis of apoptotic neutrophils

Adherent peripheral blood monocytes were cultured for 5 days in the presence of IL-10 or 1 μ M Dex. Phagocytic ability of the MDM Φ was assessed by flow cytometry. Data is shown as mean percentage phagocytosis \pm SEM for 3 separate experiments (IL-10 + Dex n=2).

* p<0.05, ** p<0.01, *** p<0.001 vs. control MDM Φ , Tukey Kramer ANOVA with post tests performed on transformed data.

Discussion

IFN- γ inhibits the GC-augmented phagocytosis of apoptotic neutrophils

One important finding reported in this chapter is that low levels of IFN- γ (10ng/ml) are able to inhibit the augmentation of phagocytosis induced by physiologically relevant levels of Dex (125nM). My data showed that IFN- γ was able to both block and reverse the pro-phagocytic phenotype within the first 24 hours of differentiation from peripheral blood monocyte to macrophage. The time-course data demonstrated that short-term culture with IFN- γ following the differentiation of 5 day Dex-treated MDM Φ failed to inhibit phagocytosis, but that when IFN- γ was included from early time points the augmented phagocytosis was inhibited. There could be two explanations for this; firstly, that the cells are only 'open' to IFN- γ inhibition of Dex-induced functional alterations during the early stages of differentiation or, alternatively, that following prolonged exposure to IFN- γ , MDM Φ were more sensitive to inhibition.

The observed inhibition of GC-induced phagocytosis by IFN- γ , together with previous studies which have shown Dex inhibition of IFN- γ signalling (Hu et al., 2003) suggests that IFN- γ and Dex may have a reciprocal ability to inhibit each other. Glucocorticoids signal via the glucocorticoid receptor and regulate the NF κ B and AP-1 transcription factors as discussed in detail in Chapter 3, leading to the suppression of pro-inflammatory gene transcription such as the cytokines IL-8 and IL-6 (Wilckens and De Rijk, 1997). IFN- γ signalling occurs by ligand binding to the ubiquitously expressed IFN- γ receptor which activates the Janus Kinase (JAK) 1 and 2. These tyrosine kinases then activate the transcription factor STAT1, shown to be responsible for the majority, but not all of, IFN- γ signalling (Stark et al., 1998). Previous investigations have demonstrated an interaction between GCs and components of the IFN- γ pathways, which have resulted in both attenuation and inhibition of the IFN- γ signal (Celeda et al., 1993; Girard et al., 1987; Stocklin et al.,

1996). GCs have been shown to interact with Jak-STAT signalling both directly, for example by GR binding to STAT-5 (Stocklin et al., 1996), and indirectly by regulating gene expression of STAT-1 in peripheral blood macrophages (Hu et al., 2003). Although outside the remit of this work, it would be of interest to investigate how IFN- γ signalling interferes and inhibits Dex-mediated functional changes in MDM Φ . It appears that early exposure of Dex-treated MDM Φ to IFN- γ is required to inhibit the Dex-induced phagocytic capacity, and this may suggest that IFN- γ acts to modify the Dex-induced differentiation pathway. As discussed, one of the major mechanisms of GC action is the alteration of inflammatory gene transcription (Barnes, 1998). IFN- γ inhibition of Dex-augmented MDM Φ phagocytosis could therefore be examined using a gene array to compare gene expression between Dex-treated and IFN- γ + Dex-treated MDM Φ . This may allow identification of genes important for phagocytosis which are altered by IFN- γ . However, IFN- γ signalling components such as JAK1, JAK2 or STAT 1 (Aaronson and Horvath, 2002) may interact with GC signalling molecules such as the GR, inhibiting GC-mediated effects. This could be tested by investigating whether there is increased binding between IFN- γ signalling components and GC signalling components.

GC inhibition of IFN- γ signalling via a reduction in STAT-1 protein levels and phosphorylation in human MDM Φ was shown to be dependent on lymphocyte co-culture and required 24 hours exposure to Dex prior to IFN- γ exposure to take effect (Hu et al., 2003). The authors proposed that Dex suppressed monocyte-lymphocyte interactions, leading to low levels of IFN- γ secretion by the lymphocytes which in turn suppressed STAT-1 production by the monocytes, rendering them less responsive to future IFN- γ signalling. Interestingly, my experiments showing that IFN- γ suppressed Dex-treated MDM Φ phagocytosis were carried out in the presence of lymphocytes, although a requirement for co-culture for IFN- γ inhibition of Dex-augmented phagocytosis was not tested. If the presence of Dex reduces monocyte-lymphocyte interactions, then my data would suggest that this interaction is not necessary for IFN- γ inhibition of Dex-augmented phagocytosis. The described indirect effect of GC inhibition of IFN- γ signalling (Hu et al., 2003) is therefore

unlikely to be significant in helping unravel the mechanism of IFN- γ inhibition of Dex-mediated phagocytosis.

A study by Lucas and colleagues used the WEHI-3 and RAW 264.7 macrophage cell lines to approximate an early and late stage of macrophage differentiation respectively, as a means of investigating IFN- γ signalling during macrophage differentiation. They demonstrated that, despite known components of the IFN- γ pathway being present in both cell lines, STAT-1 failed to be tyrosine phosphorylated in the WEHI-3 cells (Lucas et al., 1998). Results presented here demonstrate that IFN- γ was able to inhibit Dex-augmented phagocytosis only when it was added for the first 24 hours of culture, i.e. to monocytes only, suggesting that STAT-1 phosphorylation does occur in human peripheral blood monocytes. However, this would require experiments to demonstrate that IFN- γ signals via STAT-1 in MDM Φ . If IFN- γ signalling pathways are rapidly up-regulated in MDM Φ during differentiation, addition of IFN- γ after only a few hours would be predicted to inhibit Dex-augmented phagocytosis.

The effects of Dex and IFN- γ upon induction of non-phagocytic functions

Further examination of the effect of IFN- γ on Dex mediated changes revealed that Dex is dominant over IFN- γ in inducing morphological changes, adhesion status and in inhibiting the secretion of pro-inflammatory cytokines.

Dex induced expression levels of CD86, DR and CD14 were all retained in the IFN- γ /Dex co-cultures, with IFN- γ only dominant in attenuating levels of CD64.

Interestingly, expression levels of the phagocytic receptor CD14 showed no correlation with the increased phagocytic ability observed in Dex-treated MDM Φ . Although this may suggest that CD14 is not a likely candidate in Dex-augmented phagocytosis, the data does not exclude a role for CD14 in apoptotic cell recognition, as receptor expression does not necessarily equate to receptor function.

The dominance of Dex over IFN- γ in inhibiting the secretion of pro-inflammatory cytokines suggests that either during a switch from initial inflammatory responses to resolution of inflammation, where a tissue environment containing a mix of GC and IFN- γ might be envisaged, or following the administration of steroid drugs during a chronic inflammation, GCs may dampen down pro-inflammatory responses. The change in cytokine profile would then impact on future inflammatory events, such as the differentiation of infiltrating monocytes and leukocyte migration.

IL-4 and Dex induce macrophages with distinct functions

MDM Φ differentiated in the presence of either IL-4 or IFN- γ , in contrast to the Dex-treated MDM Φ , were poor at phagocytosing apoptotic neutrophils. The differential effect of IL-4 and Dex on this important macrophage function adds further weight to published evidence for diverse roles for these two populations of macrophages, that have been simply labelled as aaM Φ in many reviews and papers (Goerdts and Orfanos, 1999; Schebesch et al., 1997) (Mosser, 2003). Dex-macrophages have been shown to be divergent from IL-4 macrophages in some functions, e.g. ECM deposition (Gratchev et al., 2001a), and data presented here re-enforces this idea with incongruent levels of expression of HLA-DR, CD86 and CD14 between IL-4 and Dex-treated MDM Φ . The data endorses the previously described involvement of the IL-4 MDM Φ in the later phases of inflammation, in antigen presentation and differentiation of naïve Th cells towards a Th2 population, as well as a clear role in the healing of damaged tissue and angiogenesis. However, my data also suggest a re-definition of the role played by Dex-macrophages; they may be intimately involved in the resolution stage of inflammation, clearing apoptotic neutrophils and dampening the pro-inflammatory cytokine environment in preparation for healing of damaged tissue. Dex MDM Φ are not, however, equipped with surface receptors that would make them efficient antigen presenting cells.

The term ‘alternatively activated’, originally intended to demonstrate the point that IL-4 differentiated macrophages are not simply a deactivated cell, but have an alternative function to the classically activated macrophage, has come to include

macrophages treated with the Th2 cytokines IL-10, IL-13 as well as glucocorticoids and TGF- β . The usefulness of this categorisation is waning as the complexity of different macrophage phenotypes involved in inflammation becomes clearer. So, although both the IL-4- and Dex-macrophages could be generally described as 'pro-inflammatory resolution' macrophages they have different roles and possibly utilise divergent pathways (Gratchev et al., 2001a). It is important to appreciate that the examples of IFN- γ induced caM Φ and the IL-4 induced aaM Φ represent two extremes of what will be a continuum of phenotypes within an inflammatory site, where a complex mix of cytokines exists. The interplay between cytokines will be an important determinant of inflammatory outcome. Interestingly, Erwig and colleagues, using uncommitted rat BMDM Φ , showed that the initial cytokine exposure determined the function of the BMDM Φ , and that they became unresponsive to subsequent exposure to alternative activating signals (Erwig et al., 1998). The *in vivo* implications would be that, for MDM Φ at least, the current cytokine environment governs the differentiation and therefore the subsequent function of the MDM Φ . My data would suggest that the initial exposure of cells to either Dex or IFN- γ does not determine function, as subsequent exposure to IFN- γ or Dex further modifies MDM Φ phagocytic ability. However, simultaneous exposure of infiltrating monocytes to more than one cytokine may be expected to amplify some responses and inhibit others, in line with the demonstration of a hierarchical system of responses to some simultaneously expressed cytokines (Erwig et al., 2003; Wesemann and Benveniste, 2003).

Dex further increases IL-10 augmented phagocytosis

IL-10 significantly increased phagocytosis in MDM Φ when compared to the control population. Levels of phagocytosis did not reach those observed in the Dex-treated MDM Φ and this may reflect the induction of different pathways by IL-10 and Dex, both resulting in augmented phagocytosis. There have been some reports suggesting that Dex induces IL-10 secretion (Agarwal and Marshall, 1998; Mozo et al., 2004), and one possibility would be that the IL-10 was acting in an autocrine or paracrine

manner, inducing phagocytosis in neighbouring cells. However, IL-10 levels in Dex-treated MDM Φ supernatants were lower than those observed in control MDM Φ supernatants (Figure 6.5). In addition, the media of Dex MDM Φ following phagocytosis of apoptotic cells did not show elevated IL-10 levels either (data not shown). Furthermore, IL-10 and Dex co-cultured macrophages had a phagocytic level above that seen with IL-10 only, but below that of the Dex-treated MDM Φ . It is possible that Dex treatment may maximally stimulate phagocytosis and so further increases in phagocytic ability would not be detected. However, levels of phagocytosis were higher than in the IL-10 treated MDM Φ population and based upon these data I would speculate that IL-10 and Dex induce phagocytosis by separate mechanisms. The effect of Dex and IL-10 co-culture on phagocytosis of apoptotic neutrophils over a range of concentrations may provide further insight. Following the establishment of the concentration of IL-10 which resulted in 50% maximal MDM Φ phagocytosis of apoptotic neutrophils (mid-concentration), Dex could then be cross-titrated in, and vice versa, IL-10 could be cross titrated into the mid-concentration of Dex. In addition, experiments to block the IL-10-induced phagocytosis downstream from receptor/ligand binding would allow confirmation of whether Dex was capable of inducing augmented phagocytosis.

A dissociation between adhesion and phagocytosis

The data presented in Figures 6.6 and 6.7 show the morphology and adhesion status of the different MDM Φ populations. The three distinct phenotypes that were seen following culture under control, IL-4 or IFN- γ conditions were all over-ridden in the presence of Dex in co-cultures, with all containing a homogeneous population of small and rounded MDM Φ , which were only weakly adherent to the tissue culture plastic. The presence of Dex was shown in Figure 6.5 to result in a down-regulation in the secretion of pro-inflammatory cytokines, which may have prevented autocrine and paracrine regulation of IL-4 and IFN- γ induced morphologies in the co-cultures. The dominance of Dex over the cytokines seen in the induction of adhesion processes was also observed in the adhesion structures. The distinct podosomes

structures that were present in the control, IL-4 and IFN- γ cultures were lost in the Dex \pm IL-4 or IFN- γ cultures. It has previously been suggested that adhesion and phagocytosis may be related (McCutcheon et al., 1998), with the two processes linked by shared proteins, e.g. DOCK-180, Crk and Rac, which may cause one function to limit use in the other. I therefore hypothesised that the altered adhesion observed in Dex-treated MDM ϕ may be linked to augmentation of phagocytic capacity. However, despite a shared morphological appearance and adhesion status of the Dex and IFN- γ Dex macrophages, the IFN- γ Dex cells do not retain the phagocytic capacity of the Dex cells. These observations provide the first evidence for a dissociation between adherence status and phagocytic capacity.

Chapter 7: SUMMARY AND FUTURE DIRECTIONS

The main aim of this thesis was to examine the mechanisms by which glucocorticoids (GCs) regulate MDM Φ phagocytosis of apoptotic neutrophils. Breakdown of the normal regulation of cellular apoptotic programmes or in the controlled removal of apoptotic cells is associated with inflammatory/autoimmune disease (Botto, 1998; Haslett, 1997). For the successful resolution of inflammation, the large scale induction of apoptosis in neutrophils recruited during an inflammatory response must be paralleled by increased clearance of the apoptotic cells. Previous work examining 5 day Dex-treatment of MDM Φ demonstrated an augmentation in phagocytosis of apoptotic cells, associated with changes to the cytoskeleton and adhesion status of the macrophage (Giles et al., 2001). The hypothesis generated from this data was that adhesion and phagocytosis utilised many shared proteins and that, following Dex-induced disruption of adhesive processes, proteins were utilised in the process of phagocytosis. Data presented in this thesis both confirms and extends these studies by describing further alterations to cytoskeletal arrangement in Dex-treated MDM Φ . Importantly, this work also describes novel changes to macrophage receptor-mediated binding of apoptotic cells following Dex treatment, and an induction of PS recognition required for Dex-augmented internalisation of apoptotic cells. Thus, data presented here shows that Dex-treatment results in the promotion of multiple key stages in macrophage phagocytosis of apoptotic cells.

One of the major findings of this study was the resolution of a cation-independent 'tethering' step from a cation-dependent receptor-mediated engulfment step during phagocytosis. Dissociation of binding from internalisation has been critical for defining mechanisms of phagocytosis via other well characterised receptors, e.g. Fc and complement (Allen and Aderem, 1996; Caron and Hall, 1998; Crowley et al., 1997; Greenberg et al., 1993). For example, binding via FcR or CR does not require an intact cytoskeleton, unlike internalisation, which requires Rho GTPase- and Arp2/3- dependent actin polymerisation at the phagocytic cup (May et al., 2000).

Thus, the dissection of apoptotic cell phagocytosis into distinct binding and internalisation steps, may allow detailed examination of the processes involved. The cation-independent binding may occur through an, as yet unidentified receptor, in both the untreated and Dex-treated MDM Φ . However, Dex treatment was demonstrated to augment this initial binding step (with a 14-fold increase in the binding index) in a pH-dependent manner. The differential effect of pH on Dex-treated, but not control MDM Φ binding, may be a result of the engagement of a different receptor following Dex treatment, or it could be that Dex-treatment modifies the MDM Φ cell surface, lowering electrostatic repulsion between the MDM Φ and the apoptotic target cell. This suggestion is supported by a ~60% inhibition in Dex-augmented phagocytosis following incubation with the amino sugar, glucosamine (Giles et al., 2001). The inhibition of Dex-treated MDM Φ binding of apoptotic neutrophils at low pH may have important physiological consequences. Sites of chronic inflammation have been associated with a low tissue pH (Haslett, 1999 and references therein), which may reduce the beneficial augmentation in Dex-treated MDM Φ phagocytosis of apoptotic cells.

A key focus of any future studies would be the identification of the receptor(s) involved in this 'binding' step. Binding studies using apoptotic cells, or macrophages isolated from mice deficient in the putative recognition receptors (Botto, 1998; Platt et al., 1996), would represent one strategy for screening receptors involved. Similarly, the use of blocking antibodies or ligands could be used in parallel to identify candidate receptors (Brown et al., 2002; Chang et al., 1999; Fadok et al., 1992b; Savill et al., 1992). Screening of large numbers of hybridomas, or recombinant antibodies, may allow the identification of novel receptors that mediate apoptotic cell binding (Flora and Gregory, 1994). Subsequent expression cloning could be used to further characterise those molecules identified (Fadok et al., 2000).

Although the putative 'binding' receptor has been assumed to simply 'tether' the apoptotic cell, it could act in concert with later recruited 'signalling' receptors, with the combination dictating the anti-inflammatory cytokine synthesis associated with apoptotic cell engulfment. Therefore, following the molecular characterisation of this

receptor(s), its role as either a 'binding' receptor or in addition, as a 'signalling' receptor for apoptotic cell internalisation, could then be determined. A successful method employed by Akakura and colleagues was the transfection of the receptor of interest into a phagocytic cell line (Akakura et al., 2004). They transfected wildtype (WT) or $\beta 5$ integrin cytoplasmic tail mutant constructs (incapable of signalling), and showed that phagocytosis was associated only with the WT construct. Further studies included the co-transfection of $\beta 5$ integrin constructs with reporter constructs for signalling molecules of interest, and the demonstration of Rac-1 activation, following over-expression or ligand activation of the WT $\beta 5$ integrin construct (Akakura et al., 2004).

In this thesis, the increased binding of apoptotic cells by Dex-treated MDM Φ was shown to be paralleled by an increased cation-dependent engulfment, with the engagement of a PS recognition mechanism in addition to a novel, as yet undefined receptor mediated recognition mechanism utilised by the untreated MDM Φ . In addition, GC treatment was demonstrated to result in key changes to cytoskeletal regulation which resulted in altered adhesion, migration and phagocytosis. In terms of phagocytosis, the observed increase in Rac-1 activity may be hypothesised to be responsible for increased membrane extension required for an augmented engulfment of apoptotic cells, although roles for other signalling proteins, e.g. Cdc42 and Rho, remain to be investigated.

MDM Φ , matured in the presence of Dex, showed alterations in many of the key stages involved in the phagocytosis process, and one interesting question stemming from these studies is whether Dex-treatment augments all the processes described by 'switching on' a master switch, i.e. by altering expression of a protein or signalling pathway(s) which regulate phagocytosis. Data presented in this thesis, demonstrated that the phagocytic augmentation of apoptotic neutrophils only required the transrepressor activity of Dex. Thus, by comparing signalling molecules or receptor expression in MDM Φ treated with Dex (demonstrates transrepressor and transactivator activity) or with a GC with only transrepressor activity, changes critical to augmented phagocytosis could perhaps be identified.

My studies have also highlighted the importance of careful phenotyping of the target apoptotic cell used in a phagocytosis assay. Neutrophils induced to undergo apoptosis using different methods, resulted in differential expression of PS and potentially altered phagocytosis as a consequence. The percentage of necrotic cells present within the target cell population is also an important consideration, since phagocytosis of necrotic cells but not apoptotic cells has been reported to be associated with pro-inflammatory effects (Fadok et al., 2001). The apoptotic and necrotic neutrophils could potentially be internalised using different receptors, and therefore trigger different signalling pathways, making interpretation of data difficult.

I therefore sought to generate a homogeneous population of apoptotic cells as targets. Induction of apoptosis by exposure of cells to ultraviolet light has been used in many studies (Fadok et al., 1992b; Hoffmann et al., 2001; Ogden et al., 2001), but in my experiments always induced a significant level of necrosis in neutrophil populations. The initiation of apoptosis using TNF/gliotoxin resulted in a synchronous induction of morphological-defined apoptosis in nearly 100% of the neutrophils. This was in contrast to the overnight 'aging', where the neutrophils formed three populations; non-apoptotic, apoptotic and necrotic cells, which were difficult to further purify. A study by Ren and colleagues used plasma-Percoll discontinuous density gradient centrifugation to purify 'late apoptotic' neutrophils (distinguished from 'early' apoptotic neutrophils by the presence of nuclear degradation) from a mixed population of 'overnight' aged neutrophils (Ren et al., 2001). However, the 'early apoptotic' neutrophils (annexin V positive, PI negative) were not able to be purified from the viable neutrophils using this method. Following 'overnight aging', apoptotic neutrophils have been positively sorted from viable neutrophils, using annexin-V-biotin and streptavidin-conjugated paramagnetic beads, and cells recovered in the absence of calcium (Brown et al., 2002). However, whilst this method removes the viable neutrophils, the apoptotic and necrotic cells are still mixed. Following 'overnight aging', I attempted to separate apoptotic neutrophils from necrotic and viable neutrophils using flow cytometric cell sorting. Annexin V and PI were used to distinguish between the three populations. Following the

purification of the annexin V positive, PI negative neutrophils (a process that took several hours in order to obtain numbers sufficient for subsequent phagocytosis studies), the neutrophils were cultured for one hour at 37°C, 5% CO₂, in order to mimic culture conditions during a phagocytosis assay. The purified apoptotic neutrophils were then analysed using annexin V and PI, but it was found that approximately 60% of the apoptotic neutrophils had become necrotic, demonstrating that this method was not suitable for purifying apoptotic neutrophils.

The TNF/gliotoxin method, unlike overnight 'aging', did not require the presence of serum (data not shown) and could potentially be used to investigate roles for serum components in phagocytosis. However, it is worth noting that if the TNF/gliotoxin apoptotic neutrophils had been used as the apoptotic targets in the initial experiments investigating Dex-augmentation of phagocytosis, no augmentation would have been seen.

The observation that Dex-treated MDMΦ showed differential levels of phagocytosis between two populations of apoptotic human neutrophils, potentially rules out the suggestion that Dex-treated MDMΦ are simply better phagocytes of any particle. The use of apoptotic neutrophils induced by reagents such as Fas (Matsumoto et al., 1995) and cycloheximide (Whyte et al., 1997) may add insight by allowing the comparison of multiple targets.

Interestingly, the Dex-treated MDMΦ showed no difference in their ability to bind 'overnight aged' and TNF/gliotoxin treated neutrophils (although viable neutrophils were not bound as readily). The 'binding' step may be less discriminate than the engulfment step, serving instead to tether cells, which additional receptors then differentiate between. It would therefore be interesting to extend my studies, by investigating the binding and internalisation of different apoptotic cells, for example the phagocytosis of apoptotic thymocytes, lymphocytes and eosinophils. Cross-linking of CD44 specifically augmented MDMΦ phagocytosis of apoptotic neutrophils but not apoptotic lymphocytes (Hart et al., 1997). However, 24 hour treatment of monocytes with the GCs, methylprednisolone or Dex, augmented

phagocytosis of apoptotic Jurket cells (T cell line) and apoptotic eosinophils respectively, in addition to apoptotic neutrophils (Liu et al., 1999).

The attenuation of Dex-treated MDM Φ phagocytosis of TNF/gliotoxin treated apoptotic neutrophils when compared with 'overnight aged' neutrophils, has potential implications at the inflammatory site, where the presence of TNF- α during induction of neutrophil apoptosis may result in a failure in GC-MDM Φ recognition of these cells. The surface phenotype differences and phagocytic recognition between TNF- α (Murray et al., 1997) and 'overnight aged' neutrophils might therefore warrant further interest.

The studies presented here examined the *in vitro* effects of GCs on MDM Φ function. If the *in vitro* inhibition of GC-mediated phagocytosis by IFN- γ was also to be seen in a physiological setting, with IFN- γ inhibiting GC augmented phagocytosis of apoptotic cells, the potency of GC treatment may be reduced. Importantly, chronic inflammatory diseases such as rheumatoid arthritis (Radstake et al., 2004; Schulze-Koops and Kalden, 2001) and lung diseases such as chronic obstructive pulmonary disease (COPD) (Majori et al., 1999) have a predominant Th1 cytokine profile. Anti-IFN- γ therapy may therefore help restore the beneficial anti-inflammatory effects of GCs in the treatment of these diseases. Furthermore, any studies examining the role of GCs in the resolution of inflammation *in vivo*, for example in mouse, may fail to show beneficial effects of GC administration if the model is carried on a Th1 dominant strain, such as C57Bl/6 (Mills et al., 2000).

The down-regulation of MDM Φ surface expression of α_L , α_M and β_2 integrins (which pair to form LFA-1 and MAC-1) following Dex treatment, may affect MDM Φ interaction with the surrounding environment. LFA-1 and MAC-1 play critical roles in cell-cell adhesion and in the inflammatory response (Springer, 1990) and a down-regulation in their surface expression may result in a reduced interaction with their ligands, including the ECM molecule fibrinogen (Trezzini et al., 1988) and ICAM-1 (Diamond et al., 1990) and -2 (Damle et al., 1992), which are expressed on endothelium. This could potentially impact on migration and MDM Φ binding to

ECM. The adhesion status and cytoskeletal changes following Dex treatment were associated with an altered migratory ability. If Dex-treated MDM Φ fail to migrate towards apoptotic cells *in vivo*, then an augmentation in phagocytic ability becomes inconsequential. The chemotactic response of GC-treated MDM Φ will be of great interest, and the interplay of the cytokine milieu on this critical process should also be investigated.

Dex-induced changes to MDM Φ phagocytic ability are open to modulation by exogenous factors, namely cytokines and serum components, suggesting that the *in vitro* phenotype may well be modified in an *in vivo* setting. The requirement for serum during differentiation suggests that there is an interplay between serum factors and GCs during differentiation which will require further investigation to be fully elucidated. Examination of *in vivo* generated macrophages, differentiated in the presence of GC, failed to demonstrate an augmentation in phagocytic ability *in vitro*. One possibility is that the overnight culture of the lavaged macrophages reduced any GC effect, or that the GC-treated macrophages had cleared from the peritoneum during the 4 day period between GC administration and lavage. The investigation of an *in vivo* effect of GCs on macrophage phagocytosis of apoptotic cells may be assessed in mice, by injecting labelled apoptotic cells into the site of inflammation 24 hours after GC treatment, followed by the examination of lavaged macrophages.

In conclusion, the studies presented in this thesis have demonstrated that Dex-treatment of MDM Φ augments phagocytic ability by alteration of multiple steps of the engulfment process, as well as providing insight into the mechanism of untreated MDM Φ phagocytosis.

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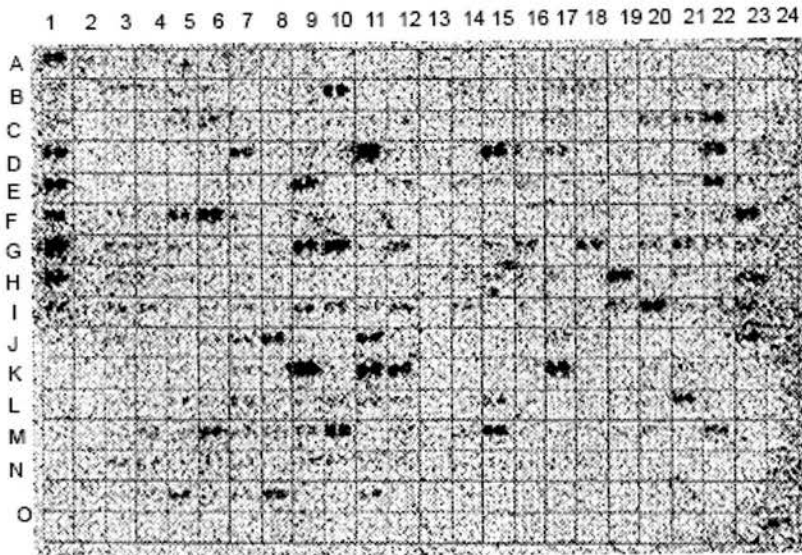
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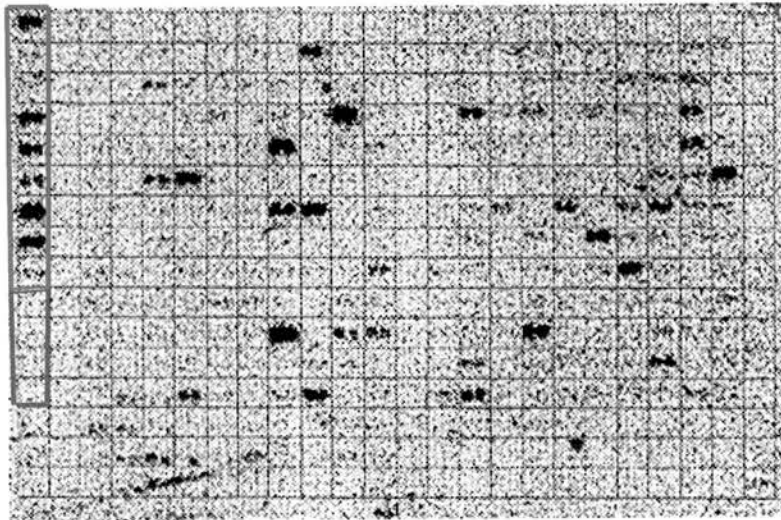
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Control Array



Dex Array

Appendix 1

Scanned arrays following incubation with radioactively labelled control and Dex-treated MDM Φ cDNA probes. The area surrounded by the boxes are housekeeping genes (red box), and negative controls (green box).

Grid Ref	Gene	Corrected Mean Control Spot Intensity	Corrected Mean Dex Spot Intensity	Control/Dex	Dex/Control
B3	Collegan IV alpha 6 subunit	0.296277916	0.214759036	1.379583001	0.724856713
B4	Collegan XVIII alpha 1 subunit	0.239702233	0.204216867	1.173763148	0.851960638
B5	Thrombospondin 2	0.266253102	0.255120482	1.043636715	0.958187831
B6	CD41 antigen	0.218362283	0.210843373	1.035661113	0.965566813
B7	Vitronectin receptor alpha subunit	0.163771712	0.262951807	0.622820257	1.605599671
B8	Semaphorin 1	0.186104218	0.253012048	0.735554768	1.359518072
B9	Matrix metalloproteinase 15	0.268238213	0.231626506	1.158063548	0.863510471
B10	Tissue-type plasminogen activator	0.622580645	0.994578313	0.625974483	1.597509208
B11	CD26 antigen	0.187096774	0.261144578	0.716449008	1.395772746
B12	TNF superfamily member 6	0.176178660	0.238554217	0.738526706	1.354047175
B14	Cadherin 4	0.261538462	0.248795181	1.051219966	0.951275691
B15	Cadherin-like protein 42	0.183870968	0.318373494	0.577532273	1.731504967
B16	Epithelial Cell Kinase	0.188585608	0.278313253	0.677601968	1.475792644
B17	Notch homolog	0.253598015	0.371385542	0.682842992	1.464465494
B18	Ninjurin 1	0.254838710	0.352108434	0.723750655	1.381691322
B19	Manic fringe homolog	0.187841191	0.410240964	0.457880143	2.183977654
B20	Beta 3-endonexin	0.177667494	0.337349398	0.526657214	1.898768257
B21	KiSS protein	0.168238213	0.320481928	0.524953824	1.904929452
B22	Rac-1	0.249875931	0.388554217	0.643091542	1.554988574
B23	RhoHP1	0.158560794	0.272590361	0.581681587	1.719153610
C3	Collegan I alpha 2 subunit	0.176923077	0.183734940	0.962925599	1.038501833
C4	Fibronectin 1	0.161786600	0.186144578	0.869144844	1.150556213
C5	Versican core protein	0.267493797	0.402710843	0.664232913	1.505496010
C6	CD11b antigen	0.369230769	0.266867470	1.383573537	0.722766064
C7	Integrin beta 1	0.218114144	0.268975904	0.810905888	1.233188728
C8	Neogenin	0.172208437	0.239457831	0.719159761	1.390511614
C9	Matrix metalloproteinase 16	0.212158809	0.263855422	0.804072198	1.243669415
C10	CD87 antigen	0.223573201	0.384337349	0.581710836	1.719067168

C11	CD27 ligand	0.226302730	0.378313253	0.598188744	1.671713168
C12	TRAIL	0.239454094	0.235240964	1.017909850	0.982405269
C14	Cadherin 5	0.177667494	0.263554217	0.674121234	1.483412701
C15	Cadherin-like protein 43	0.160049628	0.281927711	0.567697398	1.761501821
C16	Ephrin receptor A5	0.182630273	0.215060241	0.849205191	1.177571700
C17	Notch homolog 1	0.226054591	0.329216867	0.686643404	1.456360017
C18	Opioid-binding protein	0.158312655	0.342168675	0.462674309	2.161347585
C19	Patched homolog	0.152357320	0.360542169	0.422578365	2.366424983
C20	Caveolin 1	0.247642680	0.499698795	0.495583904	2.017821788
C21	MERLIN	0.264019851	0.450903614	0.585535007	1.707839818
C22	Rac-2	0.498263027	0.531024096	0.938305871	1.065750552
C23	SAS	0.171712159	0.255722892	0.671477464	1.489253256
D3	Procollagen II alpha 1 subunit	0.162034739	0.175301205	0.924321881	1.081874204
D4	Laminin alpha 4 subunit	0.151861042	0.225301205	0.674035642	1.483601071
D5	Vitronectin	0.144913151	0.215662651	0.671943663	1.488220003
D6	CD11c antigen	0.195533499	0.267168675	0.731872848	1.366357562
D7	CD18 antigen	0.389081886	0.318975904	1.219784571	0.819816895
D8	Cell adhesion kinase	0.162282878	0.235843373	0.688095985	1.453285620
D9	Matrix metalloproteinase 17	0.257320099	0.360843373	0.713107454	1.402313207
D10	Urokinase-type plasminogen activator	0.349627792	0.298493976	1.171306022	0.853747852
D11	TNF-receptor superfamily member 7	2.037965261	2.828614458	0.720481809	1.387960096
D12	FasL receptor	0.262282878	0.279216867	0.939351841	1.064563837
D14	Cadherin 6	0.209181141	0.232831325	0.898423531	1.113060784
D15	Cadherin-associated protein	0.777667494	0.729518072	1.066001684	0.938084822
D16	Ephrin receptor B1	0.224317618	0.229819277	0.976060932	1.024526202
D17	Notch homolog 2	0.280397022	0.435843373	0.643343548	1.554379465
D18	Anonin 1	0.160297767	0.298192771	0.537564228	1.860242829
D19	Smoothed homolog	0.178660050	0.410542169	0.435180752	2.297895750
D20	Caveolin 2	0.155583127	0.334939759	0.464510774	2.152802598
D21	Metastasis-associated protein 1	0.160794045	0.372590361	0.431557177	2.317190057

D22	Rho GDP dissociation inhibitor 1	0.605210918	0.674397590	0.897409669	1.114318282
D23	p21-activated kinase 1	0.268238213	0.210843373	1.272215526	0.786030338
E3	Collegan III alpha 1 subunit	0.150124069	0.208734940	0.719209106	1.390416210
E4	Laminin beta 2 subunit	0.130272953	0.238855422	0.545405049	1.833499713
E5	Heperan sulfate proteoglycan	0.149379653	0.235542169	0.634194945	1.576802226
E6	VLA-1	0.203225806	0.269277108	0.754708811	1.325014343
E7	CD61 antigen	0.179156328	0.223493976	0.801615913	1.247480226
E8	Matrix metalloproteinase 1	0.163275434	0.251506024	0.649190948	1.540378841
E9	Matrix metalloproteinase 18	0.867493797	2.057228916	0.421680733	2.371462394
E10	Alpha-2-macroglobulin	0.193300248	0.253012048	0.763996219	1.308907002
E11	CD30 ligand	0.195285360	0.273192771	0.714826234	1.398941382
E12	TNF-beta	0.264516129	0.299096386	0.884384238	1.130730238
E14	Cadherin 8	0.228784119	0.199698795	1.145645966	0.872870005
E15	Alpha N catenin	0.194292804	0.250602410	0.775303016	1.289818277
E16	Ephrin receptor B2	0.228535980	0.284638554	0.802898893	1.245486833
E17	Notch homolog 4	0.179156328	0.270481928	0.662359696	1.509753696
E18	Tight junction protein 1	0.202233251	0.234337349	0.863000504	1.158747875
E19	Sonic hedgehog	0.161042184	0.299397590	0.537887374	1.859125253
E20	CDC42 GTPase-activating protein	0.166997519	0.337349398	0.495028359	2.020086289
E21	NME4	0.183622829	0.373192771	0.492032116	2.032387659
E22	Rho GDP dissociation inhibitor 2	0.444913151	0.735542169	0.604877831	1.653226403
E23	TIAM 1	0.265756824	0.330722892	0.803563438	1.244456819
F3	Collegan IV alpha 2 subunit	0.230769231	0.203614458	1.133363678	0.882329317
F4	Laminin gamma 2 subunit	0.196029777	0.205120482	0.955681143	1.046374104
F5	Aggrecan 1	0.441687345	0.595481928	0.741730898	1.348197848
F6	LFA1A	1.137965261	1.127108434	1.009632460	0.990459439
F7	Integrin beta 4	0.235732010	0.270481928	0.871525916	1.147412809
F8	Matrix metalloproteinase 2	0.152605459	0.225602410	0.676435413	1.478337741
F9	Metalloproteinase/disintegrin	0.342431762	0.264457831	1.294844475	0.772293522
F10	Alpha-2-macroglobulin receptor-associated protein	0.310173697	0.220180723	1.408723222	0.709862651

F11	TNF-receptor superfamily member 8	0.268734491	0.198795181	1.351815926	0.739745686
F12	TNF-C	0.272952854	0.195481928	1.396307356	0.716174699
F14	Cadherin 11	0.162282878	0.171686747	0.9452226590	1.057947386
F15	Beta 1 catenin	0.179156328	0.208132530	0.860780040	1.161736976
F16	Ephrin receptor B4	0.164267990	0.183132530	0.896989683	1.114840025
F17	Delta-like protein 1	0.177419355	0.247289157	0.717457074	1.39381161
F18	Dishevelled	0.200744417	0.308433735	0.650851039	1.536449879
F19	Wnt 10b	0.158808933	0.331626506	0.478878890	2.088210655
F20	CDC42 homolog	0.151116625	0.468975904	0.322226844	3.103403763
F21	Nucleoside diphosphate kinase A	0.228287841	0.590963855	0.386297468	2.588678628
F22	p160 ROCK	0.247146402	0.544277108	0.454081934	2.202245730
F23	RHOA	0.790074442	1.122289157	0.703984741	1.420485333
G3	Collegan XVIII alpha 3 subunit	0.390322581	0.195481928	1.996719519	0.500821468
G4	Laminin beta 1 subunit	0.307692308	0.20753012	1.482639277	0.674472892
G5	Bone/cartilage small proteoglycan 1	0.233995037	0.239759036	0.975959200	1.024632996
G6	VLA2 receptor-alpha 2	0.183870968	0.213855422	0.859791004	1.163073346
G7	Integrin beta 5	0.185111663	0.238253012	0.776954134	1.287077263
G8	Matrix metalloproteinase 3	0.216129032	0.226506024	0.954186685	1.048012947
G9	TIMP1	1.00942928	1.187951807	0.849722417	1.176854912
G10	CD91 antigen	1.102729529	1.21626506	0.906652312	1.102958639
G11	CD33 antigen	0.24292804	0.205722892	1.180850793	0.846847041
G12	TNF-alpha	0.390818859	0.251506024	1.553914503	0.643536049
G14	Cadherin 12	0.194044665	0.206927711	0.937741321	1.066392167
G15	beta-catenin	0.32630273	0.271385542	1.202358559	0.831698658
G16	Ephrin A1	0.375930521	0.351807229	1.068569632	0.935830451
G27	Delta-like protein	0.158808933	0.252710843	0.628421523	1.591288592
G28	Dishevelled homolog 1-like protein	0.434243176	0.604518072	0.718329519	1.392118761
G19	Wnt 13	0.184119107	0.285542169	0.644805310	1.550855714
G20	Cytchesin 2	0.273200993	0.454819277	0.600680328	1.664779007
G21	NME2	0.333498759	0.736445783	0.452849031	2.208241448

G22	Rho-GAP hematopoietic protein C1	0.256575682	0.433433735	0.591960574	1.689301694
G23	Zyxin 2	0.336476427	0.328313253	1.0248663979	0.975739240
H3	Collegan IX alpha 1 subunit	0.357816377	0.236746988	1.511387242	0.661643801
H4	Laminin gamma 1 subunit	0.265012407	0.154216867	1.718439826	0.581923198
H5	Bone proteoglycan II	0.20719603	0.250	0.828784119	1.206586826
H6	VLA3-alpha 3	0.176923077	0.227108434	0.779024689	1.283665365
H7	Integrin beta 6	0.195533499	0.245180723	0.797507636	1.253906489
H8	Matrix metalloproteinase 7	0.166004963	0.198795181	0.835055267	1.197525528
H9	TIMP2	0.190322581	0.308132530	0.617664680	1.619001429
H10	Low-density lipoprotein receptor-related protein 2	0.180148883	0.187650602	0.960022942	1.041641774
H11	CD34 antigen	0.202481390	0.197590361	1.024753374	0.975844555
H12	TNF-receptor 1	0.212158809	0.204518072	1.037359714	0.963985768
H14	Cadherin 13	0.238957816	0.205421687	1.163255059	0.859656695
H15	Deleted in polyposis protein 2.5	0.459801489	0.280722892	1.637919467	0.610530628
H16	Ephrin A5	0.232506203	0.206024096	1.128538882	0.886101503
H17	Jagged homolog 1	0.220099256	0.300602410	0.732193916	1.365758411
H18	Frizzled homolog 2	0.185359801	0.241867470	0.766369291	1.304853954
H19	Wnt 5a	0.797766749	1.065662651	0.748610969	1.335807304
H20	NME3	0.187096774	0.316867470	0.590457500	1.693601994
H21	p21-activated kinase 2	0.222084367	0.364457831	0.609355454	1.641078280
H22	Rho6 protein	0.227047146	0.302409639	0.750793353	1.331924419
H23	Zyxin related protein	0.619602978	0.359337349	1.724293282	0.579947745
I3	Collegan VI alpha 1 subunit	0.332258065	0.262650602	1.265019236	0.790501813
I4	Laminin receptor 1	0.253101737	0.224397590	1.127916465	0.886590480
I5	Insulin like growth factor-binding protein	0.177419355	0.200602410	0.884432820	1.130668127
I6	VLA4 receptor alpha	0.212903226	0.254216867	0.837486623	1.194048923
I7	Integrin beta 7	0.202233251	0.207530120	0.974476621	1.026191884
I8	Matrix metalloproteinase 8	0.183622829	0.219578313	0.836252115	1.195811625
I9	TIMP3	0.319602978	0.224698795	1.422361777	0.703056013
I10	Insulin-like growth factor II receptor	0.325310174	0.220481928	1.475450515	0.677759091

I11	CD40 ligand	0.220843672	0.187951807	1.175001591	0.851062678
I12	TNF-receptor 2	0.332754342	0.368975904	0.901832177	1.108853760
I13	Ezrin	0.145409429	0.174397590	0.833781183	1.199355442
I14	Cadherin 14	0.276674938	0.226204819	1.223116903	0.817583338
I15	Drosophila discs large	0.197022333	0.242168675	0.813574806	1.229143273
I16	Ephrin B1	0.174937965	0.224397590	0.779589322	1.282726651
I17	Jagged homolog 2	0.209677419	0.269277108	0.778667821	1.284244671
I18	Frizzled homolog 5	0.220843672	0.251807229	0.877034680	1.140205767
I19	Wnt 8b	0.324813896	0.315963855	1.028009661	0.972753504
I20	Ezrin	0.756079404	0.935843373	0.807912334	1.237758056
I21	Paxillin	0.239702233	0.348795181	0.687229201	1.455118611
I22	Rho7 protein	0.337965261	0.250301205	1.350234254	0.740612229
I23	P37NB	0.649627792	0.380722892	1.706300845	0.586063122
J3	Collegan VI alpha 2 subunit	0.274441687	0.272289157	1.007905312	0.992156692
J4	Netrin-2	0.204714640	0.224096386	0.913511566	1.094676889
J5	KIAA0014 unclassified protein	0.179652605	0.234939759	0.764675192	1.307744791
J6	VLA5	0.199007444	0.234337349	0.849234852	1.177530571
J7	Intergrin beta 8	0.249131514	0.278915663	0.893214498	1.119551913
J8	Matrix metalloproteinase 9	0.568486352	0.259036145	2.194621732	0.455659390
J9	TIMP4	0.192803970	0.190060241	1.014436103	0.985769332
J10	Hyaluronoglucosamine	0.178660050	0.194578313	0.918190967	1.089098059
J11	CD40 antigen	0.544665012	0.284036145	1.917590500	0.521487773
J12	E-selectin	0.234739454	0.229819277	1.021408896	0.979039838
J14	Desmocollin 1A	0.172704715	0.214759036	0.804179036	1.243504189
J15	Bullous pemphigoid antigen 1	0.150124069	0.230722892	0.650668291	1.536881410
J16	Ephrin B2	0.151364764	0.234036145	0.646758066	1.546173217
J17	L1 cell adhesion molecule	0.235483871	0.332831325	0.707517151	1.413393299
J18	Frizzled homolog 9	0.190322581	0.279819277	0.680162506	1.470236880
J19	Wnt 2	0.148635236	0.279518072	0.531755369	1.880563992
J20	Focal adhesion kinase 1	0.127295285	0.311445783	0.408723740	2.446640363

J21	PYK2	0.138213400	0.321385542	0.430054814	2.325284982
J22	RhoE	0.191066998	0.283132530	0.674832374	1.481849476
J23	Hemopoietic cell kinase	0.524813896	0.428915663	1.223582959	0.817271925
K3	Collegan VI alpha 3 subunit	0.192307692	0.247289157	0.777663262	1.285903614
K4	Nidogen	0.151364764	0.194879518	0.776709455	1.287482718
K5	Insulin like growth factor-binding protein 2	0.170967742	0.258132530	0.662325441	1.509831780
K6	VLA6	0.150868486	0.249096386	0.605663089	1.651082950
K7	CD36 antigen	0.199255583	0.230722892	0.863614277	1.157924350
K8	Matrix metalloproteinase 10	0.240694789	0.238855422	1.007700756	0.992358092
K9	CD147 antigen	1.945161290	2.480120482	0.784301128	1.27502048
K10	Hyaluronan-mediated motility receptor	0.228784119	0.276506024	0.827410975	1.208589238
K11	ICAM-1	1.096774194	0.457831325	2.395585739	0.417434444
K12	CD44 antigen	0.730769231	0.517168675	1.413019130	0.707704502
K14	Desmocollin 2A	0.154094293	0.235240964	0.655048722	1.526604001
K15	Desmoplakin I & II	0.165012407	0.317469880	0.519773426	1.923915210
K16	Ephrin B3	0.183374690	0.277409639	0.661024941	1.512802224
K17	Protein tyrosine phosphatase receptor type F	0.901488834	1.392469880	0.647402753	1.544633530
K18	Frizzled-related FrzB	0.178908189	0.297891566	0.600581584	1.665052721
K19	Prostate-specific membrane antigen	0.141439206	0.300301205	0.470991137	2.123182202
K20	RHO GDP dissociation inhibitor 3	0.192555831	0.331024096	0.581697325	1.719107098
K21	Putative Rho/Rac GEF	0.231513648	0.375301205	0.616874246	1.621075944
K22	RhoB	0.151861042	0.335240964	0.452990710	2.207550791
K23	Non-receptor tyrosine kinase 1	0.201488834	0.351204819	0.573707485	1.743048549
L3	Collegan VIII alpha 1 subunit	0.207444169	0.276807229	0.749417454	1.334369776
L4	Basement membrane protein	0.151612903	0.199096386	0.761505051	1.313188926
L5	Insulin like growth factor-binding protein 3	0.214888337	0.238253012	0.901933351	1.108729375
L6	Integrin alpha 7B	0.220843672	0.236445783	0.934014003	1.070647760
L7	Semaphorin	0.299007444	0.223795181	1.336076332	0.748460231
L8	Matrix metalloproteinase 11	0.219106700	0.218373494	1.003357577	0.996653659
L9	Protein C Inhibitor	0.242679901	0.244277108	0.993461493	1.006581541

L10	Platelet-activating factor receptor	0.302233251	0.284036145	1.064066163	0.939791184
L11	CD59 glycoprotein	0.292555831	0.245180723	1.193225258	0.838064727
L12	V-CAM1	0.234987593	0.222590361	1.055695276	0.947243038
L14	Desmocollin 3A	0.166253102	0.205421687	0.809325950	1.235596116
L15	Desmoplakin III	0.263523573	0.440662651	0.598016584	1.672194427
L16	Ephrin type A receptor 1	0.153101737	0.247891566	0.617615755	1.619129679
L17	CD43 antigen	0.110173697	0.338253012	0.325713869	3.070179366
L18	Glycogen synthase kinase 3 alpha	0.167990074	0.388253012	0.432681961	2.311166379
L19	DDR2	0.162779156	0.3	0.542597188	1.842987805
L20	Integrin-linked kinase	0.169975186	0.288855422	0.588443814	1.699397590
L21	Ras-like protein TC10	0.445409429	0.690060241	0.645464559	1.549271739
L22	Rhoc	0.158560794	0.269277108	0.588838743	1.698257820
L23	ELAV-like neuron protein	0.168734491	0.383734940	0.439716257	2.274193834
M3	Collegan XI alpha 1 subunit	0.178660050	0.224397590	0.796176329	1.256003179
M4	Tenascin	0.253349876	0.313253012	0.808770758	1.236444308
M5	Insulin like growth factor-binding protein 4	0.250868486	0.237650602	1.055618979	0.947311501
M6	Integrin alpha 8	0.588833747	0.638253012	0.922571043	1.083927366
M7	Semaphorin E	0.253846154	0.219277108	1.157650042	0.863818912
M8	Matrix metalloproteinase 12	0.245657568	0.192771084	1.274348635	0.784714616
M9	Endothelial plasminogen activator inhibitor 1	0.313895782	0.282530120	1.111017052	0.900076194
M10	CD4 antigen	0.823076923	0.992469880	0.829321816	1.205804527
M11	CD72 antigen	0.212903226	0.228313253	0.932504894	1.072380431
M12	Cadherin 1	0.260049628	0.266566265	0.975553406	1.025059206
M14	Desmoglein 2	0.232258065	0.328915663	0.706132577	1.416164659
M15	Envoplakin	0.495781638	0.887048193	0.558911727	1.789191300
M16	Ephrin type B receptor 3	0.151364764	0.243072289	0.622715015	1.605871025
M17	CD146 antigen	0.156079404	0.337650602	0.462251225	2.163325799
M18	Indian hedgehog	0.181389578	0.337048193	0.538171045	1.858145304
M19	Zinc-finger DNA-binding protein	0.146153846	0.325301205	0.449287749	2.225745086
M20	MacMARCKS	0.216129032	0.368674699	0.586232342	1.705808308

M21	ras-like small GTPase TTF	0.158560794	0.262048193	0.605082570	1.652667006
M22	RhoG	0.343424318	0.457530120	0.750604828	1.332258949
M23	Comeodesmosin	0.173945409	0.424096386	0.410155369	2.438100476
N3	Collegan XI alpha 2 subunit	0.249627792	0.307831325	0.810923941	1.233161273
N4	Tenascin-R	0.232009926	0.300602410	0.771816586	1.295644611
N5	Insulin like growth factor-binding protein 5	0.193300248	0.220180723	0.877916312	1.139060736
N6	Integrin alpha 9	0.296526055	0.188253012	1.575146402	0.634861622
N7	Semaphorin III	0.244665012	0.182228916	1.342624531	0.744809868
N8	Matrix metalloproteinase 13	0.173697270	0.2	0.868486352	1.151428571
N9	Plasminogen activator inhibitor type II	0.229032258	0.177710843	1.288791689	0.775920584
N10	CD9 antigen	0.230521092	0.300602410	0.766863752	1.304012606
N11	CD82 antigen	0.231265509	0.276204819	0.837297152	1.194319122
N12	Cadherin 2	0.237717122	0.283734940	0.837814059	1.193582262
N14	Desmoglein 1	0.177667494	0.255120482	0.696406233	1.435943495
N15	Plexin-related protein	0.172952854	0.281626506	0.614121363	1.628342639
N16	CD56 ligand	0.156823821	0.270481928	0.579794083	1.724750267
N17	Contactin	0.168982630	0.256325301	0.659250684	1.516873662
N18	Lunatic fringe	0.197270471	0.288855422	0.682938441	1.464260817
O3	Collegan XVI alpha 1 subunit	0.154094293	0.236144578	0.652542158	1.532468036
O4	Thrombospondin 1	0.179900744	0.345783133	0.520270446	1.922077275
O5	Insulin like growth factor-binding protein 6+C52	0.359553350	0.463554217	0.775644653	1.289250168
O6	Integrin alpha E	0.217866005	0.322289157	0.675995455	1.479299887
O7	Semaphorin IV	0.208684864	0.207831325	1.004106880	0.995909918
O8	Matrix metalloproteinase 14	0.381885856	0.359337349	1.062750245	0.940954853
O9	Plasminogen	0.193796526	0.274397590	0.706261763	1.41590562
O10	CD19 antigen	0.167493797	0.217469880	0.770193081	1.298375725
O11	CD86 antigen	0.269727047	0.285542169	0.944613709	1.058663799
O12	Cadherin 3	0.178660050	0.244879518	0.729583474	1.370645080

Interferon γ suppresses glucocorticoid augmentation of macrophage clearance of apoptotic cells

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One of beneficial effects of glucocorticoids (GC) in inflammation may be the augmentation of macrophages' capacity for phagocytosis of apoptotic cells, a process that has a central role in resolution of inflammation. Here we define the phenotype of GC-treated monocyte-derived macrophages, comparing to IFN- γ -treated and IL-4-treated monocyte-derived macrophages and combinatorial treatment. Our data indicate that the cytokine microenvironment at an inflammatory site will critically determine monocyte functional capacity following treatment with GC. In particular, whilst GC exert dominant regulatory effects over IFN- γ in terms of cell surface receptor repertoire and morphology, the acquisition of a macrophage capacity for clearance of apoptotic cells is prevented by combined treatment. In terms of mechanism, GC augmentation of phagocytosis was reversed even when monocytes were pre-incubated with GC for the first 24 h of culture, a period that is critical for induction of a highly phagocytic macrophage phenotype. These findings have important implications for the effectiveness of GC in promoting acquisition of a pro-phagocytic macrophage phenotype in inflammatory diseases associated with high levels of IFN- γ

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1 Introduction

Macrophages play a central role in the immune system, initiating and co-ordinating both innate immunity and the adaptive immune response [1, 2]. Macrophage production of matrix, enzymes and the cytokines that regulate fibroblast migration and proliferation modulates the resolution phase of inflammation [3, 4]. In addition, the efficient clearance of extravasated leukocytes that have been induced to undergo apoptosis prevents further injury through the release of toxic or immunostimulatory intracellular contents [5]. Importantly, phagocytic clearance of apoptotic leukocytes by macrophages induces release of anti-inflammatory cytokines that promote resolution of inflammation [6] and triggers changes in macrophage behavior that promote tolerogenic responses. However, in situations where prolonged or chronic inflammatory responses occur, leukocytes may not be efficiently cleared by macrophages and consequently

undergo secondary necrosis, releasing cytotoxic granule contents and further exacerbating the inflammatory response, potentially leading to the development of autoimmunity [7]. For example, the defective phagocytosis of apoptotic cells that is observed in C1q deficiency contributes to the development of systemic lupus erythematosus [8]. Thus, defining the mechanisms that regulate this important clearance process is essential for understanding the pathogenesis of many inflammatory diseases.

A number of studies indicate that micro-environmental cues, e.g. extracellular matrix components and the cytokine repertoire, have a critical role in determining macrophage behavior [9]. It is well established that pro- and anti-inflammatory cytokines differentially activate macrophages. LPS or pro-inflammatory cytokines such as IFN- γ and TNF- α induce "classically activated" macrophages, which can be defined by production of nitric oxide and reactive oxygen species [10, 11]. These macrophages show increased potential for the engulfment and destruction of pathogenic organisms which is reflected in the repertoire of receptors expressed; these receptors include Fc and complement receptors that enable recognition and internalization of immunoglobulin- and complement-opsonized particles [12]. Conversely, in response to cytokines with anti-in-

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The first two authors contributed equally to this manuscript.

Abbreviations: GC: Glucocorticoid IDMEM: Iscove's modification of Dulbecco's modified Eagles medium MDM Φ : Monocyte-derived macrophage

flammatory potential such as IL-4 and IL-13, macrophage release of pro-inflammatory cytokines is inhibited, inducing an immunoregulatory phenotype. These macrophages are characterized by increased expression of specific pattern-recognition molecules, such as the mannose receptor [13, 14] and enhanced capacity for endocytosis and antigen presentation [15]. Arginase-1 expression is also enhanced and competes with NO synthases, increasing the metabolism of arginine to ornithine and urea, resulting in a decreased capacity for the production of reactive nitrogen species [16] and a reduced ability for pathogen clearance.

We have recently demonstrated that exposure of peripheral blood monocytes to glucocorticoids (GC), *e.g.* the synthetic glucocorticoid dexamethasone, results in differentiation towards a macrophage phenotype that exhibits a markedly increased phagocytic capacity for apoptotic cells; exposure to mineralocorticoids or sex steroids does not do this [17]. Prolonged exposure of monocytes to GC induced a homogeneous monocyte-derived macrophage (MDM Φ) phenotype consisting of small "rounded" cells with profound changes in their cytoskeletal organization and a loss of actin-containing podosomes [18].

We sought to define the cell surface phenotype and functional repertoires of human monocytes treated with GC and the cytokines IL-4 and IFN- γ . In this manuscript we present evidence that MDM Φ that have differentiated in the presence of dexamethasone exhibit distinct receptor expression patterns and functional status from either IFN- γ - or IL-4-activated MDM Φ . GC exert a dominant effect upon the morphology and adhesive status of MDM Φ following combination treatment with IFN- γ or IL-4. However, we demonstrate that IFN- γ profoundly inhibits the GC-induced capacity to clear apoptotic cells. Indeed, even after exposure of MDM Φ to GC for 24 h, subsequent exposure to IFN- γ inhibited the acquisition of a phagocytic phenotype, indicating that GC-induced alterations in MDM Φ function are reversible. Our data suggest that the cytokine milieu at an inflammatory site will critically determine whether GC induce an MDM Φ phenotype with high capacity to clear apoptotic cells; a phenotype that would favor the resolution of inflammation.

2 Results

2.1 Dexamethasone-augmented MDM Φ phagocytosis of apoptotic cells is inhibited in the presence of IFN- γ

We examined the effects of IFN- γ or IL-4, in combination with dexamethasone, on the phagocytosis of apoptotic neutrophils by MDM Φ . Dexamethasone, or dexametha-

sone in combination with IL-4, strongly increased the percentage of MDM Φ that phagocytosed apoptotic neutrophils compared with phagocytosis by untreated MDM Φ (Fig. 1). We did not observe a difference in the percentage of MDM Φ that phagocytosed apoptotic cells when comparing IFN- γ -treated and control MDM Φ . However, in the presence of IFN- γ , dexamethasone failed to augment this phagocytosis (Fig. 1).

We assessed the mean fluorescence of phagocytic MDM Φ populations to provide further information about the effects of these treatments on the number of apoptotic cells phagocytosed per MDM Φ (expressed as mean fluorescence of phagocytic cells after subtraction of the mean fluorescence of non-phagocytic cells). Dexamethasone-treated MDM Φ showed increased fluorescence when compared with untreated MDM Φ (the mean \pm SE was 655 \pm 84 compared with 407 \pm 111). Phagocytic MDM Φ from IL-4- or IFN- γ -treated populations also showed slightly higher mean fluorescences (510 \pm 163 and 650 \pm 142, respectively). Interestingly, phagocytic MDM Φ treated with dexamethasone plus IL-4 or dexamethasone plus IFN- γ had lower mean fluorescences of 455 \pm 101 and 456 \pm 106, respectively.

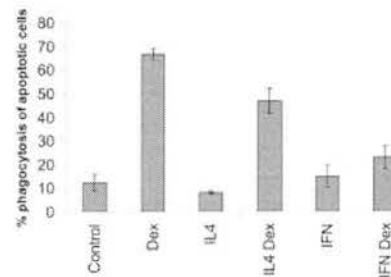


Fig. 1. Dexamethasone-induced phagocytosis of apoptotic neutrophils is reduced in the presence of IFN- γ . The phagocytic ability of MDM Φ cultured in IDMEM containing 10% autologous serum alone (control) or with combinations of dexamethasone, IL-4 or IFN- γ as described in Sect. 4.2 was determined on day 5 by flow cytometric quantification of the percentage of fluorescent MDM Φ following a 60-min incubation with CMFDA-labeled apoptotic neutrophils. Dexamethasone treatment results in a highly phagocytic population of macrophages when compared with untreated macrophages. MDM Φ treated with IFN- γ plus dexamethasone have a phagocytic ability similar to MDM Φ treated with IFN- γ alone, demonstrating that IFN- γ is dominant in determining phagocytic capacity. The results shown here represent the mean \pm S.E of five separate experiments.

2.2 Suppression of pro-inflammatory cytokine release by dexamethasone

In view of the dominant effects of IFN- γ on dexamethasone-induced augmentation of phagocytic function, we next examined the release of pro-inflammatory cytokines by treated MDM Φ . As would be expected, IFN- γ -treated MDM Φ strongly up-regulated production of TNF- α , IL-1 and IL-6 when compared with untreated MDM Φ or IL-4-treated MDM Φ (Fig. 2). Although production of most cytokines was effectively inhibited by dexamethasone alone, co-culture of MDM Φ in the presence of IL-4 and dexamethasone together produced further inhibitory effects on the production of IL-6.

In contrast to the dominant effects of IFN- γ on phagocytosis by dexamethasone-treated MDM Φ , reduced levels of TNF- α , IL-6 and IL-1 production were found for MDM Φ treated with dexamethasone in combination with IFN- γ , suggesting that dexamethasone remained able to suppress the inflammatory cytokine production that was induced by IFN- γ .

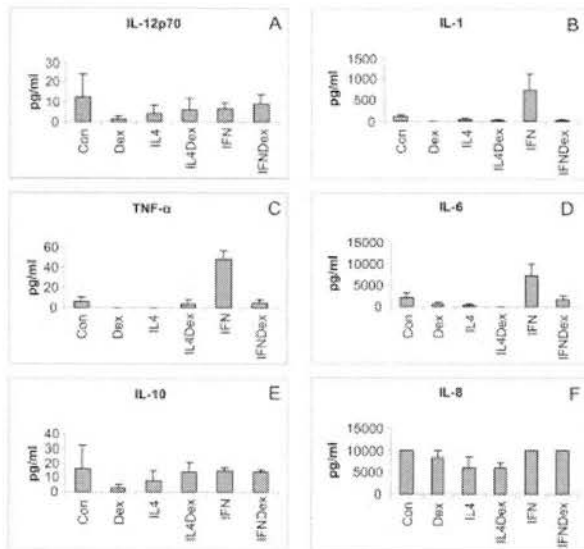


Fig. 2. Cytokine profiles of MDM Φ treated with dexamethasone \pm IL-4 and IFN- γ for 5 days. Adherent monocytes were cultured for 5 days either in 10% autologous serum alone or with combinations of dexamethasone, IL-4 and IFN- γ . MDM Φ supernatants were collected after 5 days and analyzed using an inflammation cytokine bead array kit (Becton Dickinson). Cytokine levels were determined by extrapolation from standard curves using cytokine bead array analysis software. Results shown are the cytokine levels (mean \pm S.E) from three separate experiments using MDM Φ from three different, normal donors.

2.3 MDM Φ cultured in dexamethasone exhibit distinct surface receptor profiles

We next compared cell surface receptor profiles of MDM Φ cultured with dexamethasone, IFN- γ or IL-4 alone, or with combinations of treatments. As would be expected, the high affinity receptor for immunoglobulin, CD64 (Fc γ RI), was strongly up-regulated by IFN- γ -treated MDM Φ when compared with untreated MDM Φ (Fig. 3A) and was also augmented when MDM Φ were exposed to IFN- γ in combination with dexamethasone. In contrast, CD64 was down-regulated by dexamethasone when used alone (35% reduction in levels of expression) and markedly down-regulated by IL-4 (70% reduction in expression).

Dexamethasone-treated MDM Φ expressed slightly reduced levels of HLA-DR when compared with untreated cells; MDM Φ exposed to IL-4 or IFN- γ showed increased expression compared with untreated cells (Fig. 3B). Interestingly, up-regulation of expression of HLA-DR was not observed when MDM Φ were treated with IFN- γ or IL-4 in combination with dexamethasone, suggesting a suppressive effect of dexamethasone

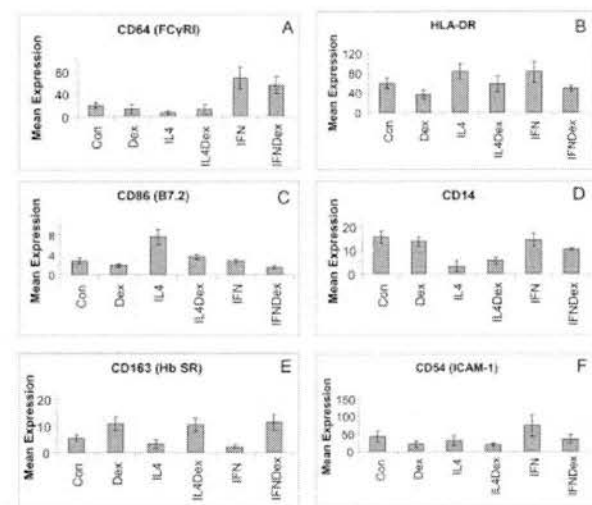


Fig. 3. Effects of dexamethasone, IL-4 and IFN- γ on macrophage cell surface receptor expression. Adherent monocytes were cultured for 5 days either in 10% autologous serum or with combinations of dexamethasone, IL-4 and IFN- γ . The surface phenotype was assessed by indirect immunofluorescence and flow cytometry using control IgG1 mAb, CD64 mAb (A), HLA-DR mAb (B), CD86 mAb (C), CD14 mAb (D), CD163 mAb (E) and ICAM-1 mAb (F). Results, corrected for the mean fluorescence intensity recorded for the IgG1 control, are expressed as the average mean fluorescence (\pm S.E.) recorded for each antibody from a minimum of three separate donors.

(Fig. 3B). IL-4-treated MDM Φ expressed approximately 3-times the levels of CD86 when compared with IFN- γ -treated or dexamethasone-treated MDM Φ (Fig. 3C). Expression of the adhesion molecule ICAM-1 on MDM Φ was increased following IFN- γ treatment, consistent with published data [19]. In contrast, both IL-4 and dexamethasone reduced ICAM-1 expression and IFN- γ -induced up-regulation of ICAM-1 was suppressed following combined IFN- γ and dexamethasone treatment (Fig. 3F).

MDM Φ expression of CD14 was not affected by dexamethasone treatment alone. In contrast, IL-4-treated macrophages exhibit markedly reduced CD14 expression, consistent with the induction of an immature “dendritic-like” cell phenotype (Fig. 3D). Reduced expression of CD14 expression was also seen following treatment with IL-4 and dexamethasone in combination, suggesting that the effects of IL-4 predominate (Fig. 3D). The divergent effects of IL-4 and dexamethasone were also found when expression of the mannose receptor was examined. Dexamethasone and IFN- γ both down-regulated expression of the mannose receptor, whereas IL-4 up-regulated expression (mean fluorescence intensities: control=58.7, IL-4=85.8, dexamethasone=18.4, IFN- γ =4.7) ($n=2$). Expression of the GC-responsive hemoglobin scavenger receptor CD163 [20] was down-regulated following culture in the presence of IL-4 or IFN- γ alone, but increased in dexamethasone-treated MDM Φ (Fig. 3E). Increased CD163 expression levels were also observed following combined treatment with dexamethasone and IFN- γ , again suggesting a dominant effect of dexamethasone.

2.4 Effects of dexamethasone and cytokines on MDM Φ morphology

Our analysis suggests that suppression of dexamethasone-augmented phagocytosis by IFN- γ is not reflected by surface phenotype alterations, with dexamethasone exerting a dominant effect. Morphological examination demonstrated that a homogeneous population of smaller, less-well-spread MDM Φ was induced by GC when compared with the heterogeneous untreated MDM Φ population (Fig. 4). When cultured in the presence of IL-4 alone, MDM Φ exhibited a highly spread, polarized morphology with pronounced cellular processes and evidence of the formation of homotypic cell aggregates (Fig. 4). A heterogeneous MDM Φ population comprising multinucleated “giant” cells together with smaller macrophages was observed in the presence of IFN- γ (Fig. 4). Culture of MDM Φ in the presence of IFN- γ plus dexamethasone, or IL-4 plus dexamethasone, yielded a population of smaller “rounded” cells similar to

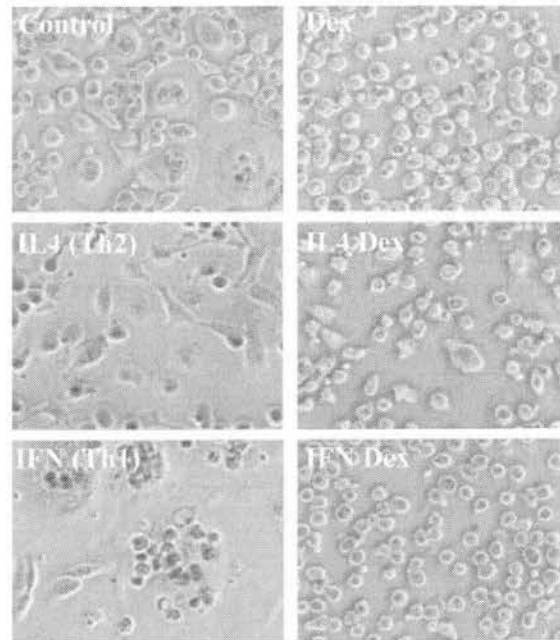


Fig. 4. Dexamethasone is dominant over IFN- γ and IL-4 cytokines in inducing cellular morphology. Adherent monocytes were cultured for 5 days on glass coverslips either in 10% autologous serum or with combinations of dexamethasone, IL-4 and IFN- γ . Representative photomicrographs depict cellular morphology using phase-contrast microscopy with a $\times 63$ objective. Control MDM Φ represent a heterogeneous population of cells containing giant, multinucleated cells as well as smaller mononuclear cells. Dexamethasone-differentiated MDM Φ form a homogeneous population of small, rounded cells. Combined treatment with either IL-4 or IFN- γ plus dexamethasone also yields a homogeneous population of small rounded cells, similar to the dexamethasone-treated population, implying that dexamethasone is dominant over both cytokines in inducing cellular morphological changes.

those seen with dexamethasone alone. Together these data suggest that dexamethasone exerts dominant effects upon MDM Φ morphology.

2.5 Dexamethasone treatment alters adhesion structures in MDM Φ

We next examined the characteristic “podosome-like” adhesion signaling complexes consisting of a punctate actin foci surrounded by a ring of paxillin, vinculin and other cytoskeletal proteins [21] that are present in MDM Φ . Untreated or IFN- γ -treated MDM Φ contain abundant podosomes, whereas these structures were absent from dexamethasone-treated MDM Φ (Fig. 5). IFN- γ -treatment appears to drive differentiation towards

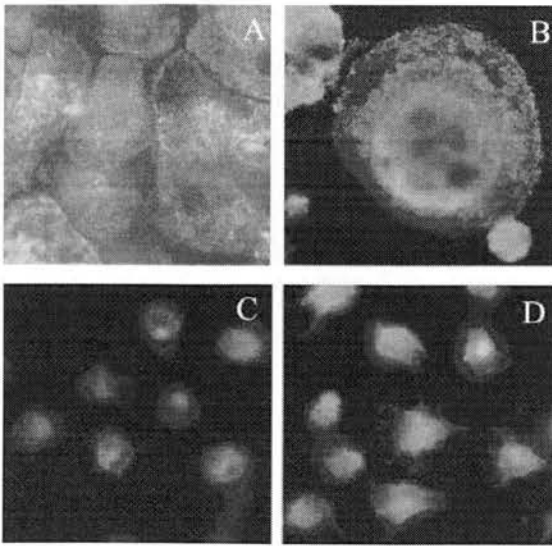


Fig. 5. Dexamethasone is dominant over IFN- γ and IL-4 cytokines in determining the adhesion status of the macrophages. Adherent monocytes were cultured for 5 days on glass coverslips either in 10% autologous serum or with combinations of dexamethasone, IL-4 and IFN- γ . Fixed and permeabilized cells were stained for F-actin using rhodamine phalloidin and paxillin (paxillin mAb and FITC-conjugated anti-mouse-IgG), to visualize cytoskeletal organization. In the representative photomicrographs shown, podosomes are characterized by a foci of actin (red) which is surrounded by a ring of paxillin (green). Untreated control MDM Φ contain abundant podosomes (A). Podosomes are also present in IFN- γ -treated MDM Φ (B). By contrast, MDM Φ differentiated in the presence of dexamethasone alone or dexamethasone plus IFN- γ (C and D, respectively) do not contain podosomes. These data demonstrate the dominance of dexamethasone over IFN- γ in determining the adherence status of the macrophages.

a multinucleated phenotype and the podosomes in these “giant” cells were particularly striking, where a cortical ring of podosomes were formed, similar to the resorption zone described for osteoclasts (Fig. 5B) [22]. Interestingly, IL-4 induces a polarized morphology with podosomes localized at the leading edge of the cell (data not shown). Podosomes were absent from MDM Φ cultured in the presence of dexamethasone (Fig. 5C) or IFN- γ plus dexamethasone (Fig. 5D), in keeping with morphological observations. Podosomes were also absent from MDM Φ treated with IL-4 plus dexamethasone (data not shown). These data confirm the dominant effect of dexamethasone in terms of both morphology and cytoskeletal organization in MDM Φ .

2.6 The mechanism of IFN- γ suppression of phagocytosis

In view of the finding that the first 24 h of culture was critical for GC-mediated augmentation of MDM Φ phagocytosis [18], we sought to investigate whether IFN- γ could override the effects of dexamethasone after this initial 24-h culture period. MDM Φ were exposed to either dexamethasone or IFN- γ for the first 24 h of culture, followed by culture for 4 days in medium alone, dexamethasone or IFN- γ . MDM Φ phagocytosis of apoptotic cells was augmented when monocytes were cultured in medium for 24 h followed by the addition of dexamethasone although less than when dexamethasone was present during the first 24 h (Fig. 6A). Addition of IFN- γ alone failed to alter the phagocytic capacity of MDM Φ when compared to untreated control MDM Φ . However, if monocytes were incubated with IFN- γ for 24 h prior to the addition of dexamethasone, augmentation of MDM Φ phagocytosis of apoptotic cells was not observed. Surprisingly, if monocytes were incubated with dexamethasone for the first 24 h of culture followed by addition of IFN- γ , the phagocytic capacity was also not augmented.

In view of the “switch off” of phagocytosis following IFN- γ treatment 24 h after dexamethasone treatment of monocytes, we next sought to test whether engagement of IFN- γ signaling in GC-treated MDM Φ would also inhibit phagocytosis. MDM Φ were treated with dexamethasone at day 0 and then IFN- γ was added on day 1, day 3 or day 6 (on day 6, it was added either 1 h or 3 h prior to the phagocytosis assay) (Fig. 6B). The results show that treatment with IFN- γ for a few hours prior to phagocytosis did not inhibit phagocytic ability when compared with MDM Φ treated with dexamethasone alone. Indeed, the suppressive effect of IFN- γ upon GC-augmented phagocytosis was less pronounced the longer the macrophages were exposed to dexamethasone before IFN- γ was added. These results demonstrate that the IFN- γ -mediated suppression of augmented phagocytosis most likely induces changes in MDM Φ phenotype rather than being a direct consequence of engagement of IFN- γ -mediated signaling pathways.

3 Discussion

One of the most important observations in our study is that IFN- γ strongly suppressed the dexamethasone-induced augmentation of apoptotic cell phagocytosis by macrophages (Fig. 1, 6A and 6B). Indeed, IFN- γ retained the ability to inhibit GC-augmented phagocytosis even when monocytes had been pre-treated with dexamethasone for 24 h. Exposure of MDM Φ to IFN- γ at later time points following dexamethasone treatment failed to

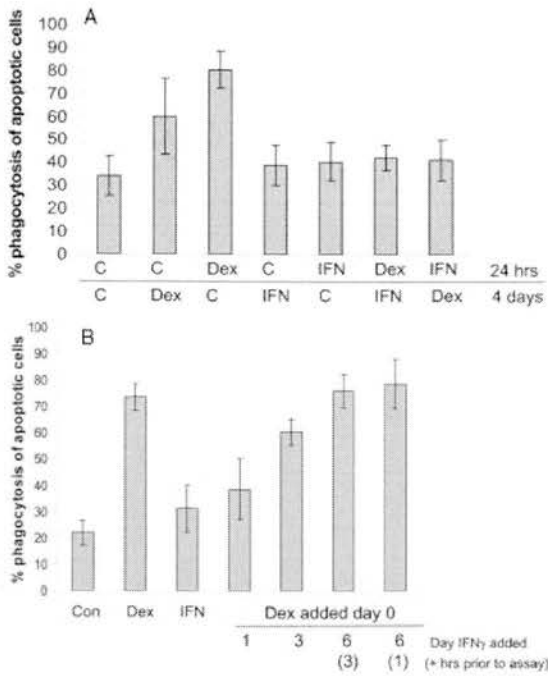


Fig. 6. IFN- γ reverses and blocks dexamethasone augmentation of MDM Φ phagocytosis of apoptotic cells. (A) Adherent monocytes were cultured for the first 24 h in IDMEM containing 10% autologous serum alone, dexamethasone or IFN- γ . Cells were washed and then treated for the remaining 4 days in IDMEM containing 10% autologous serum, dexamethasone or IFN- γ to give a checkerboard of combination treatments (24 h / 4 days). The phagocytic ability of the MDM Φ populations was then determined by flow cytometry as described in Sect. 4.5. Results are shown as the mean percentage of macrophage phagocytosis \pm S.E. from at least three separate experiments. Augmentation of MDM Φ phagocytic ability was observed following exposure to dexamethasone for the first 24 h only, or for the final 4 days of culture when compared with untreated MDM Φ . In contrast, exposure to dexamethasone followed by IFN- γ , or to IFN- γ prior to dexamethasone, resulted in a significant down-regulation in MDM Φ phagocytic ability, demonstrating that IFN- γ can both reverse and block the dexamethasone-augmented phagocytosis of apoptotic neutrophils. (B) Adherent monocytes were cultured in IDMEM containing 10% autologous serum and dexamethasone, with the subsequent addition of IFN- γ on day 1, or day 3, or 1 h or 3 h prior to the phagocytosis assay on day 6. The phagocytic ability of the MDM Φ populations was then determined by flow cytometry. Results are shown as mean percentage of macrophage phagocytosis \pm S.E. from at least three separate experiments. MDM Φ that were cultured in dexamethasone and exposed to IFN- γ on day 1 showed a suppression in phagocytic ability compared with MDM Φ treated only with dexamethasone. The addition of IFN- γ on day 3 led to only a slight reduction in phagocytic ability and MDM Φ exposed to IFN- γ for 1 h or 3 h only showed no inhibition in phagocytic ability.

reverse the augmentation of phagocytosis, suggesting that the inhibition was not due to a direct effect of IFN- γ -mediated signaling. Previous studies have also suggested that the cytokine environment may regulate macrophage phagocytosis of apoptotic cells. When human MDM Φ were given a 4-h exposure of IFN- γ after 4 days of culture, they displayed augmented phagocytosis of apoptotic cells [23]. In contrast, phagocytosis of apoptotic cells by rat bone-marrow-derived macrophages was inhibited by treatment for 48 h with IFN- γ [24]. Our data suggest that although IFN- γ -treated MDM Φ show some augmentation of phagocytosis of apoptotic cells, acquisition of a pro-phagocytic macrophage phenotype in response to GC is lost in the presence of this cytokine.

One important implication of this work is that GC may fail to stimulate macrophage phagocytosis in a Th1 environment. Thus, one of the potential beneficial effects of GC-treatment, *i.e.* facilitation of apoptotic cell clearance, may be inhibited by IFN- γ . Interestingly, a Th1 cytokine environment has been suggested to contribute to corticosteroid resistance in diseases like rheumatoid arthritis [25]. This may also impact on the use of IFN- γ to boost host defense against infection via macrophage activation following severe injury. Many trials of IFN- γ therapy have failed to show a clear improvement in patient outcome [26]. Whether IFN- γ inhibits the ability of endogenous GC to regulate macrophage phagocytosis is not known. Interestingly, expression of 11 β hydroxysterone dehydrogenase is rapidly induced during monocyte differentiation [27] concomitantly with acquisition of capacity for phagocytosis of apoptotic cells [28].

We have previously suggested that the altered adhesion observed in dexamethasone-treated MDM Φ may be linked to augmentation of phagocytic capacity. On the basis of morphological appearance, we would have predicted that co-incubation with either dexamethasone plus IFN- γ or dexamethasone plus IL-4 would give rise to a highly phagocytic phenotype. However, our data provide the first evidence for dissociation between morphology/adhesion status and phagocytic capacity. Thus, although cells treated with IFN- γ plus dexamethasone exhibit a small and rounded cell morphology without distinct podosome adhesion structures, the augmented phagocytic capacity of dexamethasone-treated macrophages is not shared by MDM Φ treated with IFN- γ plus dexamethasone.

For other characteristics of MDM Φ that we examined, dexamethasone exerted dominant effects over IFN- γ , inhibiting IFN- γ -driven pro-inflammatory cytokine production and morphological appearance. Expression of CD163 was up-regulated by dexamethasone even in the presence of IFN- γ and the IFN- γ -dependent up-

regulation of ICAM-1 and HLA-DR was attenuated by dexamethasone. Our phenotype analysis also revealed that the expression levels of the mannose receptor and CD14, both previously implicated as phagocytic receptors [29, 30], show no correlation with the increased phagocytic ability of dexamethasone-treated MDM Φ . The mannose receptor was down-regulated following GC treatment and CD14 showed no change in expression when compared with untreated MDM Φ , potentially excluding the involvement of these receptors in the augmented phagocytosis seen following GC treatment of MDM Φ .

Our data support the suggestion that monocytes treated with the GC differentiate to a phenotype distinct from the classically activated IFN- γ -treated MDM Φ or an "alternatively" activated IL-4-treated MDM Φ . We found that the mannose receptor, a well-defined marker of alternative activation [13], was expressed at higher levels on IL-4-treated MDM Φ , but was reduced on MDM Φ cultured in dexamethasone. Several other receptors exhibit differential expression on IL-4- and GC-treated MDM Φ , including HLA-DR, CD86 and CD163. MDM Φ that have differentiated in GC or IL-4 exhibit distinct phenotypes, the latter inducing a polarized appearance with many cellular processes that is very different from the "rounded" appearance of dexamethasone-treated cells. Most importantly in terms of capacity for clearance of apoptotic cells, IL-4 fails to induce a MDM Φ phenotype that is capable of efficient phagocytosis of apoptotic cells. IL-4-treated monocytes would have the potential for antigen presentation and immunomodulation (high HLA-DR and CD86), whereas dexamethasone-treated MDM Φ exhibit "anti-inflammatory" characteristics with reduced capacity for immunostimulation because of down-regulation of HLA-DR and CD86.

Although both IL-4 and GC may be involved in counteracting pro-inflammatory factors, their impact on macrophage function would have distinct consequences in the outcome of an inflammatory response. For example, discordant regulation of the capacity of macrophages for antigen presentation and apoptotic cell clearance may be critical for "safe" disposal of apoptotic and necrotic cells without the potential for "cross-presentation" of autoantigen and the induction of an autoimmune response. Together, these data suggest that the functional repertoire of IL-4- or dexamethasone-treated MDM Φ is distinct. GC promote a "tolerogenic" phenotype similar to that reported to be induced by IL-10 [31, 32]. Interestingly, like GC, IL-10 up-regulates expression of CD163, although the mechanism appears to be distinct [33]. Preliminary experiments indicate that treatment of monocytes with anti-IL-10 antibodies fails to block the development of the phenotype we observe fol-

lowing treatment with GC (S. J. Heasman, unpublished observations). However, IL-10-cultured MDM Φ show some augmentation of phagocytic capacity for apoptotic cells (data not shown), suggesting that there may be parallels in the mechanism of action of GC and IL-10.

In summary, the role that particular cytokines, steroids and lipid mediators play individually, and in concert, will critically determine the balance between pathogen clearance, resolution of inflammation, tolerance induction and wound healing. In particular, data presented here suggest that defining the interplay between GC and IFN- γ in the regulation of macrophage function may unveil novel therapeutic targets for treatment of inflammatory disease.

4 Materials and methods

4.1 Antibodies and other reagents

All chemicals were from Sigma (Poole, Dorset, GB) unless otherwise stated. Antibodies were used at saturating concentrations as determined by titration in indirect immunoassays and flow cytometry as follows: HLA-DR (clone WR18, IgG2a, used at 1/100, Serotec, Oxford, GB), CD14 (clone UCHM1, IgG2a, provided by Dr. Peter Beverley, Edward Jenner Institute for Vaccine Research, Compton, GB), C54 (clone 15.2, provided by Dr. Nancy Hogg, Cancer Research UK, London, GB), CD64 (clone 10.1, IgG1, used at 1/100, provided by Dr. Nancy Hogg), CD86 (clone BU63, IgG1, used at 1/50, Caltag, B-D Biosciences, GB), CD163 (clone Bermac, IgG1, used at 1/35, Dako, Oxford, GB), mannose receptor (clone 19.2, IgG1, Serotec), and IgG1 control (MOPC, mouse IgG1 plasmacytoma, obtained from ECACC, GB).

4.2 Cell isolation and culture

Mononuclear cells (MNC) and polymorphonuclear cells were isolated from human blood by dextran sedimentation and centrifugation over discontinuous Percoll™ (Amersham Pharmacia Biotech, Buckingham, GB) gradients as previously described [34]. The MNC (typically 15–20% monocytes by morphological analysis of cytocentrifuge preparations or flow cytometric determination on the basis of laser scatter properties and CD14 reactivity) were resuspended at 4×10^6 /ml in Iscove's modification of Dulbecco's modified Eagles medium (IDMEM; Life Technologies, Paisley, GB) and enriched for monocytes by selective adherence to 48-well (0.5 ml/well) or 6-well (4.0 ml/well) tissue culture plates for 1 h at 37°C in 5% CO₂. Adherent monocytes were washed three times and then allowed to differentiate for 5 days in IDMEM containing penicillin/streptomycin and 10% autologous serum prepared by recalcification of platelet-rich plasma. Washed adherent MDM Φ consisted of >90%

CD14-positive cells at day 5 as assessed by flow cytometry. In our hands GC did not induce monocyte apoptosis as reported by Schmidt et al. [35]. Our unpublished data suggest that autologous serum over-rides the pro-apoptotic effects of GC on monocytes. As detailed in the text, dexamethasone was added to a final concentration of up to 1 μM , whereas recombinant IL-4 and recombinant human IFN- γ (both obtained from R&D Systems, Minneapolis, USA) were used at 10 ng/ml.

Isolated polymorphonuclear cells (typically 95–98% neutrophils by morphological examination of cyto centrifuge preparations) were resuspended at 20×10^6 cells/ml cells in IDMEM and labeled with the fluorescent cell tracker dye carboxymethylfluorescein diacetate (CMFDA; Molecular Probes, Leiden, The Netherlands), 2 $\mu\text{g/ml}$ for 15 min at 37°C in 5% CO₂. Cells were then washed and cultured in 75 mm tissue culture flasks (Nunc, Fisher Scientific, Leicestershire, GB) for 18–24 h at 4×10^6 /ml in IDMEM containing 10% autologous serum to induce spontaneous apoptosis. Microscopic examination of nuclear morphology of cyto centrifuge preparations of cultured cells was used to assess levels of apoptosis and neutrophils were typically 50–60% apoptotic with 15–20% necrotic cells present, as determined by dual annexin V / propidium iodide staining and flow cytometry.

4.3 Flow cytometric determination of cytokine release

Supernatants from MDM Φ that had been cultured for 5 days were analyzed for the presence of IL-8, IL-6, IL-1 β , IL-10, TNF- α and IL-12p70 using the Human Inflammation BD Cytometric Bead Assay (Becton Dickinson, Oxford, GB) as described by the manufacturer's assay protocol. Briefly, 50 μl samples of cell supernatant were incubated with capture beads to the six cytokines and a PE cytokine detection reagent for 3 h at room temperature. Samples were then washed once and 6000 events were acquired using a FACS-Calibur flow cytometer (Becton Dickinson). Analysis of the median FL-2 fluorescence associated with bead populations defined by FL-3 labeling was made using Cellquest software (Becton Dickinson).

4.4 Flow cytometric analysis of surface receptor expression

MDM Φ obtained after 5 days of culture were washed in Hanks' balanced salt solution (HBSS) and incubated with 5 mM EDTA in Ca²⁺/Mg²⁺-free PBS on ice for 15 min to detach the cells from the wells. The detached MDM Φ were centrifuged at 220 \times g and washed in Ca²⁺/Mg²⁺-free PBS containing 0.2% BSA and 0.1% sodium azide (flow buffer). The pelleted cells were then incubated on ice for 30 min with saturating concentrations of monoclonal antibodies to cell surface determinants. The cells were then washed twice in flow buffer and incubated for a further 30 min on ice with FITC-labeled F(ab')₂ fragments of goat anti-mouse-

immunoglobulin (Dako; used at 1/50). Following two further washes in flow buffer, the labeled cells were analyzed using either an EPICS XL (Beckman Coulter, High Wycombe, GB) or FACSCalibur flow cytometer (Becton Dickinson) after acquiring 5000–8000 events per sample. Post-acquisition analysis was performed either using Cellquest (Becton Dickinson) or EXPO32 (Beckman Coulter).

4.5 Quantitation of phagocytosis of apoptotic cells

Monocytes were cultured for 5 days in 48-well plates as described above either in the presence of 10% autologous serum alone (control), or in the presence of dexamethasone, IL-4 or IFN- γ , or combinatorial treatments at the concentrations detailed in the text. MDM Φ were gently washed in Ca²⁺/Mg²⁺-free HBSS and phagocytosis of apoptotic neutrophils was then performed as described previously [36]. Briefly, MDM Φ were co-incubated with 0.5 ml of CMFDA-labeled apoptotic neutrophils at 4×10^6 /ml for 1 h at 37°C in 5% CO₂ after which the neutrophils were gently aspirated and 0.25 ml of trypsin/EDTA added. Following incubation at 37°C in 5% CO₂ for 15 min and then at 4°C for 15 min, MDM Φ were detached by vigorous pipetting. Flow cytometry was then used to determine the proportion of phagocytic macrophages (gated on the basis of forward- and side-scatter properties) and the percentage of FL-1-labeled MDM Φ was determined.

4.6 Indirect immunofluorescence analysis of macrophage cytoskeleton

Monocytes were plated on sterile coverslips in 24-well plates at 4×10^6 /ml. After 5 days of differentiation, adherent MDM Φ were fixed in 2 ml of 3% (w/v) para-formaldehyde at room temperature for 20 min. Coverslips were then washed three times in Ca²⁺/Mg²⁺-free PBS and free aldehyde groups were then quenched with 50 mM NH₄Cl/PBS at room temperature for 15 min. After three further washes in PBS, cells were permeabilized using 0.1% Triton X-100 for 4 min. Following a further three washes in PBS the coverslips were incubated for 10 min in heat-inactivated AB serum (1/10) to block non-specific antibody binding to Fc receptors. To visualize podosomes within MDM Φ , cells were then incubated on ice for 30 min with mAb specific for paxillin (BD Transduction Labs, Belgium). The cells were washed and then labeled by incubating for 30 min with an Alexa-488 goat anti-mouse-immunoglobulin antibody (1/400 in PBS; Molecular Probes). After a further three washes cells were incubated with rhodamine phalloidin (1/800 in PBS; Molecular Probes). The coverslips were then mounted onto slides using an antifadent mounting medium (Molecular Probes) and examined under oil immersion microscopy using a $\times 63$ objective using an Axiovert S100 immunofluorescence microscope with Coolsnap LCD camera and Openlab image acquisition software.

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MECHANISMS OF STEROID ACTION AND RESISTANCE IN INFLAMMATION

Glucocorticoid-mediated regulation of granulocyte apoptosis and macrophage phagocytosis of apoptotic cells: implications for the resolution of inflammation

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Abstract

Glucocorticoids represent one of the most effective clinical treatments for a range of inflammatory conditions, including severe acute inflammation. Although glucocorticoids are known to affect processes involved in the initiation of inflammation, the influence of glucocorticoids on the mechanisms by which acute inflammation normally resolves have received less attention. Apoptosis of granulocytes present at inflamed sites leads to their rapid recognition and internalisation by macrophages, a process which may be important for resolution of inflammation. However, if clearance of either eosinophils or neutrophils is impaired, these cells rapidly undergo secondary necrosis leading to release of pro-inflammatory mediators from the phagocyte, potentially prolonging inflammatory responses.

Physiologically relevant concentrations of glucocorticoids accelerate eosinophil apoptosis whilst delaying neutrophil apoptosis during *in vitro* culture. Here we discuss key pathways regulating the granulocyte apoptotic programme and summarise the effects of glucocorticoids on monocyte differentiation and the consequent changes to apoptotic cell clearance capacity. Definition of the mechanisms underlying resolution of inflammatory responses following glucocorticoid treatment may unveil new targets for modulation of inflammatory disease, allowing co-ordinated augmentation of granulocyte apoptosis together with increased macrophage capacity for clearance of apoptotic cells.

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Introduction

Whilst the effects of glucocorticoids on the events associated with the initiation of inflammation have been studied extensively (Pitzalis *et al.* 2002, Webster *et al.* 2002), the influence of glucocorticoids on the mechanisms by which acute inflammation normally resolves have received less attention. Over the past few years, we have been studying the process of resolution of inflammation, hypothesising that definition of the underlying mechanisms may allow development of new therapeutic approaches aimed at promoting the safe resolution of inflammatory responses which underlie a heavy burden of disease in the lung and other organs (Haslett *et al.* 1994) (see Fig. 1). It is now clear that neutrophil granulocytes undergo constitutive apoptosis (Savill *et al.* 1989a) at inflamed sites, a process associated with the 'disabling' of their potentially injurious

secretion responses and other effector functions including adhesion and phagocytosis (Whyte *et al.* 1993a). Surface molecular alterations, including marked down-regulation of expression of FcγRIII (Dransfield *et al.* 1994), L-selectin and uncoupling of integrins of the β2 family (Dransfield *et al.* 1995), contribute to attenuation of functional responsiveness. In addition, apoptosis-associated cell surface alterations, including phosphatidylserine exposure (Homburg *et al.* 1995), are thought to provide cues that lead to rapid recognition and internalisation of apoptotic cells by macrophages (Savill *et al.* 1989b). Importantly, phagocytic clearance of apoptotic granulocytes, in contrast to other phagocytic pathways, fails to promote the release of pro-inflammatory mediators from macrophages (Meagher *et al.* 1992), which may be important for the 'normal' resolution process. In addition, our studies have shown that macrophages (Savill *et al.* 1990, 1992)

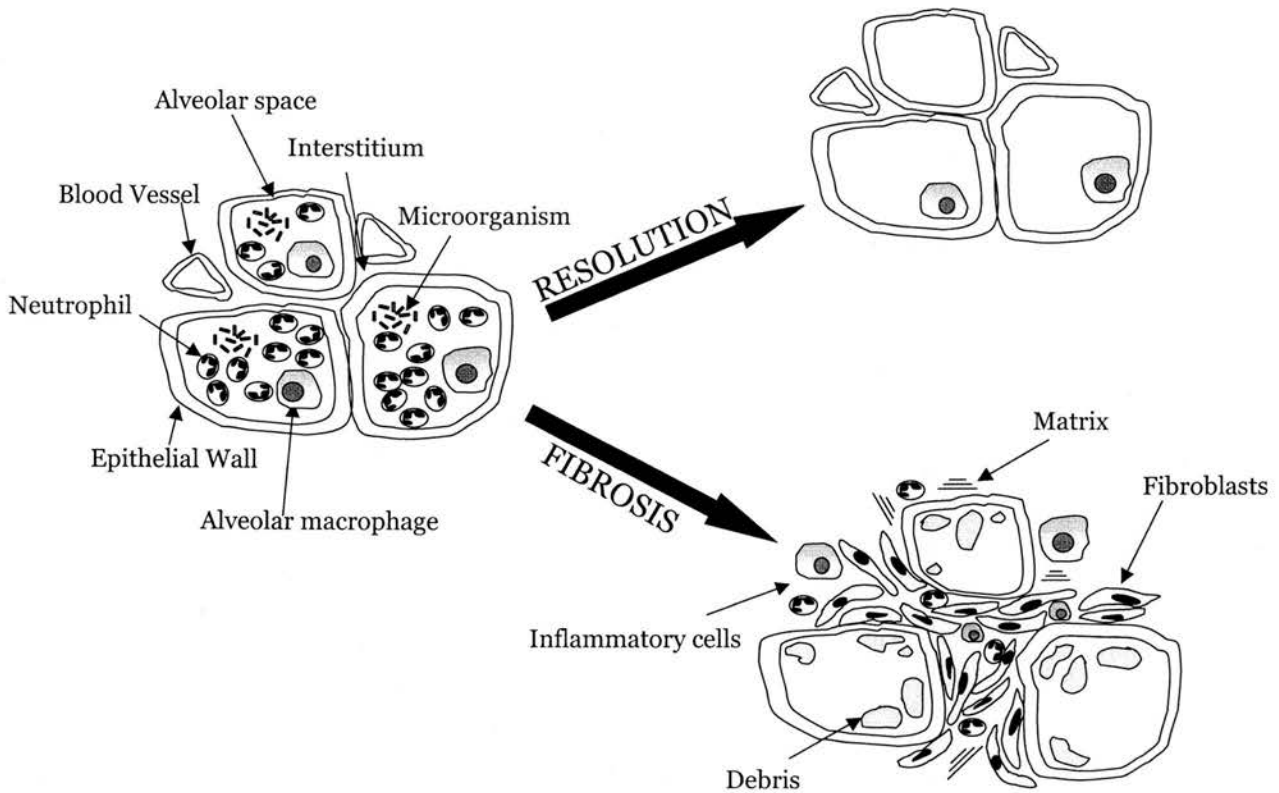


Figure 1 Schematic representation of cellular changes associated with either resolution of inflammation or development of fibrosis. Microbial infection is accompanied by infiltration of alveoli with inflammatory cells, predominantly neutrophils. Resolution of inflammation is associated with removal of microbial infection and return of tissue architecture to normal. In contrast, failure to resolve inflammation results in chronic recruitment, matrix deposition and fibroblast migration and proliferation, leading to loss of gas exchange capacity.

subsequently emigrate from the inflamed site to draining lymph nodes (Bellingan *et al.* 1996), where they may have the potential to influence activation of cells of the acquired immune system. Similarly, eosinophil granulocytes, which have been strongly implicated in tissue injury in allergic acute inflammation, are also programmed to die by constitutive apoptosis. Whilst the rate of constitutive eosinophil apoptosis is slower than that of the neutrophil granulocyte, apoptosis also leads to rapid, non-inflammatory phagocytic clearance by macrophages (Stern *et al.* 1992). However, if clearance of either eosinophils or neutrophils is impaired, these cells rapidly undergo secondary necrosis. In addition to the release of potentially toxic intracellular contents associated with necrosis, one important consequence is that macrophage phagocytosis of post-apoptotic cells leads to the release of pro-inflammatory mediators, potentially prolonging inflammatory responses (Stern *et al.* 1996).

Granulocyte deletion by apoptosis has been shown to be amenable to external regulation by lineage-specific inflammatory signals (Haslett *et al.* 1991, Stern *et al.* 1992, Lee *et al.* 1993, Whyte *et al.* 1993b, Hannah *et al.* 1995, Murray *et al.* 1997, Ward *et al.* 1997, Coxon *et al.* 1999,

Hofman *et al.* 2000), providing an opportunity for targeted therapeutic intervention. However, if triggering of apoptosis is to be considered as a therapeutic target, failure to match the apoptotic cell load to the tissue clearance capacity at an inflamed site may have deleterious consequences in terms of resolution of inflammation. Macrophage capacity for phagocytosis of apoptotic granulocytes can be rapidly regulated by exogenous factors e.g. following ligation of extracellular matrix receptors such as CD44 (Hart *et al.* 1997, McCutcheon *et al.* 1998) or prostaglandin receptors (Rossi *et al.* 1998). Thus, co-ordinated acceleration of granulocyte apoptosis at inflamed sites together with augmentation of macrophage capacity to clear apoptotic cells may be a realistic therapeutic goal (Ward *et al.* 1999a).

Glucocorticoids (GCs) represent one of the most effective clinical treatments for a range of inflammatory conditions, including severe acute inflammation. GCs have profound effects, both on granulocyte apoptotic programmes (Cox 1995) and macrophage phagocytic function (Liu *et al.* 1999). In particular, physiologically relevant concentrations of GCs, acting via the GC receptor, were found to *accelerate* eosinophil apoptosis whilst

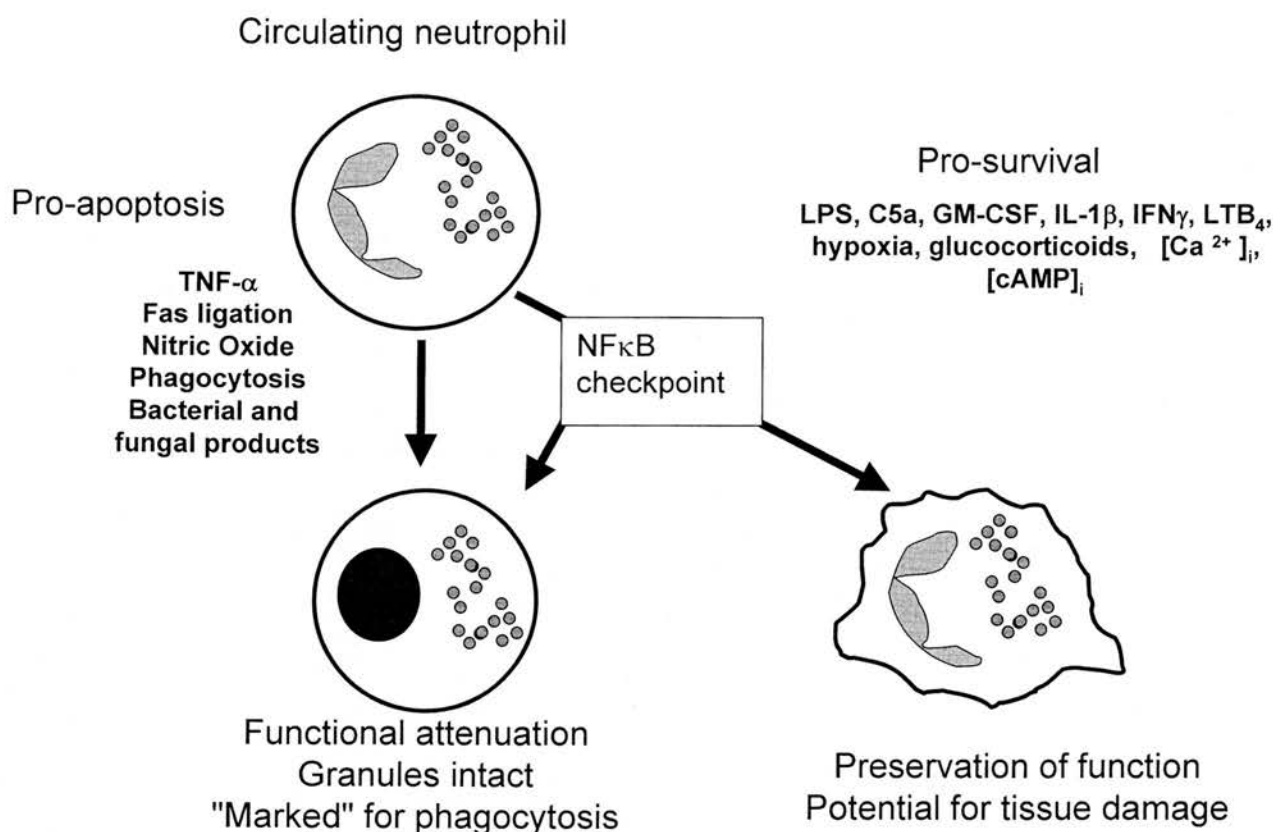


Figure 2 Neutrophils are poised on a 'knife edge' decision of life or death. A range of extracellular stimuli can promote apoptosis or survival. For those stimuli that drive survival, NF κ B activation acts as a checkpoint that can result in engagement of cell death pathways, leading to functional downregulation and preservation of membrane integrity. Apoptotic cells can then be swiftly cleared by phagocytes. IFN γ , interferon γ . LTB-4, leukotriene B4.

delaying neutrophil apoptosis during *in vitro* culture (Meagher *et al.* 1996). In this article, we will discuss key pathways regulating the granulocyte apoptotic programme following treatment with GCs, and describe recent data relating to the effects of GC on monocyte differentiation and the consequent changes to apoptotic cell clearance capacity.

Granulocytes are poised on a knife edge of nuclear factor kappaB (NF κ B)-mediated survival

It is now well established that at an inflammatory site there are many cytokines and growth factors present that provide survival signals for granulocytes with the potential to over-ride granulocyte apoptotic pathways (see Fig. 2). We have demonstrated important apoptosis-inhibiting effects of inflammatory stimuli that increase cyclic AMP levels in neutrophils (Rossi *et al.* 1995). The dramatic delay of the caspase-dependent apoptosis in neutrophils by dibutyryl cAMP (dbcAMP) was found to occur via a novel protein kinase A (PKA)-independent signalling pathway involving

maintenance of mitochondrial potential (Martin *et al.* 2001). The survival effects of dbcAMP were independent of phosphatidylinositol-3 kinase (PI3K) and MAP kinase (MAPK) activation and our data point to a novel, reversible, transcriptionally independent mechanism of action of dbcAMP that may provide opportunities to shift the balance of pro-apoptotic and anti-apoptotic proteins and hence accelerate clearance of granulocytes from inflamed sites.

One of the most potent agents known to modulate granulocyte apoptosis is bacterial lipopolysaccharide (LPS) (Lee *et al.* 1993). Most inflammatory cells sense LPS using a complex system that involves the interaction of LBP/CD14/MD-2 and Toll-like receptor 4 (TLR4) (O'Neill & Dinarello 2000, Triantafilou & Triantafilou 2002) receptors which, when engaged, trigger inter-related signal transduction pathways, including the MAPK (ERK1/2, JNK and p38), PI3K and NF κ B pathways to orchestrate innate immune responses. Detailed investigation of the role of NF κ B in control of granulocyte survival has revealed that inflammatory mediators such as LPS and granulocyte macrophage colony stimulating factor

(GM-CSF) downregulate susceptibility of neutrophils to Fas-directed death (I Murray, S O'Dea, D Harrison, C Haslett & A G Rossi, unpublished data) implying that specific pro-apoptotic regulatory pathways are overridden by NF κ B signalling pathways (Fig. 2). Although both tumour necrosis factor- α (TNF- α) and LPS act to trigger NF κ B activation in neutrophils, TNF α -mediated activation of NF κ B delays apoptosis in the majority of neutrophils not induced into early apoptosis (Murray *et al.* 1997, Ward *et al.* 1999b). The pronounced effects of protein synthesis inhibitors upon granulocyte survival supports the suggestion that NF κ B-directed gene transcription and protein synthesis of anti-apoptotic factors and powerful cytokines delays apoptosis in 'inflammatory' granulocytes.

We have demonstrated that the rate of constitutive apoptosis in both neutrophils and eosinophils was greatly accelerated by the NF κ B inhibitor and fungal metabolite gliotoxin. This effect was reproduced using other NF κ B inhibitors and suggests TNF- α -induced activation of NF κ B and production of survival proteins limits pro-apoptotic effects and may delay apoptosis at later time points. Similarly, for human eosinophils exposed to TNF- α , cytoplasmic levels of I κ B α , the inhibitory subunit of NF κ B are rapidly reduced and NF κ B is mobilised from the cytoplasm to the nucleus (Fujihara *et al.* 2002). Inhibition of TNF- α -mediated I κ B α degradation and NF κ B activation by gliotoxin treatment of eosinophils reveals caspase-dependent pro-apoptotic properties of TNF- α . Selective inhibition of eosinophil NF κ B activation may therefore represent an alternative target for inducing specific deletion of eosinophils in diseases including asthma and allergic rhinitis.

NF κ B-dependent genes may also have a key regulatory role in the pathways responsible for the metabolism of prostaglandins (PGs) in granulocytes. Although many natural prostaglandins (e.g. PGE₂, PGA₂, PGA₁, PGF_{2 α}) act either to delay apoptosis or have no effect, PGD₂ and its metabolite PGJ₂ selectively induced eosinophil apoptosis (Ward *et al.* 2002). In contrast, the sequential PGD₂ metabolites Δ^{12} PGJ₂ and 15 dPGJ₂ were found to induce caspase-dependent apoptosis in both eosinophils and neutrophils. Despite Δ^{12} PGJ₂ and 15 dPGJ₂ being known activators of peroxisome proliferator-activated receptor (PPAR)- γ , apoptosis was not mimicked by synthetic PPAR- γ and PPAR- α ligands nor blocked by an irreversible PPAR- γ antagonist, suggesting a PPAR- γ -independent mechanism (Ward *et al.* 2002). We found that Δ^{12} PGJ₂ and 15 dPGJ₂ inhibited LPS-induced I κ B α degradation and NF κ B activation, thereby triggering apoptosis. The powerful pro-apoptotic effects of Δ^{12} PGJ₂ and 15 dPGJ₂ in both eosinophils and neutrophils implies that differences in the ability of eosinophils and neutrophils to process and degrade prostaglandins may be responsible for the differential effects of PGD₂ upon granulocyte survival.

One potential limitation to the effectiveness of GCs in treatment of inflammatory diseases is that they undesirably prolong neutrophil survival (Cox 1995), increasing the potential for secretion of pro-inflammatory granule contents during inflammatory episodes. We believe that definition of mechanisms by which glucocorticoid-directed survival of neutrophils may be 'disengaged' may improve the efficacy of GCs in neutrophilic inflammatory diseases. Our preliminary data indicate that glucocorticoid-mediated delay of neutrophil apoptosis is reversed by inhibition of protein synthesis and inhibited by blockade of NF κ B (C Ward & A G Rossi, unpublished data). We suggest that GCs engage NF κ B-directed synthesis of 'survival proteins' that may be targeted to make neutrophils respond to GCs in the same way that eosinophils do.

Macrophages can be enabled for phagocytosis of apoptotic granulocytes

Apoptotic cells have potentially toxic cellular contents and autoantigens may be revealed or generated within apoptotic cells. Thus, defects in clearance of apoptotic cells would be predicted to be associated with spontaneous and/or persistent inflammatory responses and evidence of autoimmunity to intracellular antigens (Lorenz *et al.* 2000, Beutler 2001, Botto 2001, Greidinger 2001, Stuart & Hughes 2002). In support of this suggestion, spontaneous/persistent tissue inflammation and autoimmunity is observed in mutant mice with proven and probable defects in clearance of dying cells (Botto *et al.* 1998). Indeed, some patients with systemic lupus erythematosus exhibit (as yet uncharacterised) defects in macrophage phagocytosis of apoptotic cells (Baumann *et al.* 2002). As discussed above, we would predict that upregulation of macrophage capacity for 'safe' phagocytosis of apoptotic granulocytes will represent an important aspect of therapeutic strategies aimed at promoting the resolution of inflammation.

We and others have shown that macrophage phagocytosis may be rapidly modulated in response to extracellular environmental signals (Fig. 3). For example, elevation of intracellular cAMP in human monocyte-derived macrophages using the cell permeable cAMP analogue, db-cAMP, specifically reduced the phagocytosis of apoptotic neutrophils without affecting FcR-mediated phagocytosis (Rossi *et al.* 1998). Treatment of macrophages with PGE₂ resulted in rapid, transient increases in levels of intracellular cAMP and induced PKA-dependent morphological alterations indicative of changes in the adhesive status of the macrophage, including cell rounding and disassembly of 'podosome' adhesion structures containing actin, vinculin and talin that represent points of contact with extracellular matrix (Rossi *et al.* 1998). Consistent with the suggestion that adhesive interactions may influence

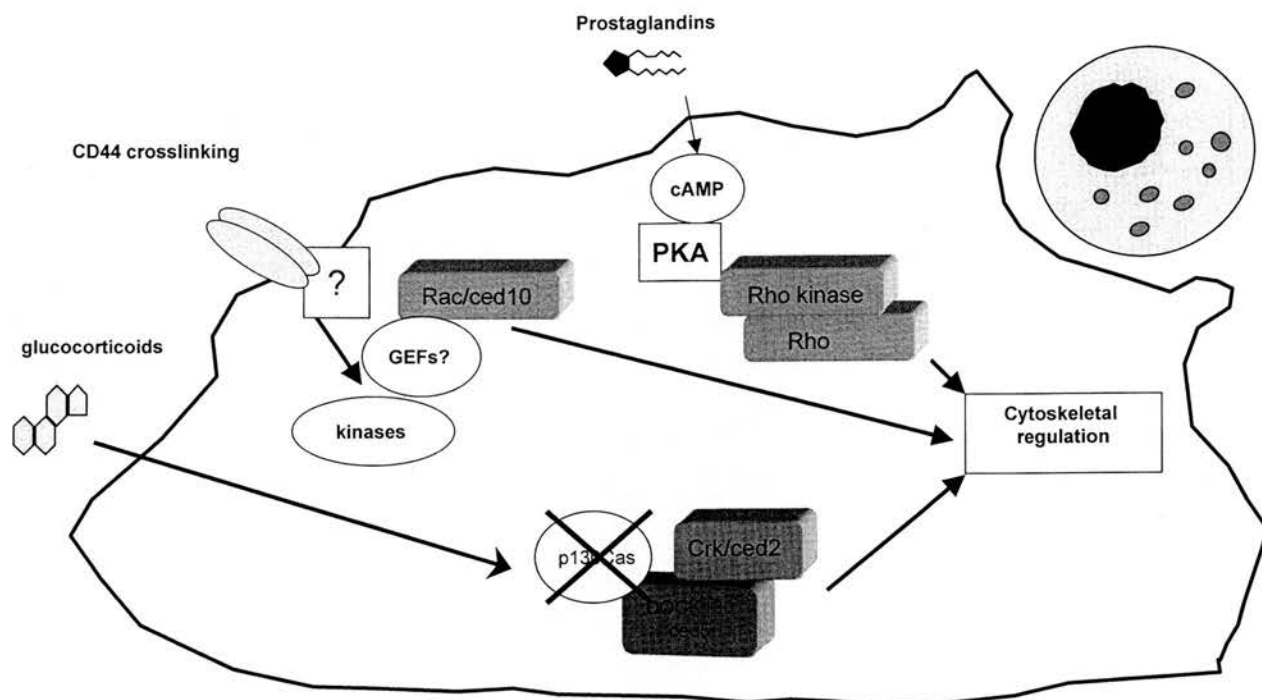


Figure 3 Schematic representation of mechanisms that regulate macrophage phagocytosis. Cross linking of CD44/matrix receptors or engagement of lipoxin receptors leads to rapid augmentation of phagocytic activity. In contrast, binding of prostaglandins to specific receptors causes cytoskeletal changes that inhibit phagocytic function. Alternatively, glucocorticoids act via glucocorticoid receptors to alter the pattern of gene expression (via either specific transactivation or transrepression) to alter cytoskeletal regulation within macrophages.

macrophage phagocytic capacity, we demonstrated that ligation of the matrix receptor CD44 rapidly and specifically increases apoptotic neutrophil internalisation (Hart *et al.* 1997). We now have very clear evidence that CD44 cross-linking is associated with the generation of intracellular signals that specifically augment clearance of apoptotic neutrophils. First, binding of Fab' fragments of CD44 antibodies does not promote phagocytosis, indicating that these reagents do not mask sites that are normally involved in negatively regulating cellular interactions in a manner analogous to the sialomucin, CD43. Furthermore, there does not appear to be 'capping' of CD44 within the membrane e.g. within uropod-like structures following cross-linking. Detailed temporal analysis of the CD44 cross-linking effects provides evidence that CD44 acts as an 'enabler' of macrophage phagocytosis, recruiting otherwise non-responsive cells. Recent data from studies of lung injury in CD44-deficient mice adds further weight to a role for CD44 in the regulation of macrophage clearance of apoptotic neutrophils in the resolution of inflammation (Teder *et al.* 2002). Our preliminary evidence indicates that engagement of specific signal transduction events following CD44 cross-linking leads to rapid changes in cytoskeletal regulation. We are currently investigating whether CD44 initiates signals that influence cytoskeletal

regulatory molecules e.g. membrane recruitment of Rac GTPase via guanine nucleotide exchange factors (GEFs) such as Tiam1 which have been shown to associate with CD44 (Bourguignon *et al.* 2000).

Glucocorticoids facilitate clearance of apoptotic cells, favouring resolution of inflammation

In contrast to the rapid effects of CD44 ligation, [cAMP]_i elevation, or lipoxins (Godson *et al.* 2000), we found that exposure of macrophages to GCs for 24 hours specifically enhanced the uptake of apoptotic leukocytes by both human and murine macrophage populations (Liu *et al.* 1999). These observations establish the capacity of GCs to promote phagocytosis of cells undergoing apoptosis, raising the possibility that anti-inflammatory effects of GCs may involve pro-phagocytic effects. Importantly, glucocorticoid-mediated enhancement of macrophage phagocytosis of apoptotic cells was not achieved by costly loss of the teleologically appropriate lack of pro-inflammatory response, failing to stimulate monocyte chemoattractant protein-1 (MCP-1) production and down-regulating interleukin (IL)-8 release by the phagocyte (Liu *et al.* 1999).

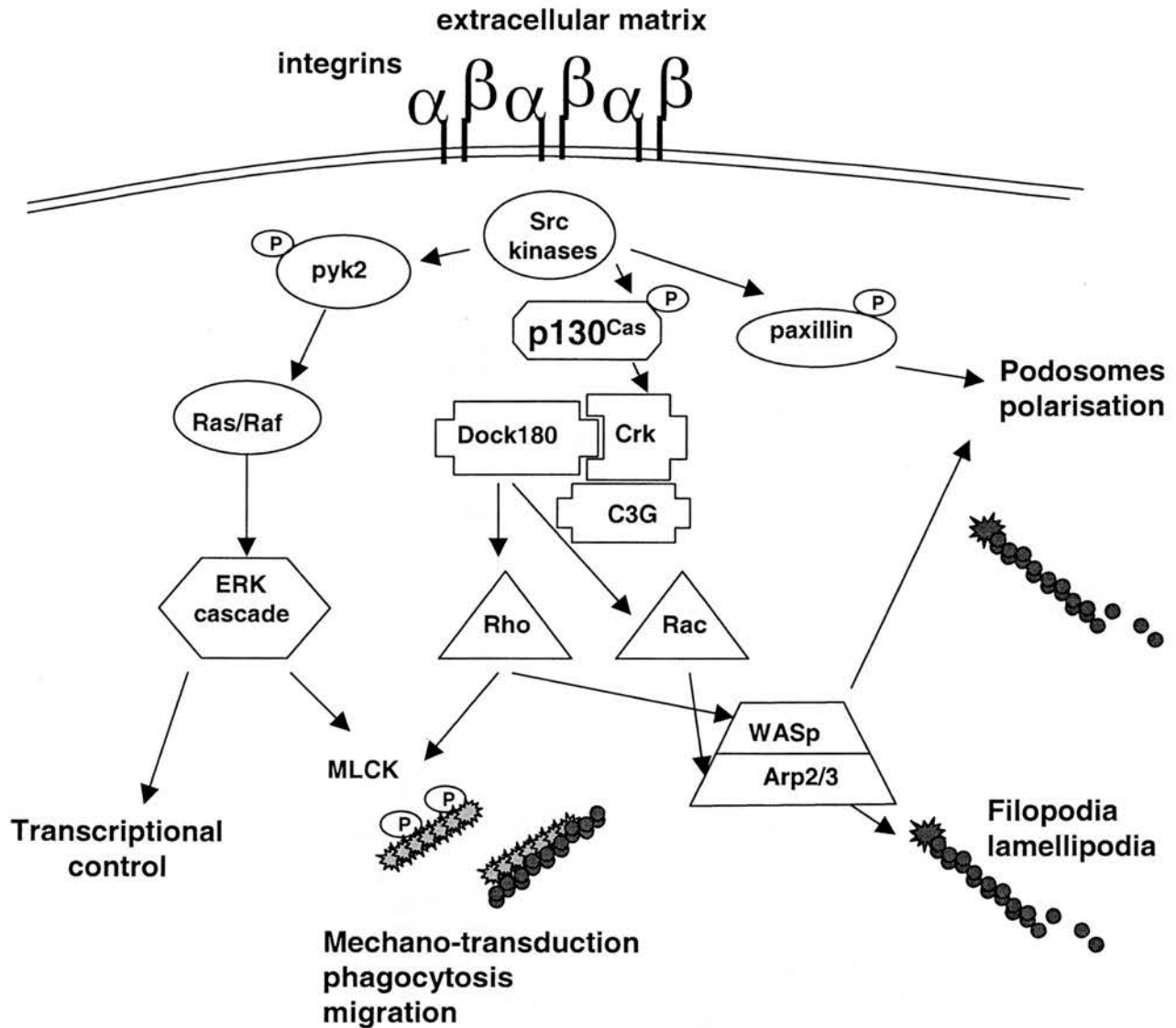


Figure 4 A central role for p130^{Cas} in adhesion signalling. Schematic representation of events occurring downstream of integrin adhesion receptor ligation. p130^{Cas} links integrin-mediated src family kinase activation to Rho family GTPases and cytoskeletal assembly necessary for adhesion, migration and phagocytosis. MLCK, myosin light chain kinase.

More recently, we have found that exposure of peripheral blood monocytes to GCs during the first 24 hours of the 5-day culture period induced a highly phagocytic monocyte-derived macrophage (MDM ϕ) phenotype. This GC-MDM ϕ phenotype was characterised by a marked morphological appearance, consisting of smaller, more 'rounded' cells with more homogeneous laser scatter properties in flow cytometric analysis (Giles *et al.* 2001). Functional and morphological homogeneity was matched by cell surface phenotype, including specific induction of expression of the haemoglobin scavenger

receptor, CD163 following GC treatment. Our data indicate that GCs acting via GC receptors have the potential to re-programme monocyte differentiation towards an 'anti-inflammatory' phenotype. In light of recent studies of apoptotic cell clearance in *Caenorhabditis elegans* (Ellis *et al.* 1991, Liu & Hengartner 1998, Wu & Horvitz 1998a,b, Chung *et al.* 2000, Reddien & Horvitz 2000, Gumienny *et al.* 2001, May 2001), we next examined key intracellular components that regulate cytoskeletal coupling following adhesion. Alterations in the morphology of GC-MDM ϕ were mirrored by changes in

cytoskeletal organisation, with a loss of paxillin and actin containing podosome structures. Tyrosine phosphorylation of paxillin and pyk2, proteins that are recruited to adhesion contacts, were not phosphorylated in GC-MDM ϕ . A particularly striking change was that GC-MDM ϕ showed decreased expression of p130^{Cas} (Giles *et al.* 2001), an adaptor protein that links integrin receptors to Rho family GTPases and the MAPK pathway (see Fig. 4). Reduced expression of p130^{Cas} would be predicted to disrupt Crk/DOCK180/ELMO complexes, which together with reduced phosphorylation of paxillin and pyk2 may have implications for control of the turnover of adhesion structures in macrophages.

Specific recruitment of p130^{Cas} to focal contacts following adhesion to matrix may mimic loss of p130^{Cas} observed in dexamethasone-treated cells and thus influence the availability of other components to drive cytoskeletal re-organisation necessary for phagocytosis. We propose that the repertoire of adhesion receptors that are engaged on the macrophage surface might therefore control phagocytic potential indirectly by releasing or sequestering key regulatory molecules like p130^{Cas} from focal adhesion complexes. Time-lapse video microscopy analysis revealed that despite the small rounded appearance of GC-MDM ϕ these cells are able to rapidly extend and retract cellular processes. Thus, although recruitment of proteins such as paxillin to podosome adhesion signalling complexes does not occur in the absence of p130^{Cas}, Rac may still drive the extension and retraction of processes required for phagocytosis. One possibility is that other p130^{Cas}-like adaptors such as HEF1 and Efts/Sin, present in macrophages may allow the recruitment of Rac/Crk/DOCK180 specifically to membranes in a manner that facilitates phagocytosis of apoptotic cells and possibly other particles. Importantly, these data raise the possibility that expression or phosphorylation of p130^{Cas} may have a negative regulatory role upon macrophage phagocytic potential. Our recent studies examining the effect of the cytokine environment on GC-mediated monocyte differentiation have shown that the Th1 cytokine interferon- γ reverses the augmentation of phagocytosis seen in the GC-MDM ϕ (K M Giles, S J Heasman & I Dransfield, unpublished data). The reduction in phagocytic ability is not accompanied by morphological changes, indicating that adhesion status and the capacity for phagocytosis can be dissociated. Further understanding of the interplay between cytokine environment and GCs in inflammation may allow the tailoring of therapies that facilitate the resolution of inflammatory disease.

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