Aston University
Birmingham

DOCTORAL THESIS

The role of the innate immune system in the clearance of apoptotic cells

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The Role of the Innate Immune System in the Clearance of Apoptotic Cells

By

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For the degree of

Doctor of Philosophy

Aston University

December 2011

Aston University

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Leanne Thomas
Doctor of Philosophy
2011

Thesis Summary

Rapid clearance of dying cells is a vital feature of apoptosis throughout development, tissue homeostasis and resolution of inflammation. The phagocytic removal of apoptotic cells is mediated by both professional and amateur phagocytes, armed with a series of pattern recognition receptors that participate in host defence and apoptotic cell clearance. CD14 is one such molecule. It is involved in apoptotic cell clearance (known to be immunosuppressive and anti-inflammatory) and binding of the pathogen-associated molecular pattern, lipopolysaccharides (a pro-inflammatory event). Thus CD14 is involved in the assembly of two distinct ligand-dependent macrophage responses. This project sought to characterise the involvement of the innate immune system, particularly CD14, in the removal of apoptotic cells. The role of non-myeloid CD14 was also considered and the data suggests that the expression of CD14 by phagocytes may define their professional status as phagocytes.

To assess if differential CD14 ligation causes the ligand-dependent divergence in macrophage responses, a series of CD14 point mutants were used to map the binding of apoptotic cells and lipopolysaccharides. Monoclonal antibodies, 61D3 and MEM18, known to interfere with ligand-binding and responses, were also mapped. Data suggests that residue 11 of CD14, is key for the binding of 61D3 (but not MEM18), LPS and apoptotic cells, indicating lipopolysaccharides and apoptotic cells bind to similar residues. Furthermore using an NF-kB reporter, results show lipopolysaccharides but not apoptotic cells stimulate NF-kB. Taken together these data suggests ligand-dependent CD14 responses occur via a mechanism that occurs downstream of CD14 ligation but upstream of NF-κB activation. Alternatively apoptotic cell ligation of CD14 may not result in any signalling event, possibly by exclusion of TLR-4, suggesting that engulfment receptors, (e.g. TIM-4, BAI1 and Stablin-2) are required to mediate the uptake of apoptotic cells and the associated anti-inflammatory response.

Acknowledgements

Completing my PhD has been a challenging journey, to say the very least. However I would not have been able to complete this journey without the aid and support of a countless number of people over the past three years. First and foremost I am grateful to my supervisor Andrew Devitt for providing me with this opportunity. Andrew's excitement for research, and scientific discussion, has allowed me to develop an understanding of the project and an appreciation of research.

I owe a great deal of gratitude to Chris Dunston for his patience, emotional support and encouragement throughout my PhD. His passion for science and sense of humour has enriched my growth as a student, a researcher and as a scientist.

I am also extremely grateful to Dave Nagel, his support and kind nature have instilled in me a true appreciation of molecular biology and therefore have contributed to the scientist I am today.

I wish to acknowledge a truly talented and hard working scientist, David Gardner, his inquisitive nature; kindness, support and scientific discussion have been indispensable.

I am thankful for my friends, Rachel Willitts, Kamila Pytel, Anne Bielemeier, Charlie Bland and especially Lois Hawkins for their friendship, support and the many cups of tea and cake we shared when times were particularly difficult.

To Jonathan, who knows nothing about macrophages but with whom I share my life, your endless love, emotional support and infectious sense of humour have paved the road to my success. I aspire to be like you in so many ways as a person and as a scientist.

Finally, I'd like to thank my parents, especially my mom for instilling in me, the confidence, and drive for pursuing my aims, no matter how high. Many times when the future looked uncertain or my goals have seemed so far out of my reach, she used the cliché, you wash an elephant "a little bit at a time".

Thank you

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Abbreviations

ABCA1	ATP binding-cassette-transporter 1
AC	Apoptotic cell
ACAMPs	Apoptotic cell-associated molecular patterns
AGEs	Advanced glycated end-products
APLT	Aminophospholipid translocase
ATP	Adenosine triphosphate
BAI1	Brain-specific angiogenesis inhibitor 1
BSA	Bovine serum albumin
cAEM	Airway epithelial growth medium
cDMEM	Complete DMEM
cDNA	Complementary DNA
cHPFM	Human pulmonary fibroblast growth medium
CR3/CR4	Complement receptor 3 and 4
CRP	C-reactive protein
cRPMI	Complete RPMI
cDMEM	Complete DMEM
CXCR4	Chemokine receptor 4
DAPI	4',6-diamidion-2-phenylindole, dihydrochloride
dH_2O	Distilled water
DAMPs	Damage associated molecular patterns
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl suphoxide
DNA	Deoxyribonucleic acid
DS	Double stimulated (VD3/PMA)
EDTA	Ethylenediaminetetra-acetic acid disodium salt
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GAS-6	Growth arrest-specific 6
GDF5	Growth differentiation factor 5
GPI	Glycosyl-phosphatidylinositol
HBS	Hepes buffered saline
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesilfonic acid]
HMDM	Human monocyte-derived macrophages
HMGB1	High-mobility group box-1 protein
HPF	Human pulmonary fibroblast
HRP	Horseradish peroxidise
HSP	Heat shock protein
ICAM-3	Intercellular adhesion molecule 3
IFN	Interferon
IL	Interleukin
LB	Luria broth
LBP	Lipopolysaccharide binding protein
LDL	Low-density lipoproteins
LOX-1	Oxidized low-density lipoprotein particle 1
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related protein
LT1	IT transLT1 transfection reagent
mAb	Monoclonal antibody
MBL	Mannose-binding lectin

mCD14	Membrane CD14
MCS	Multiple cloning sites
MFG-E8	Milk fat globule epidermal growth factor
mRAGE	Membrane RAGE
MyD88	Myeloid differentiation primary response gene (88)
NEAA	Non-essential amino acids
Neu	Neuraminidases
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Normal goat serum
NHS	Normal human serum
OD	Optical density
OPD	O-Phenylenediamine dihydrochloride
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PBS-T	PBS containing tween
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	phycoerythin
PI	Propidium Iodide
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PS	Phoshatidylserine
PSA	Prostate-specific antigen
PSR	Phoshatidylserine receptor
RAGE	Receptor advanced glycated end-products
SAP	Serum amyloid protein
sCD14	Soluble CD14
SDS	Sodium dodecyl sulphate
sfRPMI	Serum-free RPMI
siRNA	Short interfering RNA
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SP-A	Surfactant protein A
SP-D	Surfactant protein D
SR-A	Class A Scavenger receptor
sRAGE	Soluble RAGE
SR-B	Class B scavenger receptor
SRP	Serum amyloid P component
TGF-β	Transforming growth-factor β
TIM-4	T-cell immunoglobulin and mucin-containing molecule 4
TLR	Toll-like receptor
TNF-α	Tumour-necrosis factor
Tris	Tris(hydroxymethyl)methylamine
TSP-1	Thombospondin-1
Tween 20	Polyoxyethylene sorbitan monolaurate
UTP	Uridine 5’-triphosphate
VD3	Dihydroxyvitamin D3
WtCD14	Wildtype CD14

Introduction

The importance of cell loss in tissue homeostasis and embryonic development followed widespread acceptance in the early twentieth-century, that cells undergo programmed cell death (Lockshin and Williams, 1964 & Kerr *et al.,* 1972). Lockshin and Williams (1964) proposed that cell loss during development was not purely accidental but followed a controlled sequence of events. Kerr *et al.* (1972) went on to describe the uniform and morphological feature of this programmed mode of cell death and coined the term apoptosis. Apoptosis is of Greek origin and means "falling or dropping of leaves", with this analogy emphasizing the loss of cells as integral part of the cell life cycle. Kerr *et al.*(1972) distinguished apoptosis from the other mode of cell death; necrosis. Necrosis has no morphological features and is the result of tissue damage, which causes the cell membrane to rupture and the intracellular contents is be released (Kerr *et al.,* 1972). Kerr *et al.*(1972) was first to describe two stages of apoptosis that are physiological inseparable. The first stage starts with the initiation of cell death and ends with the fragmentation of the cell into membrane-bound vesicles termed apoptotic bodies. While the second stage culminates in the phagocytic clearance of the apoptotic bodies from the environment. The clearance of apoptotic cells is mediated by both neighbouring amateur phagocytes (epithelial cells and fibroblast) and professional phagocytes (macrophages and dendritic cells). Professional phagocytes are motile, highly phagocytic and uptake apoptotic cells upon contact. In contrast, amateur phagocytes are resident in tissues and are considered to delay phagocytosis upon contact with the apoptotic cell. The delay between contact and uptake of apoptotic cells by amateur phagocytes would suggest that further changes to the surface of the apoptotic cell are required to permit uptake (Parnaik *et al.,* 2000). At normal rates of apoptosis, cellular corpses are rarely observed *in vivo* as they are rapidly cleared by neighbouring amateur phagocytes. However professional phagocytes are considered to 'step in' when amateur phagocytes are overwhelmed to prevent secondary necrosis.

1.1 The importance of dealing with death

Today apoptosis is viewed as an active, protective mechanism that ensures the safe and rapid removal of cellular corpses. It is vital to maintain and regulate cell populations ensuring a balance between cell birth, from mitosis or differentiation and cell death. The importance of apoptosis in the developing embryo was recognised early, with the controlled removal of cells vital for tube formation, bone modelling along with separation of the digits and formation of the sex organs (reviewed in Vaux and Korsmeyer, 1999).

Apoptosis is also important for defence, as viral infected cells are rapidly removed limiting reproduction and spread of virus particles (Vaux and Korsmeyer, 1999).

For many years the focus of apoptosis was on the mechanisms and processes involved in dismantling the cells and the production of apoptotic bodies. Unlike necrosis, the clearance of apoptotic cells was found to suppress inflammation therefore modulating immune responses. However failed clearance is considered to result in cells lysis and subsequently uptake of apoptotic cell debris by dendritic cells, which provides an opportunity for antigen presentation and the generation of autoimmunity (reviewed by Peng and Elkon, 2011). The importance of the final stage of apoptosis, the phagocytic removal of apoptotic cells, is really highlighted in the autoimmune disease systemic lupus erythematosus (SLE). Deficiency in molecules involved in the clearance of apoptotic cells such as C1q, and Mer tyrosine kinase receptor, results in SLE, characterised by secondary necrosis and the production of auto-antibodies to intracellular antigens (Savill and Fadok, 2000). This led to the assumption that failed clearance results in the re-exposure of auto-antigens that drives autoimmunity in SLE (Casciola-Rosen *et al.,* 1994). A body of evidence has led to the generalisation that apoptotic cells are rich sources of auto-antigens. Cocca *et al.*(2002) suggests that the safe and efficient clearance of apoptotic cells by phagocytes is a tolerogenic process that prevents secondary necrosis and generation of autoantibodies, often associated with inflammation associated with SLE. The importance of removing apoptotic neutrophils in the resolution of inflammation is demonstrated in the pathogenesis of a group of vasculitides, characterised by the presence of anti-neutrophil cytoplasmic autoantibodies (Savill *et al.,* 2002). These arise following the release of auto-antigens from secondary necrotic neutrophils including myeloperoxidase and proteinase 3 (Ciavatta *et al.,* 2010).

Furthermore in some diseases, such as atherosclerosis, failed clearance is involved in the disease course following the initial onset. In atherosclerosis the capacity of macrophages to engulf apoptotic cells is decreased and the subsequent lack of clearance contributes to the formation of the necrotic core in vulnerable atherosclerotic plaques (Reviewed by Moore and Tabas, 2011). Defective clearance may also play a role in neurodegenerative diseases such as Parkinson's and Alzheimer's in which the accumulation of apoptotic cells is observed (reviewed by Elliott and Ravichandran, 2010). While this may be associated with increased cell death, the immunological consequences of failed clearance within the central nervous system is currently unknown. Inappropriate clearance can result in the loss of cells

that are required, for example loss of neurones observed in neurodegenerative diseases (Kao *et al.*, 2011). Therefore, an understanding of the mechanisms involved in the clearance of apoptotic cells has provided an understanding of autoimmunity associated with defective clearance and highlights potential manipulation for emerging therapies.

1.2 Programmed cell death

Our current understanding of the recognition and phagocytic clearance of apoptotic cells comes largely from *in vitro* studies with inhibitors that block phagocytic recognition of apoptotic cells, or, *in vivo* studies with *Caenorhabditis elegans* and the genetic defects that prevent clearance. These studies identified the gene *ced-3* as necessary for loss of 131 somatic cells by apoptosis during nematode development (Jacobson *et al.*, 1997 & Lu *et al.*, 2009). The mammalian ortholog was revealed to be IL-1β-converting enzyme otherwise known as caspase-1 (Jacobson *et al.*, 1997). Caspases are a family of cysteine proteases which function as the executioners of the apoptosis programme (Hengartner, 2000).

The characteristic morphological changes accompanying apoptosis, described by Kerr *et al.*(1972) are mediated by activation of a caspase-cascade. There are two categories of caspases based on structural similarities, initiator caspases which have protein interaction sites allowing self-activation and effector caspases are responsible for the morphological features of apoptosis (reviewed by Hengartner, 2000). Caspases which are produced as inactive zymogens and following induction of apoptosis are activated, in a cascade with initiator caspases activating effector caspases.

There are two apoptotic signalling pathways that result in caspase activation. The 'extrinsic' pathway is activated following ligation of a death receptor with TNF-α family members, for example, the FAS-ligand, which results in the assembly of a death-inducing signalling complex that activates initiator caspases (Scoltock and Cidlowski, 2004). However ligation of FAS also leads to cytochome-*c* released from mitochondria which results in caspases activation (Scoltock and Cidlowski, 2004). The 'intrinsic' pathway is activated by cellular stress and results in the assembly of the apoptosome which also activates initiator caspases (Scoltock and Cidlowski, 2004).

Effector caspases such as 3 and 6 are responsible for the early nuclear events seen during apoptosis (Hengartner, 2000). Caspase-3 is responsible for DNAase activation and

cleavage of nuclear lamin that facilitates DNA fragmentation and permits packaging into membrane-bound bodies subsequently leading to nuclear shinkage (Rao *et al.,* 1996 & Buendia *et al.,* 1999). There are a number of factors cleaved by caspase-3 that are important for detachment and dismantling of the cell, for example gelsolin, actin and catenin (Kothakota *et al.,* 1997& Lane *et al.,* 2005).

One of the major characteristic features of apoptosis is the breakup of the cell into apoptotic cell bodies a process known as blebbing. Membrane blebbing is considered to be produced following contraction of the cell during cleavage of the cytoskeleton (Coleman *et al.,* 2001). Coleman *et al.*(2001) suggested that caspase-mediated activation of ROCK I, a Rho effector kinase, is responsible for membrane blebbing, packaging and relocation of nuclear fragments into membrane-bound vesicles. It is these events of the apoptosis programme that advertise the status of the cell to surrounding phagocytes and facilitates the definitive stage, clearance by alteration of dying cell surface.

1.2.1 Apoptotic cells are dressed to impress: 'eat me' signals

It is well established that apoptotic cells display molecular markers known as 'eat me' signals that are required for phagocyte recognition and subsequent clearance. While relatively few 'eat me' signals have successfully been identified, it is accepted that these molecules can newly appear at the surface of the cell following induction of apoptosis. Known 'eat me' signals include, the externalization of phoshatidylserine (PS) and possibly the modification of already existing cell surface molecules such as ICAM 3, however such cell surface changes need to be accompanied by the loss of molecules known as 'don't eat me' signals.

1.2.1.1 Phoshatidylserine (PS)

The best characterised 'eat me' signal is the translocation of PS from the inner leaflet of the plasma membrane (Fadok *et al.,* 1998a & Krahling *et al.,* 1999). During the activation of the apoptotic programme membrane asymmetry is disrupted, resulting in the translocation of PS to the outer-leaflet of the plasma membrane, a well-defined characteristic of apoptotic cells (Fadok *et al.,* 1998a & Krahling *et al.,* 1999). In viable cells PS is restricted to the inner leaflet of the plasma membrane by an ATP-dependent aminophospholipid translocase (APLT) (Fadok *et al.,* 1998a & Krahling *et al.,* 1999). During execution of the apoptotic cell programme the APLT is inactivated and a non-specific lipid flippase is activated causing translocation of PS down a concentration

gradient to the cell surfaces within a few hours of apoptosis (Fadok *et al.,* 1998a & Krahling *et al.,* 1999). However is PS exposure alone enough to induce the phagocytic clearance of apoptotic cells? Hoffmann *et al.*(2001) found that PS exposure alone was insufficient to mediate the phagocytic uptake of apoptotic cells, suggesting that additional phagocyte receptors were required to tether the apoptotic cell to the phagocyte prior to PS-dependent uptake. This would suggest that other 'eat me' signals are required for apoptotic cell interaction with phagocytes.

1.2.1.2 Modification of viable cell surface molecules

Other 'eat me' signals may include 'functional' modification of already existing proteins such as intracellular adhesion molecule 3 (ICAM-3) present on viable cells. ICAM-3 is a member of the Ig-like superfamily expressed only on leukocytes (de Fougerolles *et al.,* 1993) and is required for T cell adhesion and activation on antigen presenting cells (Starling *et al.,* 1995 & Bleijs *et al.,* 2000). However Moffatt *et al.*(1999) suggested that following induction of apoptosis, ICAM-3 becomes modified and takes on a new role, functioning as an 'eat me' signal required for apoptotic cell adhesion in the clearance of apoptotic leukocytes. However the loss of 'don't eat me' signals may underlie the role of ICAM-3 in the clearance of apoptotic cells.

1.2.1.3 Redistribution events

The redistribution of ligands found on viable cells, are also considered as potential 'eat me' signals that can stimulate engulfment following induction of apoptosis. Calreticulin is found on the surface of most viable mammalian cells, suggested to bind thombospondin forming a co-complex with lipoprotein receptor-related protein (LRP) and induce the disassembly of focal adhesion points (Orr *et al.,* 2003, Gardai *et al.,* 2005). However following the induction of apoptosis, calreticulin expression is upregulated and redistributed to localized regions with exposed PS. Calreticulin then binds directly to LRP receptor; a highly conserved engulfment receptor that mediates the clearance of apoptotic cells (Gardai *et al.,* 2005).

Another example of a molecule that is redistributed to the cell surface following induction of apoptosis is Annexin 1 found within the inner leaflet of the cellular membrane on viable cells (Parente and Solito, 2004). During apoptosis annexin 1 is externalized and recruited to patches on the cell membrane associated with PS (Arur *et al.,* 2003) however the phagocyte receptor for annexin 1-mediated clearance is currently unknown.

1.2.1.4 Removal of Sialic acid

The surface of cells is covered in sugars which are covalently linked to membrane proteins or lipids following golgi modification. Cell surface sugars include sialic acid, which is highly negatively charged and considered the principal source of membrane charge (Weiss and Mayhew, 1967). Lutz (2004) reported that a loss of sialic acid at the surface of senescent erythrocytes may cause a reduction in membrane charge which promoted clearance. Another more recent study by Meesmann *et al.*(2010) found that desialylation of apoptotic cells also enhanced phagocytic clearance. However, no significant increase in the uptake of viable cells following removal of sialic acid was observed (Meesmann *et al.*, 2010). Therefore it seems more likely that a loss of the negative charge at the surface of apoptotic cells allows phagocyte interaction with other, discussed 'eat-me' signals. This is supported by Lutz (2004) hypothesised a reduction in membrane charge along with loss of CD47; a recognised 'don't eat me' signal which is required for clearance of erythrocytes.

1.2.2 'Don't eat me'

It is likely that the presence of 'eat me' signals are not completely absent from viable cells (Wu *et al.,* 2006 & Ravichandran, 2010). However it is currently considered that viable cells present 'don't eat me' signals at their surface which allows phagocytes to distinguish viable and apoptotic cells. These signals prevent uptake, often by mediating detachment from phagocytes but may also function by hiding 'eat me' signals present.

1.2.2.1 CD31

CD31 is a member of the immunoglobulin superfamily expressed by viable leukocytes and is involved in leukocyte migration (Muller *et al.,* 1993). Brown *et al.*(2002) found that homophilic ligation of CD31, on macrophages mediated detachment. During apoptosis the detachment signal induced by CD31 is lost and replaced with an attachment signal so the leukocyte remains tethered to the phagocyte awaiting uptake (Brown *et al.,* 2002). This again could suggest that the loss of CD31 may physically uncover already existing 'eat me' signals present at the surface of the apoptotic cells.

1.2.2.2 CD47

CD47 is an integrin-associated membrane protein expressed by most cells (Oldenborg *et al.,* 2000). CD47 was first considered as a marker of 'self' as erythrocytes taken from CD47 knockout mice are rapidly removed when placed in wild-type mice (Oldenborg *et al.,* 2000). This supports the suggestion that 'eat me' signals are present on viable cells, but the presence of 'don't eat me' signals, such as CD47 prevent uptake. CD47 on viable cells

interacts with macrophage surface protein, SIRPα and induces an inhibitory signal which prevents phagocytosis and the synthesis of proinflammatory cytokines (Oldenborg *et al.*, 2000, Brown and Frazier, 2001 & Gardai *et al.*, 2005). However during apoptosis CD47 is redistributed away from 'eat me' signals such as PS and cells lose their ability to activate SIRPα and are therefore engulfed (Brown and Frazier, 2001 & Gardai *et al.*, 2005).

1.2.2.3 CD46

Another potential 'don't eat me' signal is a loss of complement protection factor, CD46, expressed by all nucleated cells. Elward *et al.*(2005) found that a loss of CD46 during apoptosis, from various cell types resulted in C3b deposition and there by enhanced clearance. Again this supports suggestions that 'don't eat me' signals potentially mask eat me signals present on viable cells or sterically hinder phagocytes interacting with such markers. Furthermore apoptosis results in the caspase-dependent formation of membrane-associated microparticles that were found to contain CD46 (Elward *et al.*, 2005). This suggested that following apoptosis, CD46 was relocated from the surface of the apoptotic cell into apoptotic blebs.

1.2.3 To eat or not to eat?

Taken together these findings suggest that the presence of 'eat me' signals alone on apoptotic cells is not sufficient to induce phagocyte internalization. The combined appearance of 'eat me' signals with the loss or redistribution of viable 'don't eat me' signals permit the binding and engulfment of apoptotic cells by phagocytes. Findings here also suggest that viable cells have stipulated 'eat me' signals present and changes in membrane charge or loss of 'don't eat me' signals that may also function as 'self' markers, induce phagocytic clearance. Therefore cells appear to be constantly under phagocyte surveillance and have to prove that they are worthy of not being engulfed. With respect to host defence, the importance of this mechanism may be fully appreciated when cells are damaged, diseased, or senescent, prior to becoming less functional or dysplastic. However during the early stages of apoptosis, soluble factors are redistributed into microparticles. The microparticles are released into the local area and selectively function to induce phagocyte migration towards sites of apoptosis and potentially prime phagocytes for the meal ahead.

1.2.4 Flirting with phagocytes: 'Find me' signals

At normal rates of cellular turnover in tissues, free apoptotic cells are rarely observed *in situ* suggesting that the number of apoptotic cells and the capacity of phagocytes for uptake

are balanced. This led to the conclusion that apoptotic cells could advertise their cellular status prior to any morphological feature of apoptosis. Furthermore disruption of this balance, for example, by an increase in the level of apoptosis which exceeds the capacity of amateur phagocytes results in the recruitment of professional phagocytes. Lauber *et al.*(2003) and Truman *et al.*(2004) provided evidence that apoptotic cells secrete caspase-dependent soluble factors that selectively induce mononuclear phagocyte migration and are considered as 'find me' signals. Several lipid, protein and nucleotide based 'find me' signals have now been characterised of varying molecular sizes (reviewed by Ravichandran, 2010). 'Find me' signals are considered to be released and establish a chemoattractive gradient that guides phagocyte migration. However there is some debate over the range at which 'find me' signals can induce migration. Short range 'find me' signals are considered to act on local tissue residing phagocytes while long range 'find me' signals may recruit mononuclear phagocytes from the circulation (reviewed by Gregory and Pound, 2010). The range of 'find me' signals seems to be dependent on the tissue concentration and the rate of degradation.

1.2.4.1 Phospholipids

Phospholipids including lysophosphatidylcholine and sphingosine-1-phosphate have been shown to act as soluble mediators, able to induce migration of mononuclear phagocytes towards apoptotic cells (Lauber *et al.,* 2003; Gude *et al.,* 2008). In addition more complex lipid-based mediators play a role in inducing this chemotaxis. Microparticles are plasma membrane-derived vesicles, released early during apoptosis which express surface markers derived from the apoptotic cell (Segundo *et al.,* 1999,Mause and Weber, 2010 & Torr *et al.,* 2011). With a diameter ranging from 100nm to 1000nm (Mause and Weber, 2010 & Torr *et al.,* 2011) these particles are capable of diffusing through the extracellular matrix and interacting with macrophages. These particles are considered to induce chemotaxis (Segundo *et al.,* 1999) via their complement of adhesion factors and classical chemoattractants such as S1P. In addition microparticle-associated fractalkine has been shown to play a key role in recruiting phagocytes to sites of B-cell apoptosis *in vivo* (Truman *et al.,* 2008). Fractalkine is a type I transmembrane protein that acts as a chemokine and intracellular adhesion molecule that is associated with inflammatory processes within the central nervous system (Truman *et al.,* 2008). Truman *et al.*(2008) suggested that intracellular processing of fractalkine; most likely in a caspase-dependent manner resulted in its release associated with microparticles, produced during the blebbing phase of apoptosis. Inhibition of fractalkine or blockade of its macrophage receptor was

found to result in the inhibition of macrophage migration towards apoptotic cells (Truman *et al.*, 2008).

1.2.4.2 ATP/UTP

Recently, Elliott *et al.*(2009) reported the caspase-dependent release of soluble heat-stable mediators that induced migration of primary monocytes. Further analysis led to the discovery that enzymatic removal of ATP and UTP from apoptotic supernatants inhibited monocyte migration *in vivo* (Elliott *et al.*, 2009). ATP and UTP are released from the apoptotic cell via Pannexin-1 membrane channels (Chekeni *et al.*, 2010) and signal via interaction with the P2Y receptor. Furthermore Elliott *et al.* (2009) found that concentrations similar to physiological concentrations of ATP and UTP mediated monocyte migration *in vitro*. Due to the relative instability of these nucleotides and their potential uptake by neighbouring cells it may be speculated that nucleotides function to fine-tune migration over short ranges close to their target. Nucleotides at high concentrations are considered to promote inflammation, by functioning as damage-associated molecular patterns (DAMPs), however lower concentrations are thought to suppress inflammation (Elliott *et al.*, 2009). This supports the suggestion that apoptotic cells regulated recruitment of mononuclear phagocytes to sites of cell death therefore preventing unnecessary neutrophil infiltration and inflammation that potentially results in autoimmunity. This was supported by Chen *et al.*(2006) that reported neutrophils release ATP to enhance neutrophil migration as a positive feedback mechanism therefore associating ATP release with inflammation. However apoptotic neutrophils are considered to maintain membrane integrity for longer than most cells, in an attempt to avoid the release of ATP (Reviewed by Bratton and Henson, 2011). This may reduce nucleotide concentration and consequently prevent neutrophil migration to sites of cell death.

1.2.5 ‘Keep out’ signals

The absence of neutrophils and the presence of mononuclear phagocytes is often considered to define normal sites of cell death from sites of necrotic cell death (Smith, 1994, Bratton and Henson, 2011). Truman *et al.*(2004) found that apoptotic Burkitt lymphoma cells induce migration of monocytes and macrophages but not neutrophils. Considered as the first recruited line of cellular defence; neutrophils, are rapidly recruited following initiation of an innate immune response (Smith, 1994). Following phagocytosis neutrophils, use a combination of reactive oxygen species, proteolytic enzymes and anti-microbial peptides to kill (Smith, 1994). However neutrophil also damage surrounding

tissue and secrete cytokines and chemokines that regulate the inflammatory response and the adaptive immune response. Therefore neutrophils have been implicated in the pathology of many chonic inflammatory conditions. Under normal conditions neutrophil migration is regulated by signals that induce and inhibit neutrophil migration (Smith, 1994). Apoptotic cells are considered to negatively regulate neutrophil migration by secreting lactoferrin, a 'keep out' signal (Bournazou *et al.,* 2009). Bournazou *et al.*(2009) found that lactoferrin selectively inhibited neutrophil migration towards apoptotic cells in a dose-dependent manner.

Lactoferrin is an anti-microbial polypeptide released from neutrophils during inflammation (Elass-Rochard *et al.,* 1998) and has many anti-inflammatory and regulatory roles (Ward *et al.,* 2005). Elass-Rochard *et al.*(1998) found that lactoferrin competed with LBP for binding to LPS therefore inhibiting binding to CD14 and preventing proinflammatory cytokine release. Lactoferrin was found to bind to specific lactoferrin receptors present on neutrophils and inhibit cytoskeleton rearrangement that permits the contraction and retraction of neutrophils during migration (Bournazou *et al.,* 2009). It seems logical to consider that a combination of 'keep out' and 'find me' signals induce monocyte migration while preventing migration of neutrophils. Thus ensuring at each level of apoptotic cell clearance process the immune response is orchestrated and regulated. However it is also reasonable to consider that 'find me' signals could also function to prime macrophages for apoptotic cell uptake. This is supported by Truman *et al.*(2004) that found monocyte migration towards apoptotic cells was associated with the upregulation of CD14, a known tethering receptor for apoptotic cell (Devitt *et al.,* 1998 Devitt *et al.,* 2004). Furthermore fractalkine released from apoptotic cells was found to induce the upregulation of milk fat globule epidermal growth factor (MFG-EGF)(Truman *et al.,* 2008) which acts as an apoptotic cell bridging molecule that facilitates clearance (Hanayama *et al.,* 2002).

1.3 Apoptotic cell clearance: A four-course meal

The interaction between an apoptotic cell and its phagocyte, culminating in clearance, is a complex and multistage process. Hoffmann *et al.*(2001) initially described the clearance process in two stages. An initial tethering phase that shows significant redundancy as demonstrated by the persistence of apoptotic cells in the absence of inflammation in mice deficient in MBL or CD14 (Devitt *et al.,* 2004 & Stuart *et al.,* 2005). Subsequently followed by as a PS-mediated phagocytosis stage (Hoffmann *et al.,* 2001). Studies since have found that additional receptors are capable of inducing signalling for phagocytosis

including the receptor complexes calreticulin/CD91 and vitronectin/CD36 (Krieser and White, 2002 & Akakura *et al.*, 2004). Since the discovery of 'find me' signals, 'eat me' signals and a subsequent requirement for the loss of 'don't eat me' signals, an initial recognition event prior to tethering is accepted (Brown and Frazier 2001, 2002 & Grimsley, 2003). Lucas *et al.*(2006) found that TGF-β alone was insufficient to inhibit pro-inflammatory cytokine release following apoptotic cell uptake. Lucas *et al.*(2006) proposed the "kiss & tell" model, suggesting that phagocytes require additional signals provided by contact with the apoptotic cells to respond to TGF-β. Therefore phagocytes must first bind apoptotic cells, not only to promote phagocytosis and the release of TGF-β, but also to respond to TGF-β released. Taken together with the two stage model proposed by Hoffman, these suggested cell-surface signalling events were required to induce uptake along with an anti-inflammatory response. Gregory and Devitt (2004) have described the clearance process from the perspective of the macrophage, as a four stage process (figure 1): 1. Recognition, 2. Tethering, 3. Signalling events results in anti-inflammatory response and cytoskeletal rearrangements thus permitting 4. Subsequent phagocytosis of the cellular corpse.

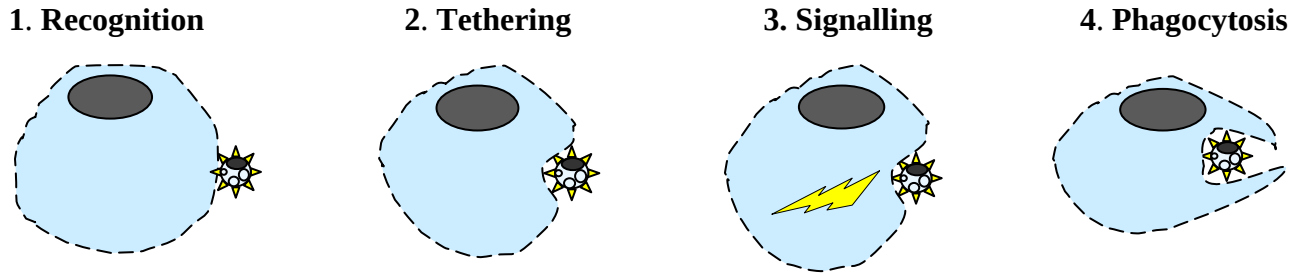

Figure 1: The four stages of apoptotic cell clearance
The interaction between an apoptotic cell and its phagocyte culminating in clearance is a complex and multistage process. From the perspective of the macrophage, the clearance of apoptotic cells is considered to occur in four stages: 1. Recognition of 'eat me' signals at the surface of the apoptotic cell which results in, 2. Tethering of the apoptotic cell to the phagocyte. 3. Induction of signalling events which initiates a characteristic anti-inflammatory response associated with apoptotic cell clearance along with cytoskeletal rearrangements which facilitates 4. phagocytosis of the cellular corpse.

1.4 Involvement of the innate immune system in the clearance of apoptotic cells

Phagocytes are considered to use a series of pattern recognition receptors (PRR) and soluble bridging molecules (figure 2), well characterised within host defence, to recognise and remove apoptotic cells (Savill *et al.*, 2002 & Monks *et al.*, 2005). Within their role in host defence, PRRs recognise evolutionarily conserved pathogen-associated molecular

patterns (PAMPs). However it is widely accepted that the innate immune system also has a role in apoptotic cells clearance suggesting that the molecular structures displayed by apoptotic cells and PAMPs share similarities. This poses the hypothetical questions: did pathogens evolve surface patterns that resemble apoptotic cells with the aim of avoiding immune surveillance or to infect other cells? Or did apoptotic cells evolve to expose ligands recognised by the innate immune system to facilitate clearance?

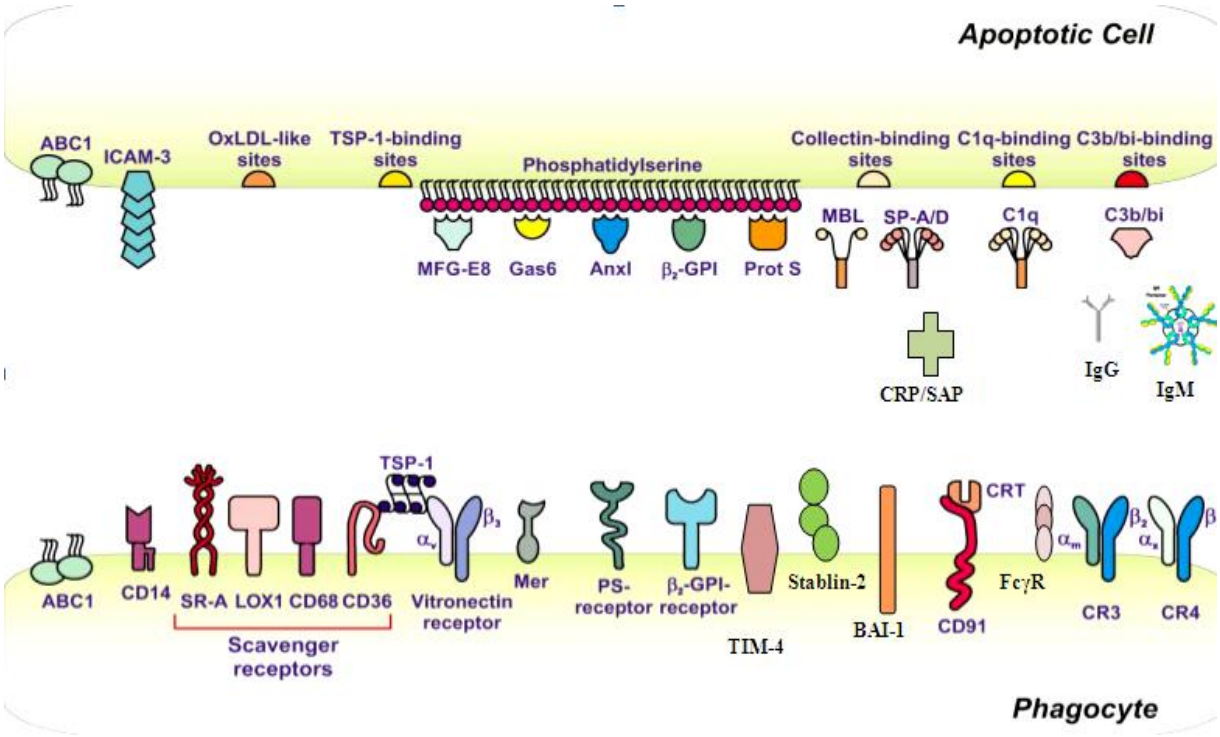

Figure 2: The super macrophage and the perfect apoptotic cell
Apoptotic cells present 'eat me' signals that interact directly or indirectly with phagocytes. Soluble pattern recognition molecules may bind to apoptotic cells as bridging molecules which then interact with phagocyte receptors. These include members of the collectins family, SPA and SPD, surfactant proteins A and D, members of the complement cascade; C1q and C3b, members of the pentraxin, C-reactive protein (CRP) and serum amyloid protein (SAP), Gas-6, growth arrest-specific 6; MFG-E8, milk fat globule-epidermal growth factor-8 and thombospondin (TSP-1). Phagocyte-associated PRRs can interact directly or indirectly with the apoptotic cell include integrin receptors; the vitronectin receptor ($\alpha_v\beta_3$) and $\alpha_v\beta_5$, complement receptor 3 and 4(CR3 & CR4) scavenger receptors; scavenger receptor A (SR-A), oxidized low-density lipoprotein particle 1 (LOX-1), CD68, and class B scavenger receptor B, CD36;CD91, CD14, FC-gamma receptor (FcγR,); phoshatidylserine receptor (PSR); β2-glycoprotein receptor, (β2GPI-R); tyrosine-kinase receptor, Mer, T-cell immunoglobulin and mucin-containing molecule, TIM family, Brain-specific angiogenesis inhibitor, BAI-1 and Stablin-2. A*dapted from Lauber et al., 2004.*1.4.1 Soluble bridging molecules and their phagocyte receptors

1.4.1.1 Complement-mediated clearance

Complement components can form a biochemical cascade that provides defence against pathogens and serves to induce inflammation, opsonise pathogens for phagocytosis or directly kill following assembly of a membrane attack complex. C1q is a member of the

collectin family and forms part of the classical complement cascade implicated in apoptotic cell clearance (Korb and Ahearn, 1997). Furthermore apoptotic cell-associated C1q is known to interact with the phagocyte receptor complex calreticulin/CD91 (Ogden *et al.*, 2001). However the importance of C1q, in the clearance of apoptotic cells, is really highlighted in the development of the autoimmune disease SLE (Kirschfink *et al.,* 1993). SLE is associated with failed clearance as a result of C1q deficiency and results in chronic inflammation and a loss of tolerance caused by the generation of autoantibodies.

The alternative complement pathway is also involved in the clearance of apoptotic cells as C3b binds to PS exposed at the surface of apoptotic cells. Apoptotic cell-associated C3 is then rapidly cleaved to form C3bi which interacts with the integrin receptors CR3 and CR4 (Mevorach *et al.,* 1998).

MBL, a member of collectin family is present in serum and binds pathogen-associated carbohydrates, activating the lectin-complement pathway and again associated with apoptotic cell clearance (Stuart *et al.,* 2005). Furthermore Stuart *et al.*(2005) reported that MBL-deficient mice showed reduced clearance of apoptotic cells *in vivo* without inflammation. This supported findings by Devitt *et al.* (2004) that suggest the binding of apoptotic cells to phagocytes could be uncoupled from the anti-inflammatory effects of clearance.

Other collectin family members include lung surfactant proteins A (SP-A) and D (SP-D) that also bind apoptotic cells and facilitate clearance in the lungs. Furthermore SP-A, SP-D and MBL are known to bind CD14 and modulate LPS responses (Chiba *et al.,* 2001). With respect to apoptotic cell clearance, SP-A, SP-D and MBL interact with the receptor complex calreticulin/CD91 to mediate uptake (Ogden *et al.*, 2001).

C-reactive protein (CRP) is a member of the pentraxin family known to bind polysaccharides and phosphocholine in pathogen-membranes and activate the classical complement pathway. Gershov *et al.*(2000) reported that CRP binds to apoptotic cells and enhances complement-mediated clearance while preventing assembly of membrane-attack complex protecting against lysis and proinflammatory consequences. Serum amyloid P (SAP) is another member of the pentraxin family associated with complement-mediated clearance of apoptotic cells.

1.4.1.2 Antibodies

IgM antibodies have been shown previously to bind apoptotic cells and function as opsonins that promote complement-dependent clearance (Quartier *et al.,* 2005). Antibodies have specific roles within the adaptive immune response tailored to foreign antigens. Antibodies function to neutralize and opsonise pathogens which facilitates activation of the classical complement pathway and enhances removal by effector cells. Quartier *et al.*(2005) found that natural IgM antibodies were required for C1q-dependent C3 deposition on apoptotic cells *in vivo*, while mice deficient in natural IgM, have been shown to develop lupus-like diseases due to failed clearance. IgG antibodies are considered to function in apoptotic cell clearance along with host defence. During erythocyte ageing a senescent erythocyte-specific antigen is displayed resulting in IgG binding which facilitates clearance (Bosman *et al.,* 2005).

1.4.1.3 Thombospondin (TSP-1)

TSP-1 is a member of the extracellular matrix protein family widely distributed in human tissues (Sid *et al.,* 2004). TSP-1 is secreted by various cells types including macrophages, fibroblasts and neutrophils (Poon *et al.,* 2009 & Moodley *et al.,* 2003). TSP-1 is implicated in various biological processes including chemotaxis, cell adhesion, proliferation and the clearance of apoptotic cells (Poon *et al.,* 2009). Savill *et al.*(1992) found that addition of TSP-1 to apoptotic neutrophils co-cultured with HMDM enhanced uptake. TSP-1 is suggested to interact with a phagocyte-associated complex; vitronectin receptor/CD36 (Savill *et al.,* 1992). CD36 has a short cytosolic signalling domain and is considered to signal using the integrin, vitronectin receptor to induce engulfment (Savill *et al.,* 1992). Furthermore Moodley *et al.*(2003) found that apoptotic fibroblasts released TSP-1 which functioned as a chemoattractant for macrophage migration suggesting that TSP-1 released from apoptotic cells also functions as a 'find me' signal. This is supported by findings that blockade of CD36 on macrophages inhibited their migration, suggesting TSP-1 may bind to CD36 and induce migration (Moodley *et al.,* 2003). TSP-1 is currently considered to forms a bridge between the apoptotic fibroblasts and the macrophage receptor complex CD36/vitronectin. With respect to amateur phagocytes, Monks *et al.*(2005) reports that mammary epithelial cells also use CD36/vitronectin receptor which potentially suggests that TSP-1-dependent clearance could also be mediated by amateur phagocytes. Therefore if amateur phagocytes express and utilise receptors also used by professional phagocytes to clear apoptotic cells, can they too be considered as 'resident professional' like resident macrophages? This ultimately suggests that it may be the receptors expressed by the

phagocyte that is responsible for its ability to efficiently clear apoptotic cells and therefore its level of profession.

1.4.2 PS-binding molecules

PS is recognised as a fundamental surface marker of apoptotic cells and as such there has been a great deal of interest in identifying its cognate phagocyte receptor (reviewed by Bratton and Henson, 2008). It was originally considered that phagocytes expressed just one universal receptor that recognised PS, termed PSR (Fadok *et al.,* 2000). This was identified using mAb 217 found to blocks the PS-dependent uptake of apoptotic cells by macrophages (Fadok *et al.,* 2000). The gene, *psr* was identified following binding of mAb217 to a phage display library of proteins from macrophages and considered to code for the PSR (Fadok *et al.,* 2000). However several studies found that the protein encoded by *psr* was primarily located in the nucleus of cells and did not function as a surface receptor (reviewed by Schlegel and Williamson, 2007, Bratton and Henson, 2008). Further work demonstrated that mAb217 showed cross-reactivity with a small sequence of protein encoded by the *psr* and was responsible for its being identified as the original PSR (reviewed by Schlegel and Williamson, 2007, Bratton and Henson, 2008). However current thinking is that there are two PS recognition mechanisms; 1. Direct PS-binding receptors, for example, the TIM family (Kobayashi *et al.*, 2007 & Miyanishi *et al.*, 2007) and 2. Indirect PS binding molecules that bridge the apoptotic cells and phagocyte facilitating clearance, for example, GAS6(Hanayama *et al.*, 2002 & Ravichandran, 2010).

1.4.2.1 Indirect PS-binding molecules

1.4.2.1.1 Growth arrest specific 6 (GAS6) and protein S

GAS6 and protein S are members of plasma vitamin K-dependent proteins (Ishimoto *et al.*, 2000). Protein S functions as an anticoagulant that inhibits protease activation in the clotting cascade (Stitt *et al.,* 1995) while GAS6 is secreted by fibroblasts during the growth-arrest state to prevent apoptosis and mediate proliferation (Ishimoto *et al.,* 2000). Gas6 and protein S bind to PS on the surface of apoptotic cells and enhance phagocytic clearance using Tyro, Axl and Mer known as the TAM family of tyrosine kinases receptors (Ishimoto *et al.,* 2000, Anderson *et al.,* 2003 & Lemke and Burstyn-Cohen, 2010). TAM family member recruit the crk-DOCK180-ELMO complex to the membrane resulting in Rac1 activation and consequently actin-dependent engulfment (Wu *et al.,* 2006). Activation of adhesion receptors including integrins such as the vitronectin receptor also results in crk-DOCK180 recruitment (Wu *et al.,* 2006).

1.4.2.1.2 MFG-E8

Hanayama *et al.*(2002) found a secreted glycoprotein known as MFG-E8 which is abundant in milk fat globules. This binds PS on apoptotic cells and bridges phagocyte interaction. MFG-E8 bound to PS, has been reported to bind to β2-integrins which includes the vitronectin receptor and mediates Rac1-dependent phagocytosis (Akakura *et al.,* 2004 & Wu *et al.,* 2006). The importance of MFG-E8 in the clearance of apoptotic cells was demonstrated by Peng and Elkon (2011) that found mice with MFG-E8 deficiency develop lupus-like diseases. Therefore this suggests that the role of MFG-E8 in apoptotic cell clearance is non-redundant.

1.4.3 Phagocyte-associated receptors that interact with apoptotic cells

1.4.3.1 PS receptors

Phagocyte interaction with PS can be direct or indirect and interaction with PS can result in signalling for corpse engulfment (Hoffmann et al., 2001). Here several potential membrane-associated PS receptors along with the signalling pathways that mediate engulfment are discussed.

1.4.3.1.1 T-cell immunoglobulin and mucin-domain-containing molecules 4 (TIM-4)

TIM-4 is a member of a the type I transmembrane family of proteins which, following expression in fibroblasts were found to enhance uptake of apoptotic cells (Miyanishi *et al.,* 2007). Furthermore blockade of TIM-4 resulted in the production of autoantibodies suggesting that the TIM-4-dependent engulfment of apoptotic cells is a non-redundant clearance mechanism. TIM members 4 and 1 have been shown to bind to PS on the surface of apoptotic cells using their immunoglobulin-domain to induce uptake. Miyanishi *et al.*(2007) also suggest that TIM family members present in microparticles, secreted by apoptotic cells may induce intracellular signalling that enhance phagocytosis when the phagocyte meets the apoptotic cells.

1.4.3.1.2 Brain-specific angiogenesis inhibitor1 (BAI1)

In vivo studies illustrated an inhibition of apoptotic cell clearance following reduced BAI1 expression (Park *et al.,* 2007a). BAI1 is a member of the adhesion-type G-protein coupled receptor family recognised to contain TSP-1-type repeats within the extracellular domain that binds PS (Park *et al.,* 2007a). Following ligation BAI1 forms a trimeric complex with ELMO/DOCK180/Rac which promotes cytoskeleton rearrangement and consequently phagocytosis (Park *et al.,* 2007a).

1.4.3.1.3 Stabilin-2

Stabilin-2 is a multidomain receptor that binds hyaluronic acid (Politz *et al.*, 2002), and bacteria (Adachi and Tsujimoto, 2002) and can function as an endocytic receptor for advanced-glycated end products (AGEs) (Tamura *et al.*, 2003). A decrease in the uptake of senescent erythocytes was demonstrated following knock-down of stabilin-2 expression by siRNA and co-incubation with PS-containing liposomes suggesting a role for stabilin-2 as a receptor for PS (Park *et al.*, 2008). Furthermore treatment of phagocytes with an agonist for stabilin-2, increased the production of anti-inflammatory cytokines to a level comparable to apoptotic cell or PS-containing liposomes (Park *et al.*, 2008). Ligation of stabilin-2 results in the recruitment of GULP and is therefore likely to influence the MEGF10/ABCA1/GULP/Rac 1 pathway leading to engulfment.

1.4.3.2 Scavenger receptors

There are many different classes of scavenger receptors of which recognise and bind different targets on Gram positive and Gram negative cells. However scavenger receptors CD36, LOX-1, CD68, SRA1, SRB1 and CLA-1 recognise oxidised lipids generated at the surface of apoptotic cells. Murphy *et al.*(2006) found that LOX-1 a C-type lectin, scavenger receptor directly binds PS in a calcium dependent-manner and facilitates clearance. Furthermore Imachi *et al.*(2000) found that the scavenger receptors SRB1 and CLA-1 were used by 'amateur' phagocytes to facilitate apoptotic cell clearance in the thymus.

1.4.3.3 CD14

The gene encoding CD14 is located on chromosome 5 and is composed of two exons that codes for a single 1.4kb transcript which once translated produces a 356 amino acid protein (Ziegler-Heitbrock and Ulevitch, 1993). Post-translation modifications include glycosylation at O-and N-linked sites and the addition of a hydrophobic glycophosphatidylinositol (GPI) tail to the C-terminal that anchors the protein in the cell membrane (Zielgler-Heitbrock and Ulevitch, 1993). CD14 functions as a PRR expressed on monocytes, macrophages, granulocytes and B cells (Ziegler-Heitbrock and Ulevitch, 1993). Pugin *et al.*(1998) used THP-1 stably transfected with a membrane CD14 expressing plasmid and isolated CD14 within the Triton X-100 insoluble-fraction suggesting that CD14 is located in microdomains within the plasma membrane.

CD14 expression is upregulated during monocyte maturation and was therefore originally considered a marker of macrophage differentiation (Ziegler-Heitbrock and Ulevitch, 1993).

Surface expression of CD14 is upregulated on both professional and amateur phagocytes, following LPS stimulation, with the exception being alveolar macrophages (Fearns *et al.*,1995). CD14 also exists as a soluble form found in serum and is upregulated following LPS stimulation (Fearns *et al.,* 1995). Soluble CD14 (sCD14) is generated following proteolysis of membrane associated receptor (mCD14) or released prior to cleavage of the C-terminus and subsequent addition of GPI tail (Bufler *et al.,* 1995). While the exact role of sCD14 in innate immunity is unknown, sCD14 is reported to convey LPS responsiveness to cells that do not express mCD14, for example, endothelial cells (Frey *et al.,* 1992).

CD14 is known to bind an array of endogenous and exogenous ligands most notably, LPS and promotes inflammation (Wright *et al.,* 1990). However other microbial ligands, include, peptidoglycan (Dziarski *et al.,* 1998) and lipoteichoic acid (Cleveland *et al.,* 1996). CD14 also binds endogenous ligands including heat shock proteins (Kol *et al.,* 2000a), β amyloid (Liu *et al.,* 2005), pulmonary surfactant proteins (Sano *et al.,* 2000), rat mannose-binding lectin (Chiba *et al.,* 2001), minimally-modified-LDL (Miller, 2002) and apoptotic cells (Devitt *et al.,* 1998).

1.4.3.3.1 Role of CD14 in LPS binding

The binding of LPS to CD14 generates inflammatory responses, characterised by the activation of NF-κB-dependent genes causing the expression of proinflammatory cytokines; including TNF-α and chemokines (Haziot *et al.,* 1996). CD14-deficient mice were reported to be highly resistant to septic shock indicating that CD14 mediates inflammatory responses (Haziot *et al.,* 1996). However a decrease in the dissemination of bacteria was also observed, suggesting that CD14 facilitates the spread of infection too(Haziot *et al.,* 1996). LPS are the major component of the outer membrane of the cell wall of Gram negative bacteria and is released during growth, lysis or on exposure to human serum (Aurell and Wistrom, 1998 & Zhang *et al.,* 1994a). This amphiphilic molecule is composed of three subunits (figure 3); a highly conserved lipid A region that anchors the whole molecule to the bacterial cell membrane, covalently attached to a core oligosaccharide and the O-antigen which protrudes into the surrounding area (Rietschel *et al.,* 1994). The core polysaccharide and O-antigen vary in composition depending on the serotype of bacteria (Rietschel *et al.,* 1994). However lipid A is responsible for biological activity of LPS and while this region is highly conserved, slight variations between bacterial strains are responsible for the potency of LPS (Rietschel *et al.,* 1994).

Due to the chemical nature of LPS, in aqueous solutions such as blood and tissue culture medium, aggregates known as micelles form, with LPS monomer transfer to CD14 being slow (Aurell and Wistrom, 1998 & Fenton and Golenbock, 1998). The binding of LPS to CD14 is enhanced by the serum glycoprotein LPS-binding protein (LBP) which is a lipid transferase that destabilises aggregates and transfers monomers of LPS from micelles to CD14, producing a CD14-LPS receptor complex (Tobias *et al.,* 1986 & Hailman *et al.*, 1994). *In vitro* data suggest that LBP is necessary for LPS responsiveness however *in vivo* data from LBP-deficient mice suggests LPS response is unaffected (Wurfel *et al.,* 1997). Furthermore Youn *et al.*(2008) found that HMGB1 bound directly to LPS and facilitated transfer to CD14. Formation of LPS:CD14 complex recruits TLR4 associated with MD-2 to the membrane along with associated signalling molecules, for example, MyD88 (da Silva Correia *et al.*, 2001 & Triantafilou *et al.*, 2002).

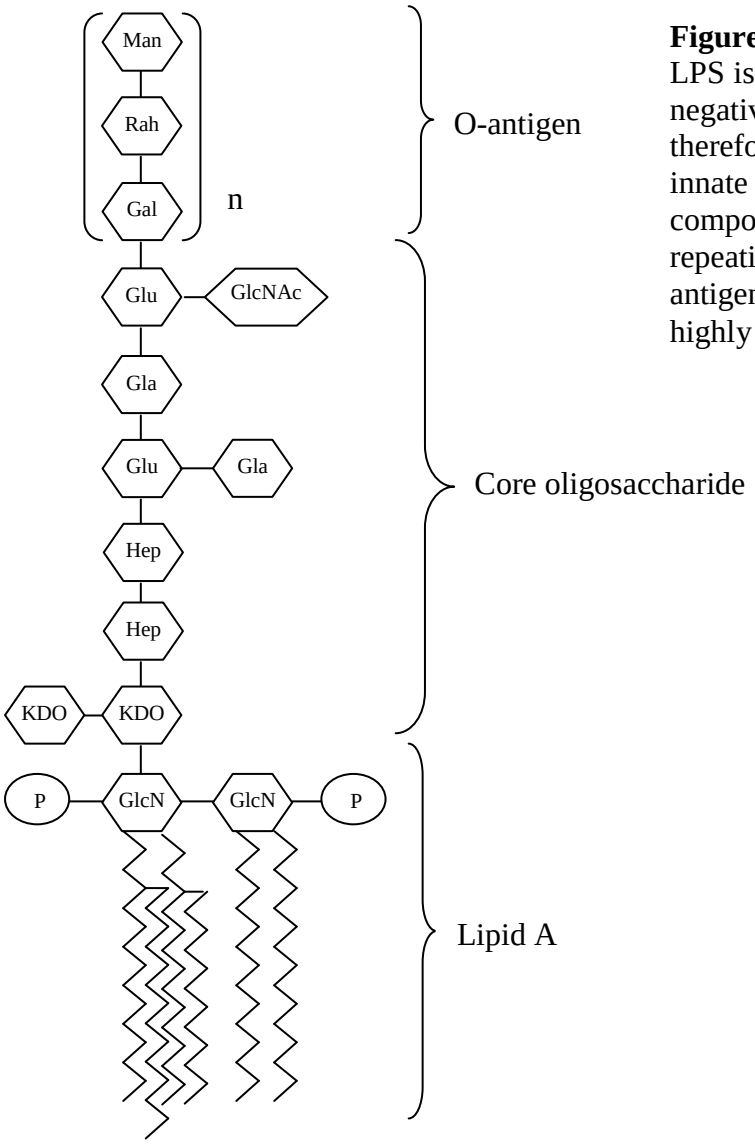

Figure 3: Structure of LPS
LPS is the major component of Gram negative bacterial cell wall and therefore a potent activator of the innate immune system. LPS is composed of three main subunits, a repeating sugar unit known as the O-antigen, a core oligosaccharide and a highly conserved lipid A region.

1.4.3.3.2 Signalling partner

Membrane CD14 does not contain a cytosolic signalling domain therefore CD14 interacts with different signalling partners to confer ligand-dependent responses (reviewed by Bosshart & Heinzelmann, 2002). The CD14-LPS complexes activate TLR4 (figure 4) to propagate the signal causing the activation of NF-κB-dependent proinflammatory genes (Hoshino *et al.,* 1999 & Miyake, 2004). Studies conducted with TLR4-deficient mice or mutated TLR4 domains were hyporesponsive to LPS therefore confirming the requirement of TLR4 in LPS signalling (Hoshino *et al.,* 1999). Shimazu *et al.*(1999) revealed the requirement of the accessory protein, MD-2, required for glycosylation and cell surface expression of TLR4 (Nagai *et al.,* 2002). TLR4 propagates the signal by interaction with the adaptor molecules myeloid differentiation factor 88 (MyD88) or a MyD88-independent pathway which activates the adaptor molecule (TRAM) and TIR-domain containing adaptor including interferon-beta (TRIF) (Takeda and Akira, 2004). Activation of these pathways results in the activation of the transcription factor NF-kB which is responsible for the expression of proinflammatory genes (review by Miyake, 2004).

Isolation of microdomains containing CD14 by Triantafilou *et al.*(2002) along with SDS-PAGE and immunoblotting revealed the presence of other LPS binding molecules including Heat shock protein (Hsp) 70 and 90 (Byrd *et al.,* 1999 & Wallin *et al.,* 2002). Following LPS stimulation Triantafilou *et al.,* 2002 found that along with TLR4, other LPS-binding proteins including chemokine receptor 4 (CXCR4) and growth differentiation factor 5 (GDF5) were recruited and formed a CD14-independent LPS receptor (da Silva Correia *et al.,* 2001). Previous studies have shown that incubation with CD14-blocking mAb only partially inhibit LPS-induced binding or responses (Troelstra *et al.,* 1997 & Gessani *et al.,* 1993). Monocytes and macrophages express scavenger receptors, β2 integrins and L-selectins which are also bind LPS (reviewed by Fenton and Golenbock, 1998& Peppelenbosch *et al.,* 1999). However these receptors are reported to have a low affinity for LPS therefore a high concentration of LPS is required for activation (Peppelenbosch *et al.,* 1999).

Figure 4: LPS binds to CD14 and activates TLR4
LPS aggregates into micelles in aqueous solutions therefore LBP is required to destabilise aggregates and transfer monomers of LPS from micelles to CD14, producing a CD14-LPS receptor complex. CD14-LPS complexes activate signalling partner, TLR4 to propagate an proinflammatory signal resulting in the transcription NF-κB-dependent genes (Hoshino *et al.*, 1999 & Miyake, 2004).

1.4.3.3.3 CD14 binds apoptotic cells

The clearance of apoptotic cells is currently known to be more than simply waste disposal as originally considered. Voll *et al.*(1997) highlighted the immunomodulatory effects of apoptotic cell clearance by macrophages. Furthermore Fadok *et al.* (1998, 2002) found that apoptotic cell uptake by macrophages, actively inhibited production of tumour necrosis factor-α (TNF-α) and other proinflammatory cytokines following stimulation with zymosan or LPS. Fadok *et al.*(1998) established that the immunosuppressive effects produced by apoptotic cell uptake could be inhibited by anti-transforming growth factor-β (TGF-β) antibodies (Fadok *et al.*, 1998b). The lead to the suggestion that interactions formed between the apoptotic cell and the interface of the macrophage; known as the phagocytic synapse, produced a dominant anti-inflammatory response. This anti-inflammatory macrophages response is characterised by the expression and release of the TGF-β and immunomodulatory cytokines such as IL-10 (Fadok *et al.*, 1998b).

Flora and Gregory (1994) tested a range of mAbs, against monocyte and macrophage surface antigens, for their ability to inhibit the *in vitro* binding and phagocytosis of apoptotic cells. These studies identified mAb 61D3 which could block the binding of apoptotic cells to cultured macrophages. Analysis of the epitope recognised by 61D3 by Devitt *et al.*(1998) identified this epitope as the LPS receptor, CD14. The transient expression of CD14 by COS-1 then identified the role of CD14 in the binding and

phagocytosis of apoptotic cells. Devitt *et al.* (1998) demonstrated that LPS interaction with macrophages induced the release of the pro-inflammatory mediator TNF-α, whereas macrophages interacting with apoptotic cells did so without such consequences. Furthermore Devitt *et al.*(2004), investigated the importance and level of redundancy of CD14 in apoptotic cell clearance *in vivo* using CD14-knockout mice. In these mice persistent apoptotic cells were found in tissues, including the thymus, spleen and lung suggesting a defect in apoptotic cell clearance related to their CD14 deficiency (Devitt *et al.,* 2004). The persistence of these apoptotic cells was for the first time, without inflammatory consequences (Devitt *et al.,* 2004). This suggesting that the anti-inflammatory response produced by macrophages following apoptotic cell uptake, could be uncoupled from the initial binding events (Devitt *et al.,* 2004). Therefore CD14 is currently considered to function as a tethering receptor, required for the binding of apoptotic cells.

CD14 was the first PRR identified with a role in the clearance of apoptotic cells and quickly led to the discovery that other PRRs were also involved in apoptotic cell clearance (e.g C1q and MBL). Furthermore this led to the suggestion that apoptotic cells display apoptotic cell-associated molecular patterns termed ACAMPs considered to resemble PAMPs (Gregory and Devitt, 2004). In the case of CD14, Gregory and Devitt (2004) suggested that the ACAMP recognised by CD14 are LPS-like. This was suggested following findings by Devitt *et al.* (1998) that 61D3 and another CD14-blocking mAb, MEM18, blocked both LPS responses and apoptotic cell binding. Therefore suggestion that the epitope for LPS and apoptotic cells within CD14, are the same or closely associated (Devitt *et al.,* 1998). This promoted interest into the mechanisms employed by CD14 to produce two contrasting ligand-dependent responses.

1.4.3.4 Another PRR involved in the clearance of apoptotic cells?

Recent findings have implicated another innate immune molecule, the receptor for non-enzymatically glycated and oxidised proteins (RAGE) in the clearance of apoptotic cells by phagocytes (Liu *et al.,* 2008, Banerjee *et al.,* 2010). RAGE shares much similarity with CD14, both receptors bind exogenous and endogenous ligands and have been implicated in innate immunity (Lin, 2006, Foell *et al.,* 2006 & Bianchi, 2009). Furthermore RAGE, like CD14 also forms complexes with LPS and employs TLR4 to promote inflammation (Bianchi, 2009, Park *et al.,* 2004, Youn *et al.,* 2008, Hofmann *et al.,* 1999 & Ziegler-Heitbrock and Ulevitch, 1993). Ligation of RAGE also activates the transcription factor NF-κB, sequentially promoting the expression of proinflammatory cytokines and

chemokines (Chavakis *et al.,* 2003,Schmidt *et al.,* 2000). However with previous suggestions that ACAMPs share similarities with PAMPS, along with previous findings that CD14 also binds and facilitates the removal of apoptotic cells, the role of RAGE in the clearance of apoptotic cells is of interest.

1.4.3.4.1 RAGE

RAGE was first identified by Neeper *et al.*(1992), as a cell surface receptor for the products of non-enzymatically glycated and oxidised proteins (AGEs). Later RAGE was identified to recognise other families of ligands, including members of the S100/calgranulins, amyloid fibril and high mobility group box-1 (HMGB1) (Schmidt *et al.,* 2000 & Bucciarelli *et al.,* 2002). The structures of these ligands are heterogeneous and crystallography revealed that the ligands of RAGE share common structural characteristics with all of the three dimensional structures having multiple β-sheet conformations (Schmidt *et al.,* 2000 & Bucciarelli *et al.,* 2002). Therefore RAGE functions as a PRR expressed at low levels by most cell types, including neurons, glioma, endothelial, smooth muscle cells, and professional phagocytes (Park *et al.,* 2004 & Lutterloh *et al.,* 2007). The involvement of RAGE in innate immunity was first suggested by Liliensiek *et al.*(2004) who found RAGE-deficient mice displayed normal physiology but were protected against septic shock as seen in CD14-deficient mice. This protective effect could be reversed following the reconstitution suggesting a direct role for RAGE in innate immune responses (Liliensiek *et al.,* 2004 & Lutterloh *et al.,* 2007). The RAGE gene is located within the major histocompatibility class III locus, known to code for other members of the innate immune system (Xie *et al.,* 2008).

1.4.3.4.2 RAGE binds HMGB1

The presence of HMGB1 outside the cell is reported to function as a damage-associated molecular pattern (DAMP), considered as an endogenous molecule that signifies tissue damage and activates the innate immune system. Necrotic cells passively release HMGB1 due to loss of membrane integrity and HMGB1 binds with high affinity to RAGE which promotes inflammation (Scaffidi *et al.,* 2002). During activation of the apoptosis programme, HMGB1 becomes bound to chromatin therefore sequestering this DAMP signal (Scaffidi *et al.,* 2002). Macrophages have also been reported to secrete HMGB1 as a proinflammatory cytokine considered to function though RAGE ligation (Scaffidi *et al.,* 2002). A study by Liu *et al.*(2008) found that preincubating apoptotic neutrophils with HMGB1 prior to co-culturing with mouse peritoneal macrophages inhibited the phagocytic uptake of apoptotic cells. Furthermore HMGB1 was found to compete with MFG-E8 for

binding to the vitronectin receptor expressed by phagocytes (Friggeri *et al.*, 2010). However loss of C-terminus of HMGB1 diminished both the inhibitory effects on apoptotic cell clearance and ability to bind RAGE (Friggeri *et al.*, 2010). Therefore suggesting that this innate immune receptor; RAGE, may also function in the clearance of apoptotic cells.

1.5 Aims & objectives

The established role for CD14 in both LPS response and apoptotic cell clearance implies that CD14 is capable of producing two distinct ligand-dependent macrophage responses. Therefore this project seeks to use a series of point-mutants to dissect the role of CD14 in the binding of LPS and apoptotic cells and seeks to establish an understanding of the molecular mechanisms underlying the involvement of CD14 in the non-inflammatory clearance of apoptotic cells. Previous findings suggest that CD14 employs the same or closely associated region of CD14 to interact with PAMPs and ACAMPs therefore there are several possible scenarios in which CD14 ligation with PAMPs and ACAMPs can induce divergent macrophage responses (figure 5).

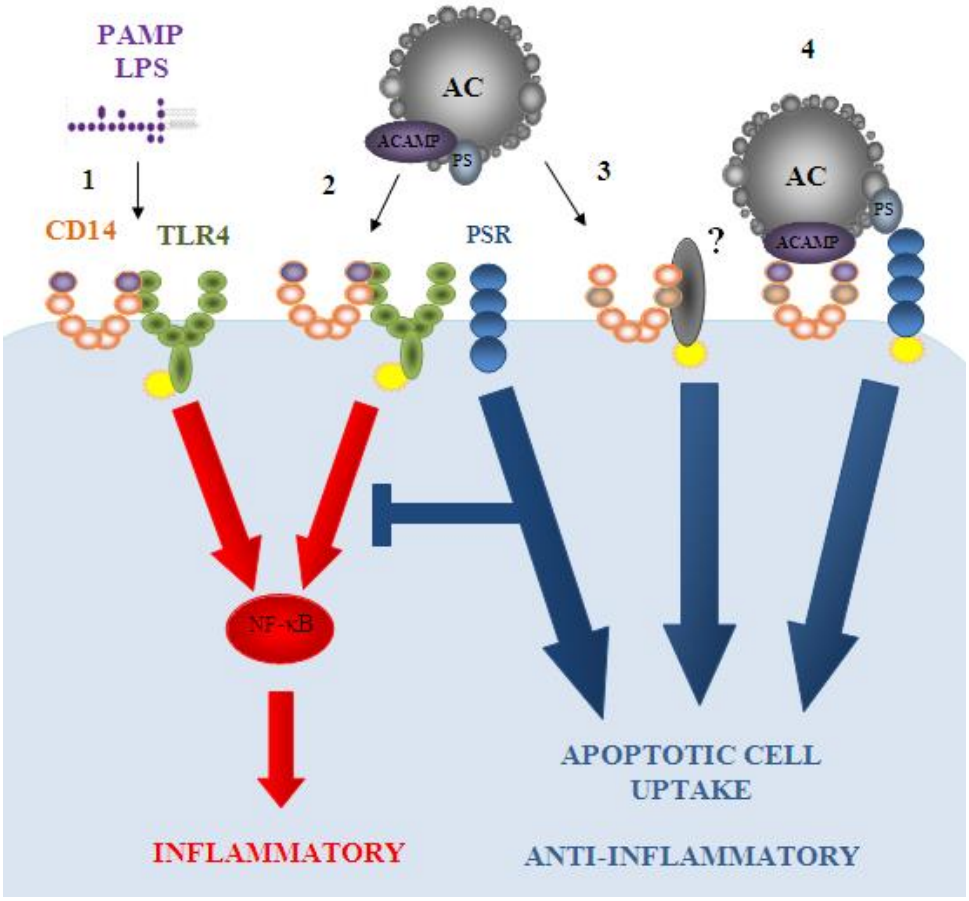

Figure 5: Possible scenarios involved in CD14-dependent responses
CD14-dependent response to LPS: **1.** LPS binds CD14 (purple domains) and induces a pro-inflammatory signal (red arrows) though association with TLR4, resulting in NF-κB translocation and an inflammatory response. Possible CD14-dependent responses to apoptotic cells: **2** ACAMP on apoptotic cells bind to CD14 in an identical manner to LPS (purple domains) and induce a pro-inflammatory signal as seen in scenario one. However pro-inflammatory signalling is suppressed following further phagocyte receptor ligation (e.g. PS-PS receptor) which produces a dominant anti-inflammatory signal (blue arrows). **3** ACAMP binding to CD14 differs from LPS binding (grey domains), thereby dictating the recruitment of an alternative signalling partner to CD14 resulting in anti-inflmmatory signalling **4.** CD14 here functions very simply just to tether the apoptotic cell to the phagocyte. The tethering effects of CD14 may be mediated thought ACAMP interaction with LPS binding regions of CD14 (purple domains) or another region of CD14 (grey domains), but interaction does not result in signalling. Therefore interaction of the apoptotic cell with another phagocyte receptor independently mediates corpse uptake and the anti-inflammatory response.*Adapted from Gregory and Devitt, 2004*

The hypotheses underlying this project are: **1. Apoptotic cells present ACAMPs that share structural similarities with PAMPs, which in the case of CD14 are considered to be LPS-like structures. 2. ACAMP and PAMP binding to CD14 is indistinguishable, however apoptotic cells bind to CD14 in a manner that re-programmes the downstream responses to be non-inflammatory or even anti-inflammatory**.

Therefore the specific aims and objectives of this project are:

1) Given the knowledge of LPS binding, a series of mutants with single amino acid changes within key LPS binding regions of CD14 will be used to address apoptotic cell binding and will compare and contrast ligand binding.
2) However if the same regions of CD14 are employed to bind LPS and apoptotic cells, this project will seek to identify the point at which CD14 signalling pathways diverge.
3) Given findings that amateur phagocytes use receptors also expressed by macrophages to clear apoptotic cells, this project aims to characterise the function of CD14 in the clearance of apoptotic cells by a series of amateur phagocytes.
4) Given CD14, an LPS binding molecule, binds to apoptotic cells this project seeks to address if another LPS binding molecule, RAGE can also bind. Therefore this project aims to produce soluble RAGE to function as a competitive decoy for membrane-bound RAGE in order to assess the role of RAGE in the clearance of apoptotic cells.

Materials & Methods

2.1 Equipment

Cryo-freezing container NALGENE (Fisher, Loughborough,UK

Eppendorf Centrifuge 5810R (DJB Labcare Ltd, Buckinghamshire, UK)

Flow cytometer Quanta SC (Beckman Coulter High Wycombe, UK)

Flow cytometric analysis software VenturiOne (Dinnington, Sheffield, UK)

Haemocytometer Double cell standard (Camlab Ltd, Cambridge, UK)

Inverted fluorescence microscope Zeiss Axiovert 200M (Hertfordshire, UK)

Luminometer (Berthold detection systems, Phorzhein, Germany)

Nano drop 1000 spectrophotomer (Thermoscientific, Loughborough,UK)

Plate reader EL800 (BioTek Potton, Bedfordshire, UK)

RENA air 50 (Chalfont, PA, USA)

Thermocycler Gene Amp (Applied Bio systems Paisley, UK)

UVP transilluminator (Cambridge, UK)

2.2 Reagents

All general laboratory consumables were purchased from Sigma Aldrich (Dorset, UK) unless otherwise specified.

2.2.1 Antibodies

Goat anti-mouse IgG (whole molecule)-FITC (GAM-FITC), goat anti-human IgG (Fc specific)-FITC, mouse IgG1 (MOPC 21), goat anti-rabbit-PE and goat ant-mouse-PE Sigma Aldrich (Dorset, UK). Mouse IgG2a (MOPC 173), TLR4 (clone HTA 125)-PE, Mouse IgG2a-PE, Rabbit polyclonal antibody to RAGE Abcam (Cambridge, UK). Rabbit immunoglobulin fraction (normal) negative control Dako (20mg/ml) (Ely, Cambridgeshire, UK). ECL-anti-rabbit IgG horseradish peroxidise (anti-rabbit-HRP), ECL anti-mouse horseradish peroxidise (anti-mouse-HRP), Streptavidin horseradish peroxidise (Streptavidin-HRP), Amersham biosciences (Cambridge, UK). Rabbit anti-*Escherichia coli* LPS anti-serum AbD serotec (Oxford, UK)**.**

2.3 Tissue culture

2.3.1 Tissue culture reagents

All tissue culture reagents were purchased from PAA Laboratories Ltd (Yeovil, Somerset, UK) unless otherwise specified.

2.3.2 Tissue culture media and solutions

Suspension cell growth medium (cRPMI): RPMI 1640, penicillin (final concentration of 100U/ml) and streptomycin (to a final concentration of 100µg/ml) , 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS).

Adherent cell growth medium (cDMEM): DMEM, penicillin (final concentration of 100U/ml) and streptomycin (to a final concentration of 100µg/ml), 2 mM L-glutamine and 10% (v/v) FCS.

Growth medium for oral epithelial (H400) cells: DMEM/F12 (gift kindly received from Professor Ian Chapel, Birmingham Dental School), penicillin (final concentration of 100U/ml) and streptomycin (to a final concentration of 100µg/ml), 2 mM L-glutamine and 10% (v/v) FCS.

Human pulmonary fibroblast growth medium: Fibroblast growth medium (Promocell, Heidelberg, Germany), supplemented with the antibiotic Normocin (Promocell) (final concentration of 100µg/ml), 2 mM L-glutamine, 15% (v/v) FCS and the manufacturer's supplement.

Growth medium for Calu-3 cells: DMEM/F12 (Promocell), 15% (v/v) FCS, Normocin (final concentration of 100µg/ml) and 2 mM L-glutamine.

Airway epithelial growth medium: Airway epithelial growth medium (Promocell), 5% (v/v) FCS, Normycin (final concentration 100µg/ml), 2 mM L-glutamine and the manufacture's supplement.

Cell freezing medium: 5% (v/v) Dimethyl sulphoxide (DMSO) in FCS.

Wash buffer for flow cytometry: 0.1% (w/v) BSA in PBS.

Fixative for flow cytometry staining: 0.1% (w/v) BSA, 1% (w/v) formaldehyde in PBS

Apoptotic cell interaction assay medium: sfRPMI containing 0.2% (w/v) BSA

Jenner-Giemsa buffer (50x): Solution A:100mM sodium dihydrogen orthophosphate dihydrate. Solution B:100mM disodium hydrogen phosphate. Solution B is added to solution A to pH 5.6 and diluted in dH_2O to x1 solution.

Cell lysis buffer: 5µl/ml protease inhibitor cocktail (Sigma Aldrich, Dorset UK) (broad spectrum of protease inhibitors including 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin) 0.5% (v/v) Triton X-100 (Sigma Aldrich, Dorset UK) in PBS.

2 x HEPES buffered saline: (HBS) 8g sodium chloride, 0.2g 1.5mM disodium hydrogen phosphate, 6.5g HEPES diluted in dH_2O to pH7.0 then made to 500ml solution.

2.3.3 Cell lines

2.3.3.1 Suspension cell lines

Mutu: EBV-positive Burkitt lymphoma cell line (Gregory *et al.,* 1991). Supplied courtesy of Professor Christopher Gregory (MRC Centre for Inflammation Research, Edinburgh University).

THP-1: Human monocytic leukaemic cell lines.

U937: Human monocytic leukaemic lymphoma cell line.

Jurkat: human T cell leukaemia cell line.

2.3.3.2 Adherent cell lines

HEK293: An adherent adenovirus transformed human embryonic kidney cell line (Graham *et al.,* 1977).

COS-1: African green monkey kidney cell line; derived from CV-1 by transformation with origin defective SV-40 mutant (Jensen *et al.,* 1964).

H400: Human oral squamous carcinoma cell line cultured in complete DMEM/F12 (Prime *et al.,* 1990). These cells were kindly received from Ian Chapel from School of dentistry, University of Birmingham.

HeLa: Human cervical epithelial cells taken from a carcinoma transformed by human papillomavirus 18 sequences.

MCF-7: Mammary epithelial cell line from human breast adenocarcinoma (Soule *et al.,* 1973).

Beas-2B cells: Normal human bronchial epithelial cell line transformed by SV40 T-antigen (Reddel *et al.,* 1988). Received with thanks from Dr Lindsay J. Marshall (School of Life & Health Sciences, Aston University, UK).

HPF: A primary human pulmonary fibroblast cell line received from Dr Lindsay J. Marshall (School of Life & Health Sciences, Aston University, UK). The cells were cultured in cHPFM

Calu-3: Human sub-bronchial gland cell line from submucosal adenocarcinoma origin. Received from Dr Lindsay J. Marshall (School of Life & Health Sciences, Aston University, UK). This cell line was cultured in cDMEM/F12.

2.3.4 Tissue culture

All cell lines were routinely cultured every 2-3 days and maintained in a 37°C, 5% CO_2 humidified incubator. Suspension cells were cultured by gently resuspending the cells and replacing 70-80% of the culture medium with fresh, prewarmed cRPMI. Adherent cell

lines were cultured by rinsing the monolayer first with PBS (PAA) then with trypsin/EDTA (PAA). Following incubation at room temperature for 10-15min, the cells were resuspended and 90% of the culture medium replaced with prewarmed cDMEM unless specified in section 2.3.3.2.

2.3.4.1 Freezing Cells

Only healthy cultures of cells with high viability were frozen down. Cells harvested as outlined in section 2.3.4 were centrifuged and resuspended in freezing medium. 1ml aliquots were transferred to labelled cryovials and placed in a cryo-freezing container at -80°C to allow freezing to be controlled to -1°C/min. Cryovials were then transferred to nitrogen vapour for long-term storage.

2.3.4.2 Thawing Cells

Cryovials of cells were stored in nitrogen vapour. Non-adherent cells were thawed rapidly at 37°C in a water bath. Once defrosted, cells were transferred to a 25ml tube and gently mixed (in a drop-wise manner) with 10ml prewarmed growth medium. Cells were centrifuged gently (300xg for 5 min) and the pellet resuspended in fresh growth medium to remove cryoprotectant. Adherent cells lines were similarly thawed but cryoprotectant was removed by changing culture medium following cell adhesion.

2.3.5 Differentiation of THP-1 into macrophage-like cells

Differentiation of THP-1 into macrophage-like was induced with 100nM dihydroxyvitamin D3 (vitamin D3) (Biomol, Exeter, UK), 250nM phorbol 13-myristate12-acetate (PMA) (Sigma Aldrich, Dorset, UK) or both (vitamin D3/PMA) for 72h at 37°C in 5% CO_2 incubator.

2.3.6 Immunofluorescence staining of cells

2.3.6.1 Indirect Immunofluorescence staining

$2x10^5$ cells were washed in flow buffer and incubated with 100µl of primary antibody for 30min on ice. All antibodies were used in excess or at saturating concentrations. Cell surface CD14 expression was detected with undiluted tissue culture supernatant 61D3, 63D3 or the IgG1:κ (10µg/ml) isotype-control MOPC 21. Cell surface expression of RAGE was detected with (1µg/ml) polyclonal anti-RAGE antibody or the isotype-control pre-immune rabbit serum (1µg/ml). Following incubation cells were washed twice with flow buffer before incubation with 100µl secondary-conjugated antibody (goat anti-mouse

FITC/PE (1:50) or goat anti-rabbit FITC (1:50) for at least 30min on ice. Cells were washed and fixed in 1% (w/v) formaldehyde in PBS prior to analysis.

2.3.6.2 Direct Immunofluorescence staining

Cell surface TLR4 was determined using direct immunofluorescence staining with TLR4 (HTA 125)-PE or IgG2a/κ isotype-control. $2x10^5$ cells were washed with flow buffer and incubated with 2μL of HTA125 or the isotype-control at saturating concentrations for 30min on ice. Following incubation cells are washed twice with flow buffer and fixed in 1% (w/v) formaldehyde in PBS prior to analysis.

2.3.6.3 Immunofluorescence staining of apoptotic cells

Mutu were induced to apoptosis as outlined in section 2.3.9.1. Following incubation $2x10^5$ cells were washed in flow buffer and incubated with 100μl of supernatant taken from cells transfected with sRAGE, PSA or with polyclonal anti-LPS polyclonal antibodies (1:1000) or negative control normal pre-immune rabbit serum (10μg/ml) in flow buffer for 30 min on ice. Following incubation, cells were washed and bound sRAGE detected with (1μg/ml) polyclonal anti-RAGE antibodies or isotype-control pre-immune rabbit serum (10μg/ml) in flow buffer for 30 min on ice. Cell were washed prior to incubation with (1:50) anti-Rabbit-PE in flow buffer for 30 min on ice, washed then fixed in 1% (w/v) formaldehyde in PBS prior to analysis

2.3.7 LPS responsiveness of THP-1-derived macrophages

THP-1 were seeded to wells in 24 well plates and stimulated to differentiate as outlined in section 2.3.5. To allow for the growth of THP-1 and VD3 cells, these cells were reseeded post differentiation at $6x10^5$ cells per well. All THP-1-derived macrophages were challenge with lipopolysaccharide (LPS) (from *Escherichia coli* O111:B4 purchased from Sigma Aldrich, Dorset UK) in the presence of 10% (v/v) human serum (from male AB plasma, purchased from Sigma Aldrich, dorset UK) and mAbs (as appropriate) for 4h. TNF-α release in phagocyte-culture supernatants was quantified using an anti-human TNF-α capture ELISA (R&D systems, Minneapolis, USA)

2.3.7.1 LPS binding to THP-1-derived macrophages

THP-1 were induced to differentiate with vitamin D3 for 72 hours. Following differentiation, cells were washed with 5mM EDTA in PBS to remove any adherent cells from culture flask. $2x10^5$ cells were incubated with LPS-FITC in PBS containing 10% (v/v) normal human serum in flow buffer for 15 min on ice. LPS binding was inhibited by

preincubating cells with CD14-blocking mAb MEM18 (10µg/ml) (Juan *et al*, 1995) prior to LPS-FITC incubation. Cells were washed and fixed in 1% formaldehyde in PBS prior to flow cytometric analysis.

2.3.8 LPS responsiveness of HeLa

Following LT-1-mediated transfection (2.3.10.3), mock-transfected HeLa or HeLa expressing mCD14 were seeded in triplicate at a density of $3.5x10^5$/well in 96-well plates and incubated overnight to allow for attachment. Cells were stimulated with 200µl of either medium alone or LPS in sfDMEM containing 10% NHS at 1µg/ml, 10µg/ml or 100µg/ml for 24 hours. Following incubation, cell supernatants were collected and frozen at -20°C until analysis. Cells were washed and cytosolic IL-8 was collected following incubation with 200µl of lysis buffer for 30 min at 37°C. Cytosolic supernatants were collected and frozen at -20°C until analysis.

2.3.9 Apoptotic cell phagocyte interaction assay

2.3.9.1 Generating apoptotic cells

For the preparation of apoptotic cells, Mutu were induced to undergo apoptosis by exposure to 100mJ/cm^2 UV radiation and incubated for 18-24h at 37°C in 5% CO_2 atmosphere. Apoptotic cells were resuspended in assay medium prior to the assay and cell death estimated by flow cytometry where cells were gated based on side scatter into viable and apoptotic populations. 18-24 hours following the induction of apoptosis, Mutu resembled secondary necrotic cells in terms of propidium iodide and annexin IV staining.

2.3.9.2 Phagocyte interaction assay

THP-1 were seeded to four well glass slides and stimulated to differentiate as outline in section 2.3.5. Following differentiation medium was removed and phagocytes co-cultured with apoptotic cells for 1h at 37°C in the presence or absence of sRAGE or mAbs (e.g. 61D3, 63D3 or an isotype-control MOPC 21). Slides were washed three times in cold PBS, prior to fixation with methanol (30 min) and staining with Jenner-Giemsa. Interaction of apoptotic Mutu was assessed by light microscopy and values scored as a percentage of phagocytes interacting with apoptotic cells (minimum number of phagocytes counted 200)

2.3.9.3 Amateur phagocyte interaction assay

Amateur phagocytes were seeded at $2x10^4$ cells on four well glass slides and left to adhere overnight. Apoptotic cells were incubated with epithelial cells for 1h at 37°C in the presence or absence of 61D3 or 63D3 in apoptotic cell interaction assay medium. Slides

were washed three times in cold PBS, prior to fixation with methanol (30 min) and staining with Jenner-Giemsa. Interaction of apoptotic Mutu was assessed by light microscopy and values scored as a percentage of phagocytes interacting with apoptotic cells (minimum number of phagocytes counted 200)

2.3.9.4 Mapping apoptotic cell binding to mCD14

HeLa were transiently transfected, using LT-1 as outlined in section 2.3.10.3 with plasmids encoding mCD14 point mutants. 24h post-transfection, cells were re-seeded at $1x10^4$per well to four well glass slides and left to adhere overnight. Prior to the apoptotic cell interaction assay slides were placed at $4^{\circ}C$ for 30 min then washed in cold assay medium to reduce the rate of interaction. Apoptotic cells were prepared as outlined in 2.3.9.1 and resuspended in cold assay medium. $1x10^6$ apoptotic cells were added to each well at room temperature for 5 min. Slides were then washed in cold PBS, prior to fixation in methanol (30 min) and staining with Jenner-Giemsa stained. Interaction of HeLa with apoptotic Mutu was assessed using light microscopy from a sample size of at least 200 cells and the percentage of HeLa cells interacting with apoptotic cells estimated.

2.3.9.5 Jenner-Giemsa staining of cells

Jenner and Giemsa stains (both purchased from Oxoid, Basingstoke, UK) were diluted with 1x Jenner-Giemsa buffer. The slides were incubated in a 1:3 dilution of Jenner stain for 4.5min. Slides were washed in Jenner-Giemsa buffer and incubated in 1:10 dilution of Giemsa stain for 9.5min. Slides were dried by aspiration and mounted with D.P.X. (BDH reagents, Lutterworth, Leicestershire, UK). Interaction with apoptotic Mutu was assessed by light microscopy and values scored as a percentage of phagocytes interacting with apoptotic cells.

2.3.10 Transient transfection of cells

The transient transfection of mammalian cells was conducted several ways depending on the cell line used, transfection efficiency required, use of protein expressed and cost. The transient transfection of HEK293 was performed using either calcium phosphate or LT-1 transfection reagent and generally used to produce soluble protein for harvest. The transient transfection of HeLa and COS-1 was performed using LT-1 transfection and used to produce cells expressing surface-bound CD14 mutants. With every transfection, a mock-transfection (no DNA) was conducted as a negative control and a reporter assay (GFP) was conducted to demonstrate transfection efficiency then cells were analysed by flow cytometry.

2.3.10.1 $CaPO_4$–mediated transfection

250mM $CaCl_2$ (Fisher, Loughborough, UK) containing 2 µg plasmid DNA was added (drop-wise manner) to an equal volume of 2xHBS (see section 2.3.2 for details) with continuous agitation by aeration. The precipitate was incubated at room temperature then added to $3x10^5$ HEK293 suspended in 2ml of cDMEM. Cells were plated and cultured for 72h at 37°C, 5% CO_2 in a humidified atmosphere. When producing soluble proteins 24h after transfection the medium was replaced with sfDMEM to avoid contamination with serum.

2.3.10.2 DEAE-Dextran-mediated transfection of COS-1

COS-1 were seeded in 6 well plates at density of $3x10^5$cells/ well and incubated for a short period to allow cells to adhere. Following adhesion cells were washed with sfDMEM then transfection mix added (5µg DNA, 500µl/ml DEAE-Dextran (Sigma), 100µM chloroquine diphosphate and volume was made up with sfDMEM) and incubated until vesicles formed. Cells were then washed in 10% (v/v)DMSO in PBS for 2min and resuspended in cDMEM.

2.3.10.3 LT-1-mediated transfection

24h prior to transfection HeLa or COS-1 were plated in 6 well plates at a density of $3x10^5$ per well. Following preliminary experiments it was found the ratio 5µl:2µg (HeLa) or 3µl: 2µg (COS-1) of LT1 reagent to DNA produced optimum transfection efficiency for 6 well plate HeLa or COS-1 transfection. LT-1 was mixed with 250µl of sfRPMI and 2µg of plasmid DNA was added, transfection mixture was then incubated (15min at room temperature) to allow complexes with DNA to form. Following incubation the transfection mixture was added (drop-wise) to the 6 well plates and the cells were cultured for 24-72h at 37°C, 5% CO_2 in a humidified atmosphere.

2.3.10.3.1 Transfecting HeLa with NF-κB reporter

HeLa were seeded at $1.5x10^4$/well in a 96 well plate 24 h prior to transfection. In separate eppendorfs, 100ng of NF-κB reporter plasmid DNA (Promega) was mixed with 100ng of mCD14 plasmid DNA, 9µl of sfRPMI then 0.3µl of LT-1 added and the mixture was incubated for 30min (room temperature). Following incubation the transfection mixtures were added (drop-wise) to each of the 96 wells and cultured for 24h at 37°C, 5% CO_2 in a humidified atmosphere prior to stimulation.

2.3.11 NF-κB reporter assay

An NF-κB reporter containing firefly luciferase upstream of NF-κB response element was used to map the LPS responsiveness of membrane CD14 within HeLa transfectants.

Following transfection of HeLa with NF-κB reporter (section 2.3.10.3.1) the medium was removed and cells were incubated with 100µl of sfDMEM containing 10µg/ml of LPS (supplemented with 10% (v/v) NHS), apoptotic cells or medium alone (control wells). Plates were incubated for 5h at 37°C and luciferase activity was detected by addition of 100µl of One-Glo (Promega) to each well and incubated at room temperature for 3min. The luminescent signal generated was measured at room temperature using a luminometer.

2.4 Enzyme Linked Immuno-sorbent Assay (ELISA)

2.4.1 ELISA solutions

TNF-α ELISA block solution: 1% (w/v) BSA, 5% (w/v) sucrose and 0.05% (w/v) sodium azide in PBS.

IL-8 block buffer: 1% (w/v) BSA in PBS

IL-8 diluents: 0.05% (v/v) Tween 20, 0.1% (w/v) BSA in PBS

ELISA buffer: 0.05% (v/v) Tween 20 in PBS.

ELISA wash buffer: 0.9% (w/v) saline supplemented with 0.05% (v/v) Tween 20

LPS solution: 10µg/ml (w/v) of LPS with 10% (v/v) NHS in PBS.

2.4.2 Indirect ELISA

2.4.2.1 Detection of soluble RAGE

In order to detect sRAGE within supernatants following transfection, 100µl of undiluted culture supernatants was added, in triplicate, directly to ELISA plates and incubated at 4°C overnight. Plates were washed with ELISA wash buffer and 100µl of polyclonal anti-RAGE antibody (1µg/ml, excess concentration) or pre-immune rabbit serum (1µg/ml) incubated for 1h at 37°C. Plates were washed and 100µl anti-rabbit-HRP (1:1000 in PBS (T)) incubated for 1h at 37°C. Bound HRP was detected by addition of OPD, the reaction was stopped by 50µl of 1M hydrochloric acid and the absorbance read at 490nm.

2.4.2.2 Detection of soluble RAGE binding to LPS

In order to detect soluble RAGE binding to LPS, 100µl of (10µg/ml excess concentration) LPS was added in quadruplet, directly to the ELISA plate and incubated at 4°C overnight. Plates were washed with ELISA wash buffers and blocked with 300µl of 0.05% (v/v) Tween in PBS for 2h at 37°C. Following incubation plates were washed and incubated with 100µl of supernatant taken from HEK293 transfected with a plasmid coding for sRAGE or control (prostate-specific antigen, PSA) for 2h at 37°C. Bound RAGE was detected with 100µl polyclonal anti-RAGE antibody (1µg/ml) or pre-immune rabbit serum (1µg/ml) incubated for 1h at 37°C. Plates were washed and 100µl anti-rabbit-HRP (1:1000

in PBS (T)) incubated for 1h at 37°C. Bound HRP was detected by addition of OPD, the reaction was stopped by 50µl of 1M hydrochloric acid and the absorbance read at 490nm.

2.4.3 Capture ELISA

2.4.3.1 TNF-α ELISA

In order to detect human TNF-α produced by THP-1-derived macrophages following LPS stimulation, ELISA plates were coated with (2µg/ml) TNF-α capture antibody diluted in PBS, overnight at 4°C. The ELISA plate was blocked for 1h at 37°C by the addition of 100µl of TNF ELISA block. After washing three times with ELISA wash buffer, 100µl of cell supernatant or known concentrations of recombinant TNF-α were added to each well and incubated for 2h at 37°C. Plates were washed and incubated with 100µl of TNF-α detection antibody (200ng/ml in PBS (T)) for 2h at 37°C. Plates were washed and 100µl streptavidin-HRP (1:2000 in PBS (T)) was incubated for 1h at 37°C. Bound HRP was detected by the addition of 100µl of OPD and the reaction was stopped by the addition of 50µl of 1M hydrochloric acid. The plates were read at absorbance 490nm.

2.4.3.2 IL-8 ELISA

In order to detect IL-8 produced by HeLa following LPS stimulation, ELISA plates were coated with (5µg/ml) IL-8 capture antibody diluted in PBS overnight at room temperature. Plates were blocked for 1h at room temperature by the addition of 300µl of IL-8 ELISA block. After washing four times with ELISA wash buffer, 100µl of cell supernatant or known concentrations of recombinant human IL-8 were added to each well and incubated for 2h at room temperature. Plates were washed and incubated with 100µl of IL-8 detection antibody (250ng/ml in diluents) for 2h at room temperature. Plates were washed and 100µl avidin-HRP (1:2000 in PBS (T)) was incubated for 30min at room temperature. Bound HRP was detected with the addition of 100µl of OPD and the reaction was stopped following the addition of 50µl of 1M hydrochloric acid. The plates were read at absorbance 490nm.

2.4.3.3 Soluble CD14-Fc ELISA

In order to map 61D3/MEM18 binding to sCD14 point mutants ELISA plates were coated with (5µg/ml) sheep anti-human-Fc in PBS at 4°C overnight. 100µl of sCD14 mutants were added to wells in triplicate and plates incubated for 1h at 37°C. Plates were washing and mAbs added in excess: (1:100) 61D3, MEM18 (8µg/ml), 63D3(1:100) or MOPC 21 (10µg/ml) for 1h at 37°C. Any binding was detected with anti-mouse-HRP (1:2000 in PBS-T) for 1h at 37°C. Bound HRP was detected with the addition of 100µl of OPD and

the reaction was stopped following the addition of 50µl of 1M hydrochloric acid. The plates were read on a plate reader at absorbance 490nm.

2.4.3.4 LPS-binding ELISA

In order to map the binding of LPS to sCD14 point mutants, ELISA plates were coated with 100µl of 5µg/ml of sheep anti-human-Fc in PBS at 4°C overnight. 100µl of sCD14-Fc mutant was added to wells in triplicate and plates incubated at 37°C for 1h. Plates were washed with ELISA wash buffer and non-specific binding of LPS blocked with 300µl of goat serum overnight at 20°C while control wells were incubated with ELISA buffer. 100µl of LPS solution (10µg/ml excess concentration) was added to each mutant and incubated at 37°C for 1h, again control wells were incubated with ELISA buffer. Soluble CD14 mutants were detected with excess amount of 63D3 ((1:100) positive control) or MOPC 21(10µg/ml) negative isotype-control) while LPS binding was detected with anti-LPS antibody (1:2000 in PBS-T) for 1h at 37°C. 63D3 and MOPC 21 binding was detected with anti-mouse HRP (1:2000) while anti-LPS binding was detected with anti-rabbit HRP (1:1000). Bound HRP was detected with the addition of 100µl of OPD and the reaction stopped following the addition of 50µl of 1M hydrochloric acid. The plates were read on a plate reader at absorbance 490nm.

2.4.4 Competition ELISA

2.4.4.1 MEM18 and biotinylated-61D3

In order to establish the independent binding of MEM18 and 61D3 to CD14 a competition ELISA conducted. Prior to competition ELISA, both MEM18 and biotinylated-61D3 were titrated to establish required concentrations (figure 6). Following titration ELISA plates were coated with 100µl of 5µg/ml of anti-human-FC in PBS at 4°C overnight. Plates were washed and 100µl of soluble wtCD14 (1:10 in PBS-T) was added to wells in triplicate and incubated at 37°C for 1 h. All concentrations of MEM18 were made to double-concentration in PBS-T and 50µl added to appropriate wells followed immediately by 50µl of competing mAb (61D3-biotin final concentration of 100ng/ml) and incubated for 1h at 37°C. Plates were washed and incubated with streptavidin-HRP (1:2000) for 1 h at 37°C. Bound HRP was detected with the addition of 100µl of OPD and the reaction stopped by the addition of 50µl of 1M hydrochloric acid. The plates were read on a plate reader at absorbance 490nm.

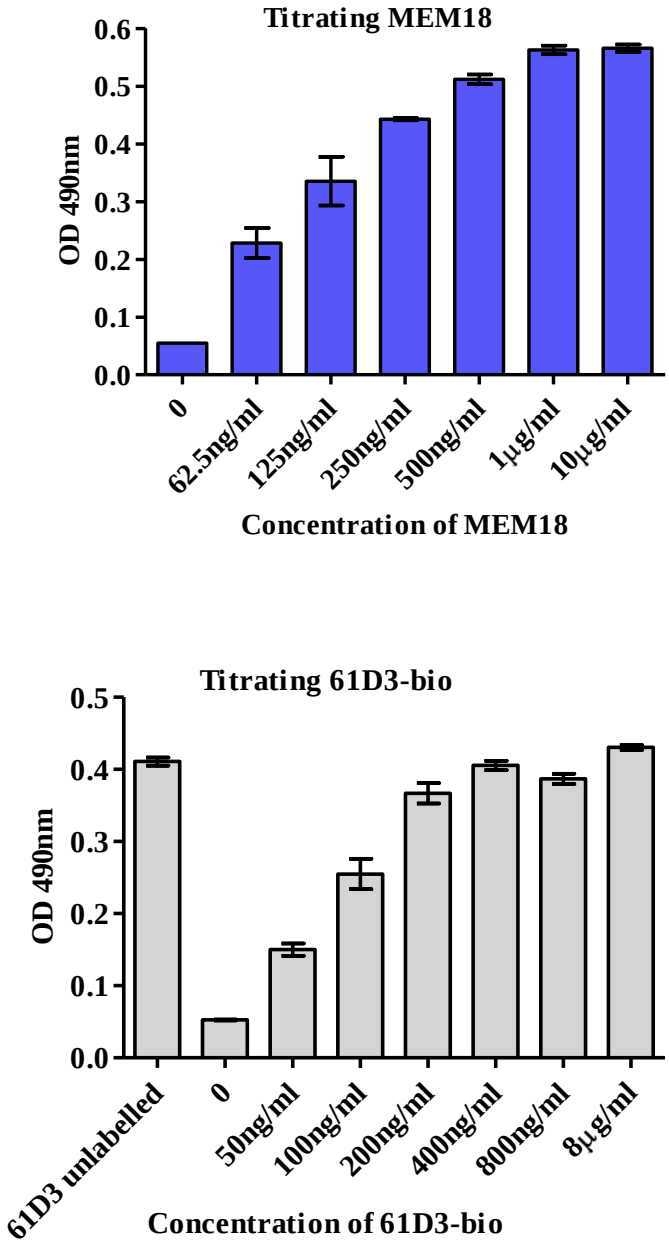

Figure 6: Titrating MEM18 and biotinylated-61D3 for competition ELISA
Soluble wtCD14-Fc was immobilised from supernatant by capture with 5µg/ml of sheep anti-human Fc for 1h at 37°C. Following incubation, the indicated concentrations of 61D3 (biotin) or MEM18 was added and plates incubated for 1h at 37°C. Plates were washed and incubated with streptavidin-HRP (1:2000 for detection of biotin) or anti-mouse-HRP for 1h at 37°C. Bound HRP was detected with the addition of 100µl of OPD and the reaction stopped by the addition of 50µl of 1M hydrochloric acid. The plates were read on a plate reader at absorbance 490nm. Results are mean ± SD.

2.5 Molecular Biology

2.5.1 Molecular Biology reagents

Receptor for Advanced Glycation End-products (RAGE) DNA was a gift from Dr Susan Staddon (Aston University). pSecTag expression plasmids, One Kilobase DNA ladder, Zeocin, and pSecTag expression plasmids were purchased from Invitrogen (Renfrew, UK)

2.5.2 Molecular Biology solutions

TE buffer: 10mM Tris pH 8.0 containing 1mM EDTA.

LB broth: 1% (w/v) Bacto Tryptone (Oxoid, Basingstoke, UK), 0.5% (w/v) Bacto yeast extract (Oxoid), 0.5% (w/v) sodium chloride in dH_2O.

LB agar: 1.5% (w/v) Bactoagar (Oxoid) in LB broth.

GTE: 50mM glucose (Fisher), 25mM Tris pH 8.0, 10mM EDTA solution and autoclaved.

Bacterial cell lysis buffer: 10% (v/v) sodium dodecylsulphate solution (SDS) (Fisher), 2% (w/v) sodium hydroxide in dH_2O.

2.5.3 Bacterial cell lines

Novablue single competent cells (Novagen, Nottingham, UK): were purchased as ready use competent cells for transforming plasmid DNA

***E. coli* DH5α cells:** Competent DH5α cells were a gift from Dr David Nagal (Aston University) and used for transforming plasmid DNA.

2.5.4 Preparation of DNA

2.5.4.1 Primers design

All primers were purchased from Functional Genomics (Birmingham, UK)

RAGE forwards primer: 5’ CAG GTA CCG CTC AAA ACA TCA CAG 3'

RAGE untagged reverse primer: 5’ CAC TCG AGC AGA GCC TGC AGT TG -3'

RAGE tagged reverse primer: 5’ CAC TCG AGA GCC TGC AGT TG 3'

2.5.4.2 General PCR and colony PCR

All PCR reactions were performed in 50µl reaction volumes, containing 50ng of DNA or a bacterial colony, 50pmol of reverse and forward primers (25pmol/µl) and 25µl of AcciSure (Bioline, London, UK). All PCR reactions were carried out in a PCR machine employing a

standard PCR cycle of 30 cycles at the following temperatures: 94°C for 30 secs, 58°C or 30 secs and 72°C for 1min.

2.5.4.3 Gel electrophoresis

1% (w/v) agarose gels were prepared using electrophoresis grade agarose (Fisher) and 1X TAE buffer with the addition of eth idium bromide at a final concentration of 0.5µg/ml to stain DNA for visualisation (DNA samples were mixed with 6X gel loading buffer and loaded into wells; 5µl of 1 kb DNA ladder was also loaded into gel). Gels were electrophoresed 100 Volts, 150 Watts 250 mA and viewed using a UVP transilluminator.

2.5.5 Isolation of DNA from gel (freeze/squeeze or purification kit)

DNA to be purified was separated on a 1% agarose gel and bands excised using a sharp clean scalpel. The gel slices were frozen in liquid nitrogen then were wrapped in Parafilm, gently squeezed to thaw and the liquid collected (freeze/squeeze). One tenth the extraction volume of 3M sodium acetate and twice the extraction volume of 100% ethanol was added. The precipitated DNA was pelleted at 14,400xg for 5min, dried by aspiration and resuspended in 50µl of TE buffer then stored at -20°C until required. DNA was also isolated from gels using a purification kits (Qiagen,Crawley,UK) in accordance with the manufacturer's instructions.

2.5.6 DNA Quantitation

DNA concentration was estimated by comparison to quantitative DNA molecular ladder using agarose gels prepared as outlined above or with the use of a nanodrop machine.

2.5.7 Restriction enzyme digestion

DNA for digestion was incubated with the desired restriction enzymes (Promega, Southampton, UK) diluted with the appropriate buffers in 20µl reaction volumes prior to incubation at 37°C for at least 2h. Following digestion restriction enzymes were either heat inactive or DNA isolated from a gel (as outlined in section 2.5.5) or using phenol-chloroform extraction. Purification of plasmid DNA by phenol-extraction was carried out following DNA precipitation by the addition of an equal volume of phenol chloroform: isoamyl alcohol which was then vortexed to form an emulsion before being centrifuged (14,400xg 5min). The upper aqueous phase was removed and the process repeated to prevent DNA loss. DNA was recovered by ethanol precipitation.

2.5.7.1 Antarctic Phosphatase

Following digestion, DNA was treated with Antarctic phosphates (Biolabs, London, UK) to remove 5'phosphate groups and prevent self-ligation. One tenth the volume of Antarctic phosphatase reaction buffer was added for 1-5µg of digested DNA. 5 units of Antarctic phosphatase were added and incubated for 15min at 37°C then heat-inactivated for 15min at 65°C prior to down-stream manipulations.

2.5.8 Ligation reactions

Ligations were performed in 20µl reaction volumes in (1X) T4 ligation buffer (Promega), at a 3 molar ratio of insert DNA to plasmid DNA and T4 DNA ligase (Promega). Ligation reactions were incubated for 16h at 14°C.

2.5.9 Sequencing reactions

Sequencing reactions were carried out in conjunction with Birmingham University Functional Genomics Laboratory and performed in accordance with supplier's instructions.

2.5.10 Transformation of competent bacteria

2.5.10.1 DH5α cells

Competent DH5α cells were thawed on ice, mixed with pcDNA3 plasmids (10ng) and incubated on ice for 20-30min prior to heat shock (1min and 20 secs at 37°C) then returned to ice for 5min. Sterile LB broth (900µl) was added and the cells cultured (50min at 37°C) to allow for expression of antibiotic resistance conferred from transformed plasmid. Aliquots of transformed bacterial cells containing pcDNA3 plasmid were spread on LB agar plates containing 50µg/ml of ampicillin and incubated at 37°C overnight.

2.5.10.2 Novoblue cells

Novoblue single competent bacterial cells were used for the growth of pSec Tag 2 plasmid and therefore selected by zeocin resistance (50µg/ml). Novoblue cells were transformed in accordance with the manufacturer's instructions.

2.5.11 Small scale Plasmid Preparation ('Mini prep')

1.5ml of overnight liquid culture was pelleted (14,400xg for 5min) and the bacterial cells resuspended in 200µl of GTE. Bacterial cells were then lysed with the addition of 400µl bacterial cell lysis buffer and incubation on ice. Genomic DNA was removed with the addition of 300µl of 3M potassium acetate (Fisher), incubation (5min on ice) to allow precipitation and removed by centrifugation (14,400xg for 5min) leaving the plasmid DNA

in solution. Plasmid DNA was precipitated from solution with the addition of 600µl of ice-cold isopropanol then centrifuged (14,400xg for 5min), the remaining pellet was washed with 70% (v/v) ethanol, dried by aspiration and resuspended in 20µl TE buffer.

2.5.12 Large scale plasmid preparation ('Maxi prep')

Plasmid DNA was recovered from bacterial cells grown in 50ml selective liquid cultures grown overnight using the pure yield plasmid midi prep system (Promega) in accordance with the manufacturer's instructions.

2.6 Preparation of RAGE

2.6.1 Preparing the expression plasmid pSec Tag2

pSec Tag 2 A, B and C are 5.2kb expression plasmids (Invitrogen, Paisley.UK) and vary by the reading frame that the insert is subcloned into. The plasmids contains an Ig κ-chain leader sequence upstream of the MCS, allowing secretion and a *myc* epitope and six histidine residues that allow detection and purification of the product downstream MCS (figure 7). All three plasmids were used to ensure that the RAGE insert would be inserted in the correct reading frame allowing the transient expression of sRAGE in HEK 293 cells, whilst also providing an out of frame control plasmid.

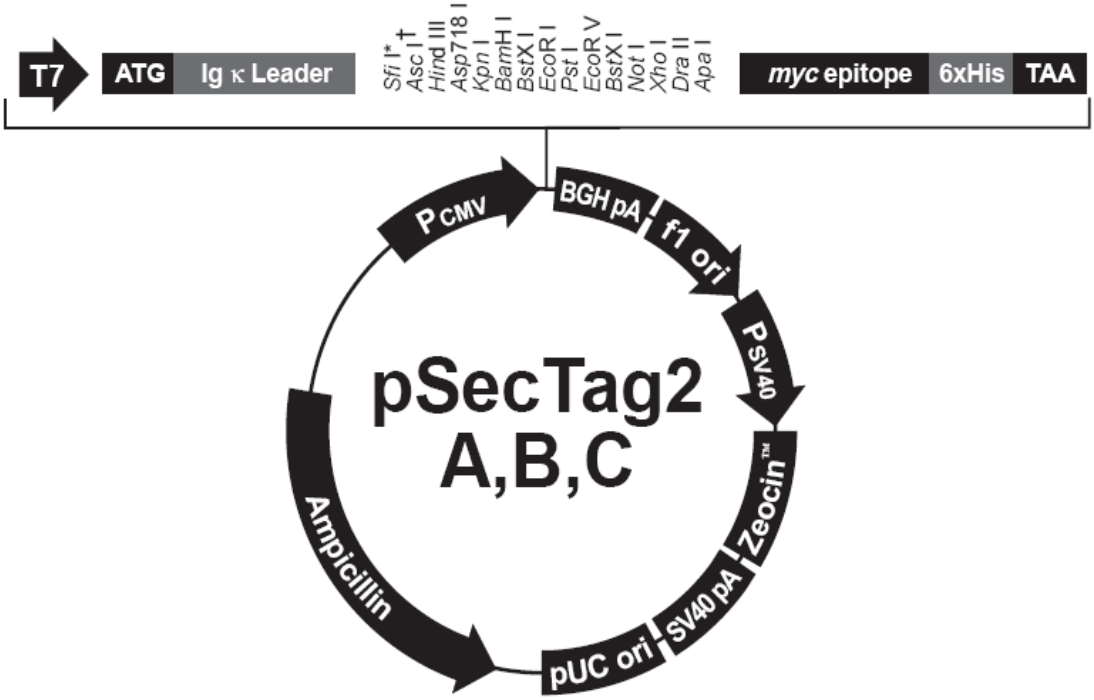

Figure 7: Map of expression plasmid pSec Tag 2
The plasmid contains an Ig κ-chain leader sequence upstream of the multiple cloning sites (MCS) allowing secretion of cloned product. Downstream of the MCS are myc-epitope and histidine tag for purification and detection. The plasmid confers both zeocin and ampicillin resistance for antibiotic selection.

All expression plasmids were digested with the restriction enzymes *Kpn* I and *Xho* I and the enzymes removed using phenol-chloroform extraction to prevent further digestion. The plasmid extracted was run on a 1% agarose gel to ensure digestion (figure 8).

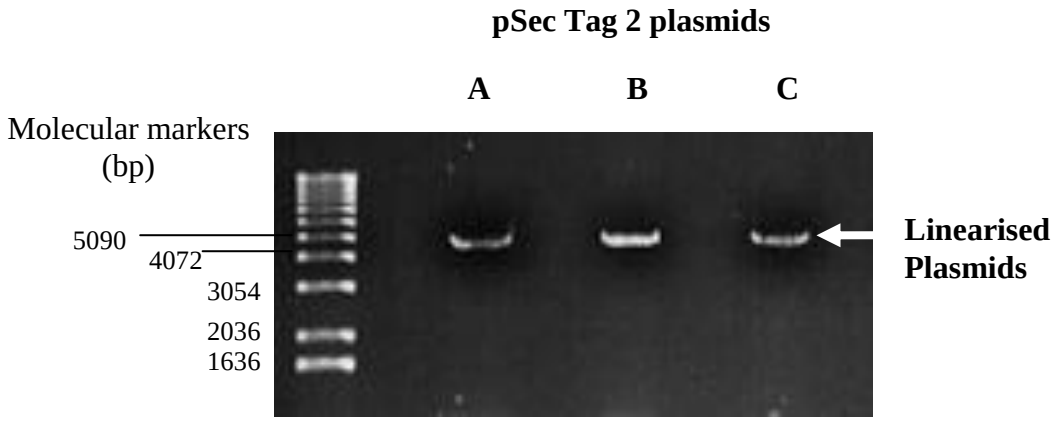

Figure 8: Agarose gel analysis of purified, digested pSec Tag2 plasmids
Expression plasmids pSec Tag 2 A, B and C were digested with *Kpn* I and *Xho* I. Following digestion, the plasmid DNA was extracted and samples were run on a 1% agarose gel. The molecular ladder used was a 1kb ladder

The expression plasmids prior to digestion are often supercoiled, which form thick, slightly smeared bands on agarose gel. However the results in figure 8 show clear single bands for the pSec Tag 2 expression plasmids that corresponds to the marker at 5090bp. The bands are linear and slightly less than 5.2kb therefore the agarose gel analysis suggests that all the plasmid has been digested and successfully isolated from restriction enzymes.

2.6.2 Amplification of RAGE

The cDNA encoding for human wildtype mRAGE (figure 9A) was kindly received from Dr Susan Staddon (Aston University). As shown in figure 9A human wildtype RAGE contains a signal sequence, an extracellular-ligand binding domain, a transmembrane domain and a short cytosolic tail. Forwards and reverse primers (section 2.5.4.1) were designed, directed against the extracellular ligand-binding domain of RAGE cDNA not including the leader sequence or the transmembrane domain thus allowing secretion of the protein following sub-cloning into expression plasmid. The predicted size of this fragment of RAGE cDNA was 930bp.

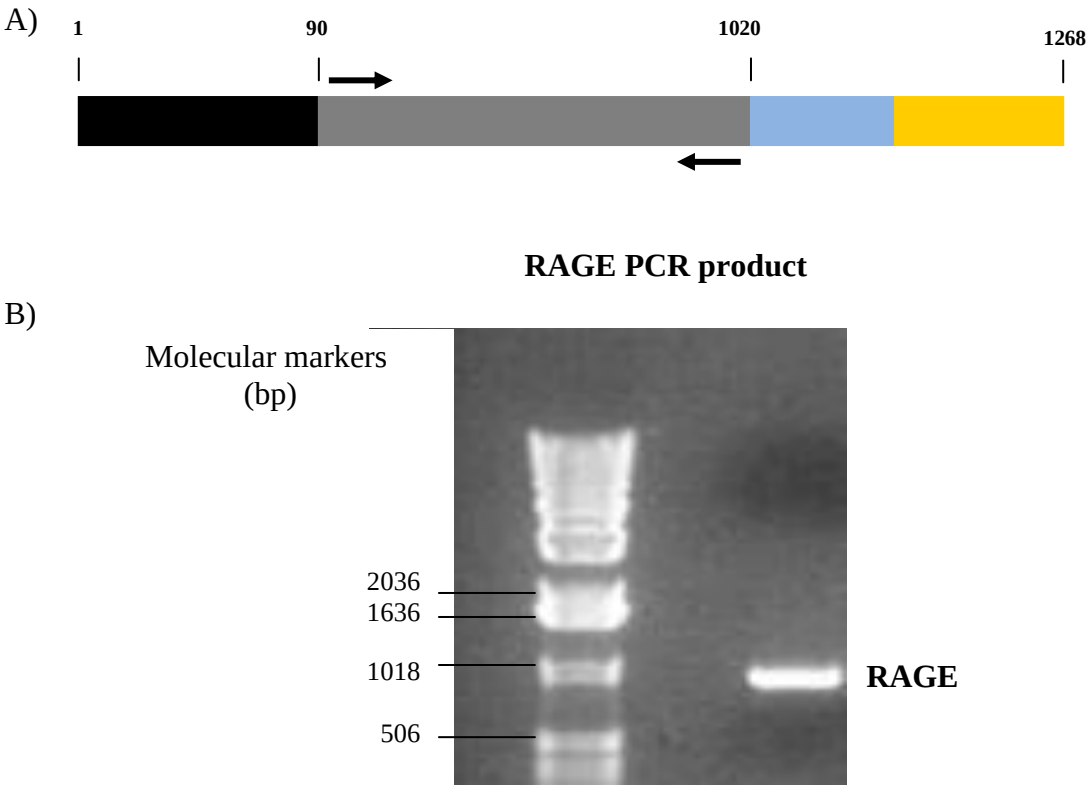

Figure 9: Human wildtype mRAGE cDNA and generation of sRAGE
A. Membrane-associated RAGE DNA contains a signal sequence (black), an extracellular ligand-binding domain (grey), one transmembrane domain (blue) and a short cytosolic tail (yellow). Primers (arrows) were designed, directed against the extracellular region of RAGE generating a PCR product estimated at 930bps in length. **B.** The PCR product was run on a 1% agarose gel to verify the size of the product in comparison with the expected size of RAGE 930bp. *(The molecular ladder used was a 1kb ladder*)

The predicted length of RAGE PCR product is 930bp and analysis of the PCR-product on the agarose gel in figure 9B shows a single band just below the marker at 1018bp suggesting the PCR reaction amplified the region of interest. The PCR product was extracted from the gel in figure 9B and digested with *Kpn* I and *Xho* I generating sticky ends that permit directional ligation within the digested pSec Tag2 expression plasmids digested and isolated.

2.6.3 Sub-cloning RAGE into pSec Tag 2 expression plasmids

Ligation of the purified RAGE PCR product with digested pSec Tag 2 expressions plasmids occurred at a molar ratio of 3:1. Following ligation the product was transformed into competent Novablue cells and selected on agar plates containing the antibiotic zeocin. Individual resultant colonies were grown over night and small scale preparations of DNA

purified. To confirm the identity of colonies containing the RAGE insert, plasmid DNA was isolated and digested with *Kpn* I and *Xho* I (figure 10).

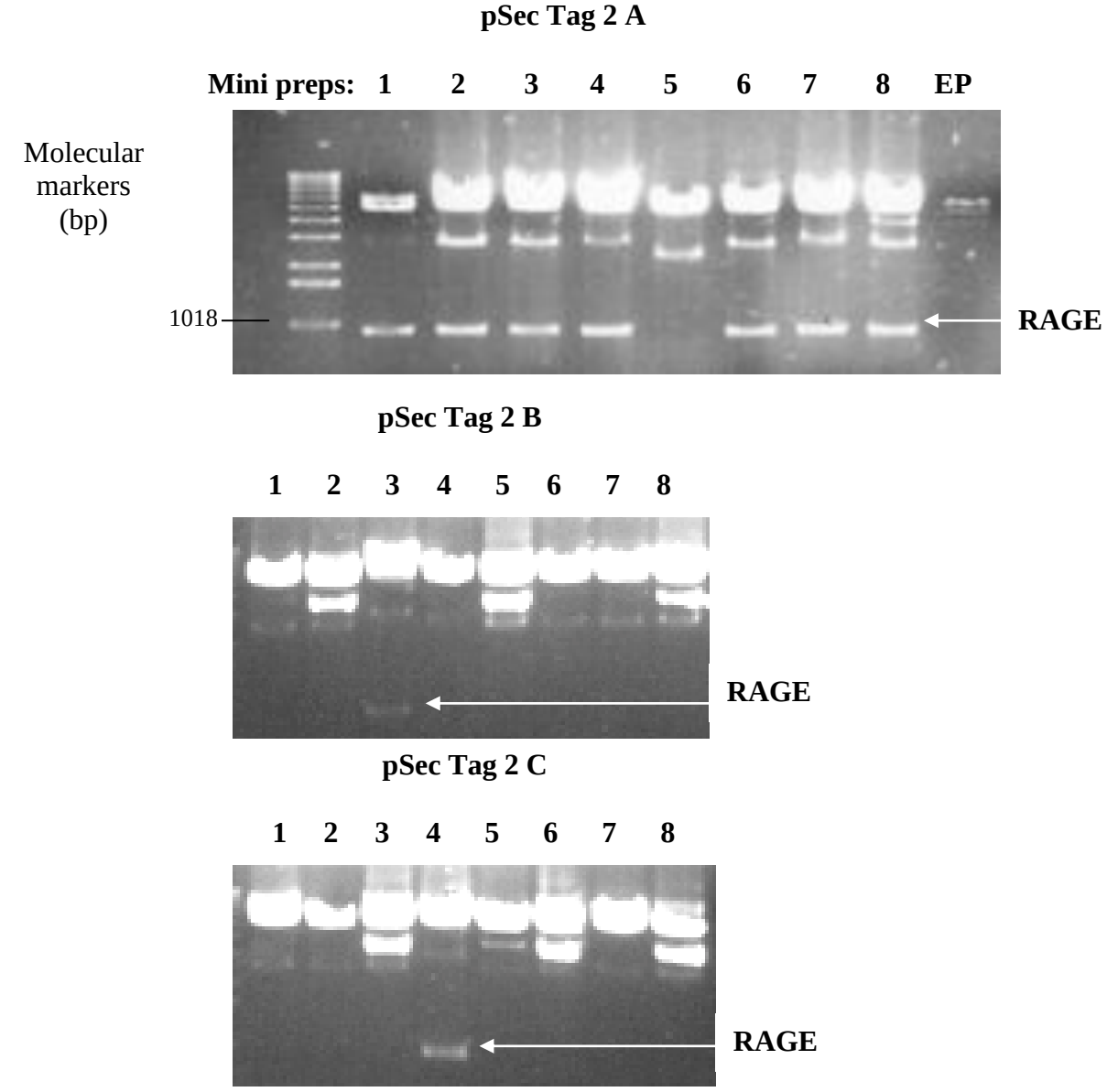

Figure 10: Digestion of ligation products for detection of RAGE insert
Competent cells were transformed with pSec Tag 2A, B and C containing the RAGE insert. Bacterial cells were cultured, plasmid DNA purified and digested with *Xho* I and *Kpn* I to confirm the presence of the RAGE insert. (8 colonies for each plasmid (pSec Tag 2 A, B, C were prepared). The predicted RAGE insert size is 930bp.

Agarose gel analysis of the midi preps in figure 10 was used to identify bacterial cultures containing plasmid with RAGE insert. Samples from cultures A2, B3 and C4 were used to inoculate 50mL Zeocin-selective cultures for large scale preparations of plasmids and sequencing. Having identified colonies of interest and prepared plasmids containing the putative insert in each of the reading frames (pSec Tag 2 A, B and C) it was necessary to assess the ability of the clones following transfection to produce sRAGE. Sequence

analysis predicted clone B would produce sRAGE protein whilst A and C were cloned out of frame.

2.6.3.1 Indirect ELISA for detection of sRAGE

To establish which plasmid contained sRAGE within the correct reading frame, each of the plasmids isolated from cultures A2, B3 and C4 were transiently transfected into HEK293 and cultured for 72 hours. Following incubation cell culture supernatants were collected and the presence of sRAGE confirmed by indirect ELISA (figure 11).

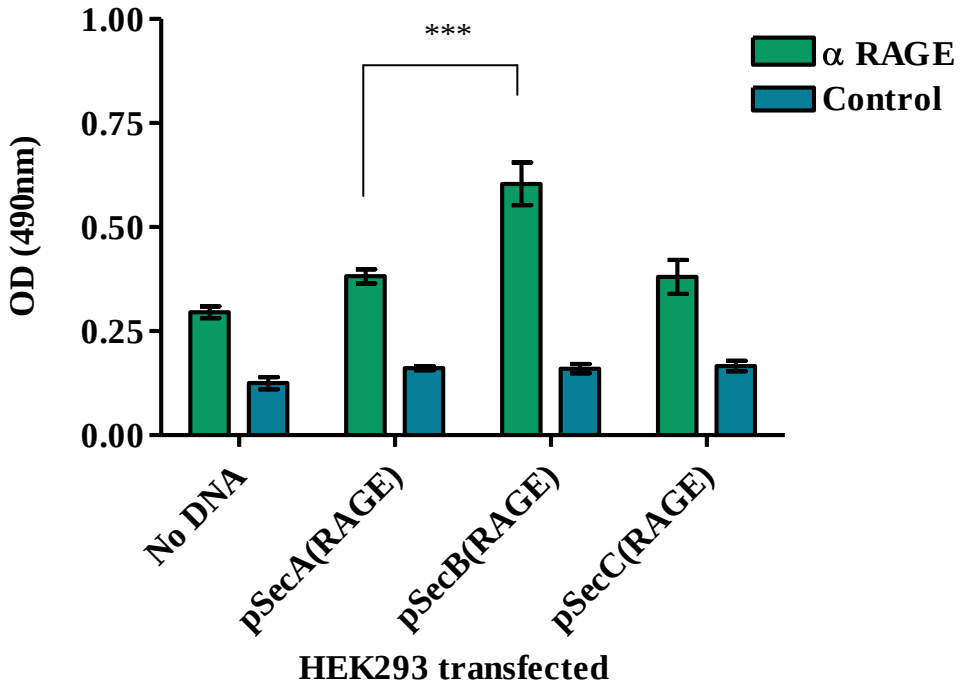

Figure 11: Detection of sRAGE by indirect ELISA
HEK293 were transiently transfected using calcium phosphate with 2µg of pSec Tag 2 A, B and C containing the RAGE insert or no DNA. Cells were cultured in sfDMEM for three days and the supernatants collected for analysis. ELISA plate were coated with 100µl of supernatant and incubated at 4°C overnight. Following washing the plate was incubated with rabbit polyclonal anti-RAGE antibody or negative control pre-immune rabbit serum (control). Any binding was detected using anti-rabbit HRP conjugated antibody and the ELISA plate was developed with OPD and read at 490nm. The data shown are the mean ± SD of triplicate well and is representative of two independent experiments. *Statistical analysis was conducted using ANOVA followed by Bonferroni post-test. (***P<0.001).*

Soluble RAGE was detected in supernatant taken from HEK293 transfected with pSec Tag 2B (figure 11). Further sequence analysis of the sRAGE insert confirmed that the N-terminus of the RAGE insert was in frame to allow expression but the reverse primer resulted in an out of frame C-terminus. Therefore the RAGE protein expressed did not contain c-myc epitope or polyhistidine tags making purification and detection difficult. The reverse primer was redesigned and sRAGE subcloned into pSec Tag2B, expression was

verified following sequence analysis and ELISA following transfection. The control plasmid pSec Tag2/PSA which expressed the prostate-specific antigen upstream of c-myc epitope and polyhistidine tag was also transformed ready for transfection.

2.6.4 Cloning of tagged RAGE

To produce sRAGE with the polyhistidine tags for purification and detection, another reverse primer was designed and used to clone sRAGE into expression plasmid PSec Tag 2B, in frame with the C-terminal producing the myc-epitopes and tags. The PCR product was run on agarose cell to confirm that the PCR reaction had amplified the RAGE DNA and that it was the expected size (figure 12).

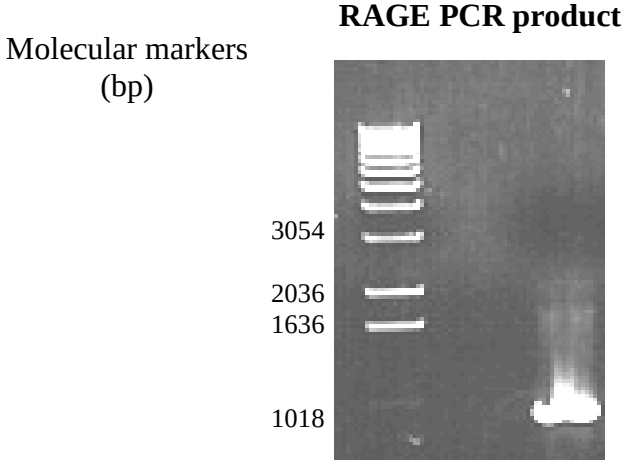

Figure 12: Producing tagged RAGE: Agarose gel analysis of PCR product
The forwards primer and a new reverse primer were used to amplify the extracellular domain of RAGE. The PCR product was run on a 1% agarose gel to ensure the PCR reaction was successful and had produced a single band of the expected size.

The gel in figure 12 shows a single band for RAGE PCR product just below the molecular marker at 1018bp which corresponds with the expected size of 930bp of RAGE therefore suggesting that PCR reaction was successful and that RAGE has been digested. The RAGE PCR product was isolated from the gel, purified and then sequentially digested with *Xho* I and *Kpn* I.

2.6.4.1 Preparation of plasmid (only pSec Tag 2B)

The expression plasmid pSec Tag 2B was digested with restriction enzymes *Xho* I and *Kpn* I then subsequently treated with Antarctic phosphatases (2.5.7.1) to remove the phosphate from the 5' end nucleotide of the plasmid therefore preventing self-ligation.

2.6.4.2 Cloning tagged RAGE

Following ligation (2.5.8) the plasmids were transformed in DH5α cells (2.5.10.1) and selected for by ampicillin resistance as this plasmids also contains an ampicillin resistance gene. Colony PCR (2.5.4.1) was conducted using T7 promoter and BGH reverse primers and the results revealed which bacterial colonies contain the plasmid with the RAGE insert (figure 13).

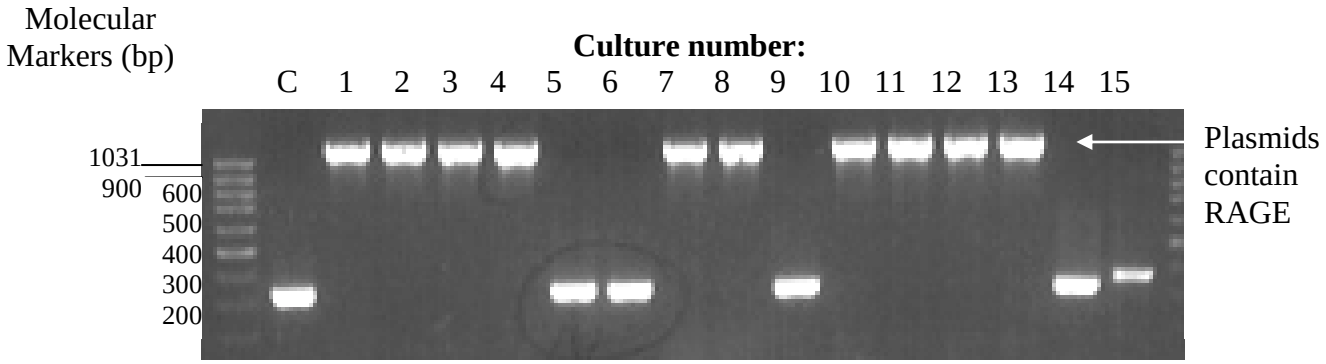

Figure 13: Gel analysis of colony PCR
DH5α were transformed with pSec Tag 2B containing the RAGE insert then cultured with ampicillin selection. Several colonies were screened using colony PCR for the presence of the RAGE insert using T7 promoter and BGH reverse primers. (C is the empty plasmid, and the bands circled represent self-ligation of plasmid).

The PCR products from bacterial colonies that contain plasmid and insert will generate products over 1031bp in size, those bands that are around 300bp in size are empty plasmid products. Therefore the results suggested that cultures 1-4, 7, 8 and 10-13 contained the inserts. One of the colonies containing the RAGE insert was used to prepare mini preps (2.5.11) and the DNA isolated was sequenced (2.5.9) then a large scale preparation of the plasmid produced (2.5.12).

2.6.5 Preparing control plasmid, pSec Tag2/PSA

The control plasmid pSec Tag2/PSA expressed and secreted the prostate-specific antigen (PSA) upstream of *c-myc* epitope and the polyhistidine tag. The control plasmid was first transformed into DH5α cells (2.5.10.1) and selected on ampicillin plates. Single colonies were used to inoculate 50ml cultures for large scale plasmid preparation (2.5.12) ready for transfection into mammalian cells.

2.7 Preparing membrane CD14 mutants

2.7.1 Human CD14-Ig plasmids received

A number of plasmids encoding for CD14-Fc constructs that contained single amino acid replacement within the NH_2-terminus of CD14 were generated and kindly received from

Richard Darveau (University of Washington, USA) (Table 1).The plasmids received were constructed by subcloning the extracellular region of wtCD14 upstream of cDNA encoding H, CH2 and CH3 domains of human IgG1, into the expression plasmid CDM7B. The mutations were then introduced at specific site by oligonucleotide-directed mutagenesis (Cunningham *et al.,* 2000)

Human fusion proteins received	Location of mutation (LPS binding regions
Wt CD14	wildtype
E11A E11D E11R	1st LPS binding region
R14A R14K	Outside 1st LPS binding region
E37D E37Q	3rd LPS binding region
D59A D59N	4th LPS binding region

Table 1: CD14-Fc constructs received from R. Darveau (Washington Univ)
pcDNA 3.1 (+) expression plasmid cut with the restriction enzymes *Hin*d III and *Xba* I to allowing insertion of hCD14 and Ig. Restriction enzymes *Hin*d III and *Xho* I were used to replace 5' fragment of wild-type CD14 with mutated fragments producing mutated soluble CD14-Fc proteins.

2.7.2 Making CD14 mutants membrane-bound

Membrane wtCD14 has previously been subcloned into pcDNA3 and supplied courtesy of Christopher Gregory (figure 14). Absence of the Fc tag will allow the addition of a glycosylphosphatidylinositol tail onto the C-terminus of the protein during post-translation (Bufler *et al.,* 1995) and therefore will anchor CD14 to the membrane of the mammalian cells transfected.

A. Membrane Wt human CD14 plasmid

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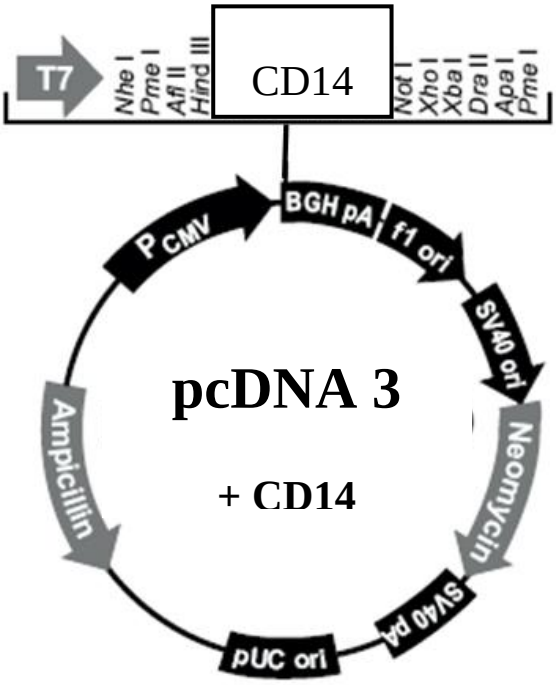

B. Subcloning membrane-bound CD14 point mutants

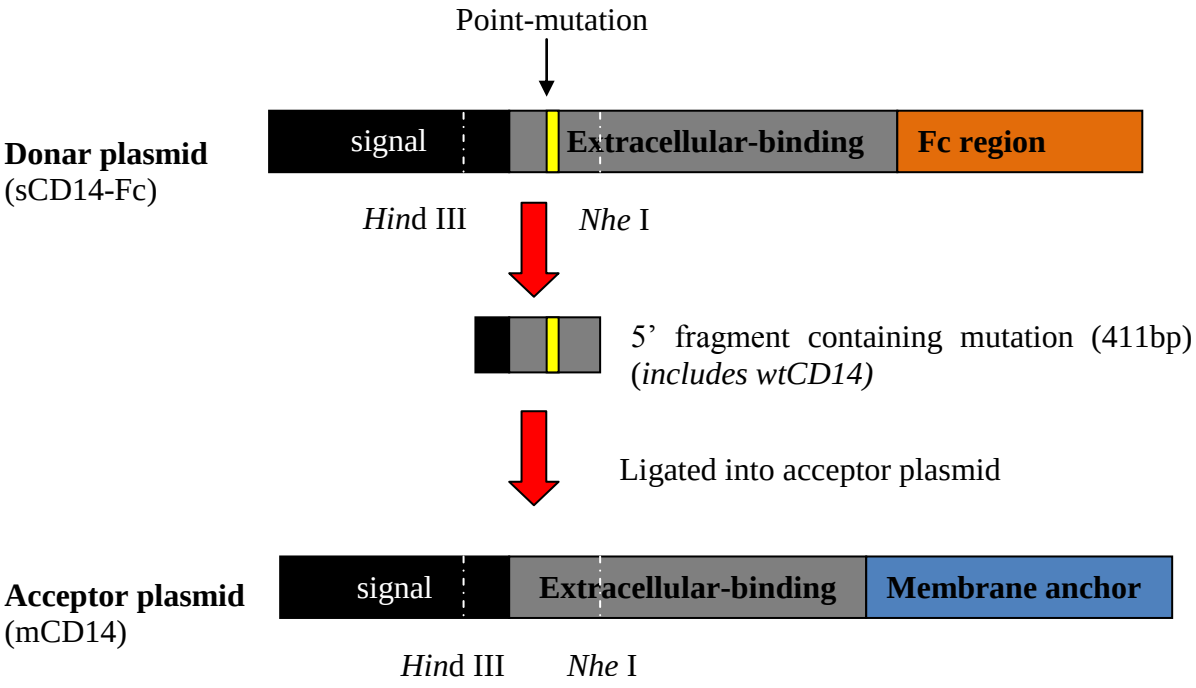

Figure 14: Subcloning wildtype and mutant mCD14
A. Devitt *et al.*(2004) previously used human CD14 ligated into pcDNA3 which following transfection results in the addition of glycosylphosphatidylinositol tail therefore anchoring CD14 to the membrane. B. To generate membrane-bound CD14 point mutations, restriction enzymes *Hin*d III and *Nhe* I were consecutively used to excise the 5' mutated region from sCD14-Fc plasmids and were ligated into a similarly cut mCD14 vector.

2.7.2.1 Preparing the acceptor plasmid

1µg of the mCD14 plasmid DNA (figure 14a) was digested with 5 units of *Hin*d III and 4 units of *Nhe* I in Buffer M (Roche) overnight at 37°C (figure 15).

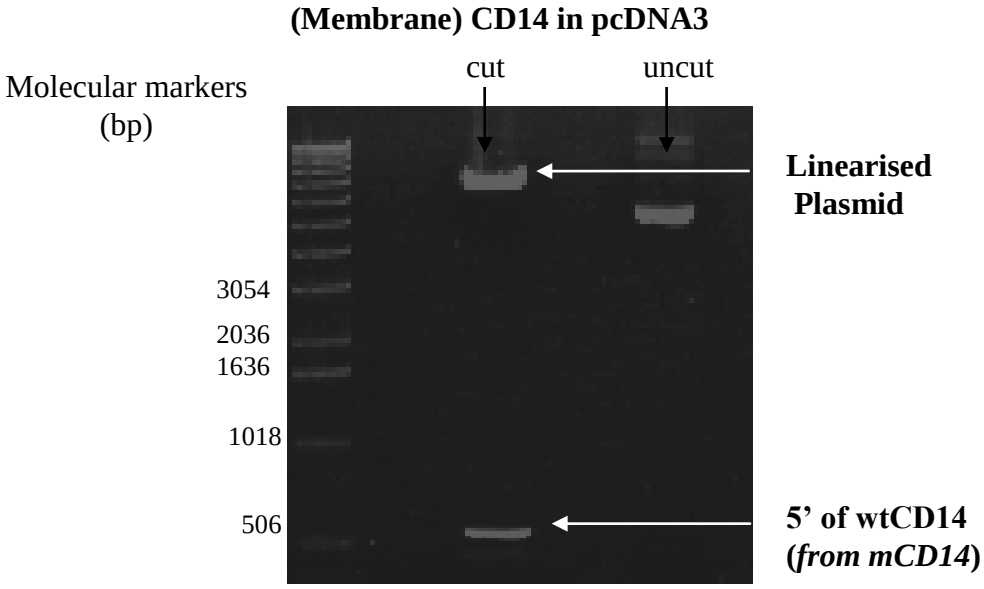

Figure 15: Digesting the mCD14 (acceptor plasmid)
1µg of the mCD14 plasmid was digested with *Hin*d III and *Nhe* I overnight at 37°C then run on a 1% agarose gel to ensure digestion. Uncut and digested mCD14 pcDNA3 was run alongside each other for comparison. The molecular ladder used was 1kb ladder.

The mCD14 plasmid is approximately 6785bp and following digestion with *Hin*d III and *Nhe* I a small segment of CD14 DNA containing the mutation, estimated at 411bps in length was excised. Following digestion the acceptor plasmid was treated with Antarctic phosphatase to prevent self-ligation and reforming of the original WT-expressing plasmid. Antarctic phosphatase removes the phosphate on the 5' digested mCD14 plasmid; to ensure only plasmids containing mutated CD14 inserts produce colonies. The digested mCD14 plasmid was then gel purified to remove contaminating restriction enzymes to allow future ligation.

2.7.2.2 Purification of 5'fragments for subcloning

5µg of wildtype and mutant sCD14 plasmids were digested with *Hin*d III and *Nhe* I as outlined above, and incubated at 37°C overnight (figure 16).

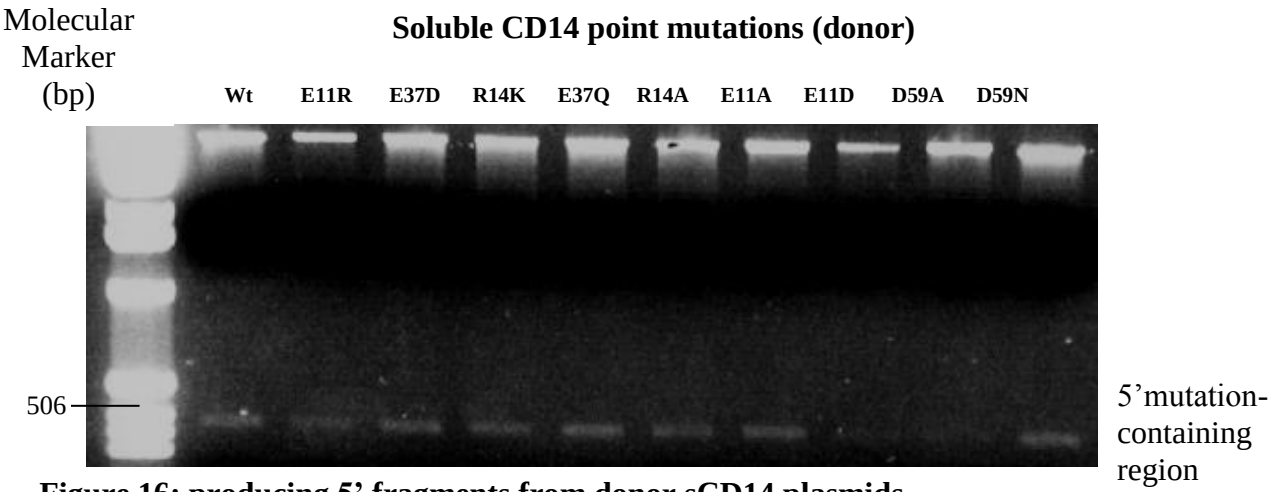

Figure 16: producing 5' fragments from donor sCD14 plasmids
1µg of donor wt CD14 and point-mutants was digested with *Hin*d III and *Nhe* I overnight at 37°C. Following incubation DNA was ethanol precipitated and run on a 1% agarose gel to ensure digestion. The molecular ladder used was 1kb ladder.

Once digestion had been determined, DNA was ethanol precipitated, resuspended in a smaller volume of water to allow all DNA to be loaded and run on a 1% agarose gel. The lower bands corresponding to the mutated 5' fragment was excised from the gel and purified using Quiagen gel purification kit to remove contaminating restriction enzymes.

2.7.2.3 Subcloning membrane-bound CD14 point mutants

Having excised the 5' fragment from donor plasmids (containing point-mutations) the next stage was ligation (section 2.5.8). Following ligation plasmids was transformed into DH5α cells (section 2.5.10.1) and colonies selected by ampicillin resistance. Colony PCR (section 2.5.5.2) was then used for sequencing the plasmids to verify the 5' fragment within mCD14 plasmids and also for large scale preparations of plasmids (section 2.5.12).

Results

Results 1: A THP-1-derived macrophage model system

3.1 Introduction

Macrophages are multifunctional cells that play a vital role in both the regulation of immune responses and tissue homeostasis (Hou *et al.,* 2000 & Mosser and Edwards, 2008). Interaction between macrophages and pathogens has been extensively studied, with many advances in the understanding of macrophage-host defences. However the macrophage is also considered the professional scavenger of apoptotic cells (Gregory and Devitt, 2004) and therefore has a vital role in homeostasis. The engulfment of apoptotic cells is crucial for many biological processes including, resolution of immune responses, tissue modelling or simply, removal of cells that are diseased or damaged (reviewed by Mosser and Edwards, 2008 & Kozmar *et al.,* 2010). In order to carry out these roles, macrophages are armed with a series of PRR; well characterised for their participation in host defence, but also identified as key receptors in the clearance of apoptotic cells (Reviewed in Savill *et al.,* 2002 & Grimsley, 2003). Furthermore apoptotic cell uptake by macrophages has been shown to actively inhibit inflammation and produce anti-inflammatory mediators (Voll *et al.,* 1997, Fadok *et al.,* 1998b & Savill *et al.,* 2002). This has led to the suggestion that apoptotic cell clearance by macrophages can shape the immune response and induce resolution (reviewed by Bratton and Henson, 2011).

The prototypic PRR, CD14 is well characterised for its ability to recognise PAMPs, most notably, LPS (Wright *et al.,* 1990 & Ziegler-Heitbrock and Ulevitch, 1993). Ligation of CD14 with LPS mediates inflammation, characterised by the release of proinflammatory cytokines including TNF-α (Cuschieri *et al.,* 2006). CD14 has also been implicated in the removal of apoptotic cells by macrophages, first demonstrated by transiently expressing CD14 in COS cells, which caused an increase in their phagocytic ability to uptake apoptotic cells (Devitt *et al.,* 1998). This suggested that CD14 is involved in the assembly of two distinct ligand-dependent macrophage responses. **The aim of this chapter is to characterise the role of CD14 in macrophage responses to LPS and apoptotic cells, in order to establish an understanding of the molecular mechanisms employed by CD14 to generate two distinct ligand-dependent macrophages responses.**

3.2 Phenotypic characterisation of THP-1-derived macrophages

THP-1 are a human monocytic leukaemia cell line that, following differentiation, produce macrophage-like cells, which are commonly used to model macrophage function (Schwende *et al.,* 1996, Park *et al.,* 2007b & Daigneault *et al.,* 2010). Here three different cell differentiation strategies were used and in each case the resultant macrophage-like cells were characterised. Cells were characterised phenotypically for changes in morphology, adherence and CD14 expression but also functionally, with respect to LPS-responsiveness and ability to bind apoptotic cells in a CD14-dependent manner. Monocyte differentiation was induced by treatment of the cells with 1,25-dihydroxyvitamin D3 (VD3), phorbol-13-acetate (PMA) or both (double-stimulation, DS) for 72 hours.

3.2.1 Morphology

Differentiation of monocytes to macrophages involves a number of morphological changes. Such changes include an increase in cell size and adhesion, a reduction in nuclear: cytoplasmic ratio and an increase in granularity, caused by an increased number of membrane-bound organelles (Hou *et al.,* 2000 & Daigneault *et al.,* 2010). Here microscopic analysis was used to detect changes in morphology and profound differences between the various differentiation treatments were noted (figure 17). PMA and double-stimulation induced extensive morphological changes, including an increase in cell size and shape. Differentiation induced cells to spread, become irregular in shape and produce extensions from the cells known as pseudopodia. Microscopic analysis also illustrated an increase in cytoplasm which was consistent with a reduction in nuclear to cytoplasmic ratio. No consistent difference in cell morphology was observed between vitamin D3 and untreated THP-1 (figure 17a).

With respect to adhesion, cells were considered adherent if they were resistant to washing by pipetting with growth medium. THP-1 and vitamin D3 macrophages were semi-adherent and removed by washing. Differentiation induced with PMA or double-stimulation caused an increase in cell adhesion and the removal of these cells required EDTA treatment and extensive washing.

Flow cytometric analysis was used to determine differences in volume and granularity between the THP-1-derived macrophages (figure 17b). Again PMA and double-stimulated macrophages consistently demonstrated an increase in electronic volume which correlated

with an increase in side scatter, suggesting increased volume and granularity. Also differentiation of macrophages is reported to cause a slight increase in autofluorescence (van Haarst *et al.,* 1994). The flow cytometric analyses suggested that differentiation of THP-1 caused an increase in the autofluorescence of unstained cells, with PMA and double-stimulated macrophages being substantial more fluorescent (figure 17c). Taken together, these data suggest that PMA and double-stimulated macrophages were larger in volume with increased granularity, characteristic traits of macrophages (Natale and McCullough, 1998 & Daigneault *et al.,* 2010). Increased granularity is caused by a greater number of organelles such as lysosomes and mitochondria, and secretory vesicles, allowing macrophages to be functionally active effector cells (Natale and McCullough, 1998 & Daigneault *et al.,* 2010).

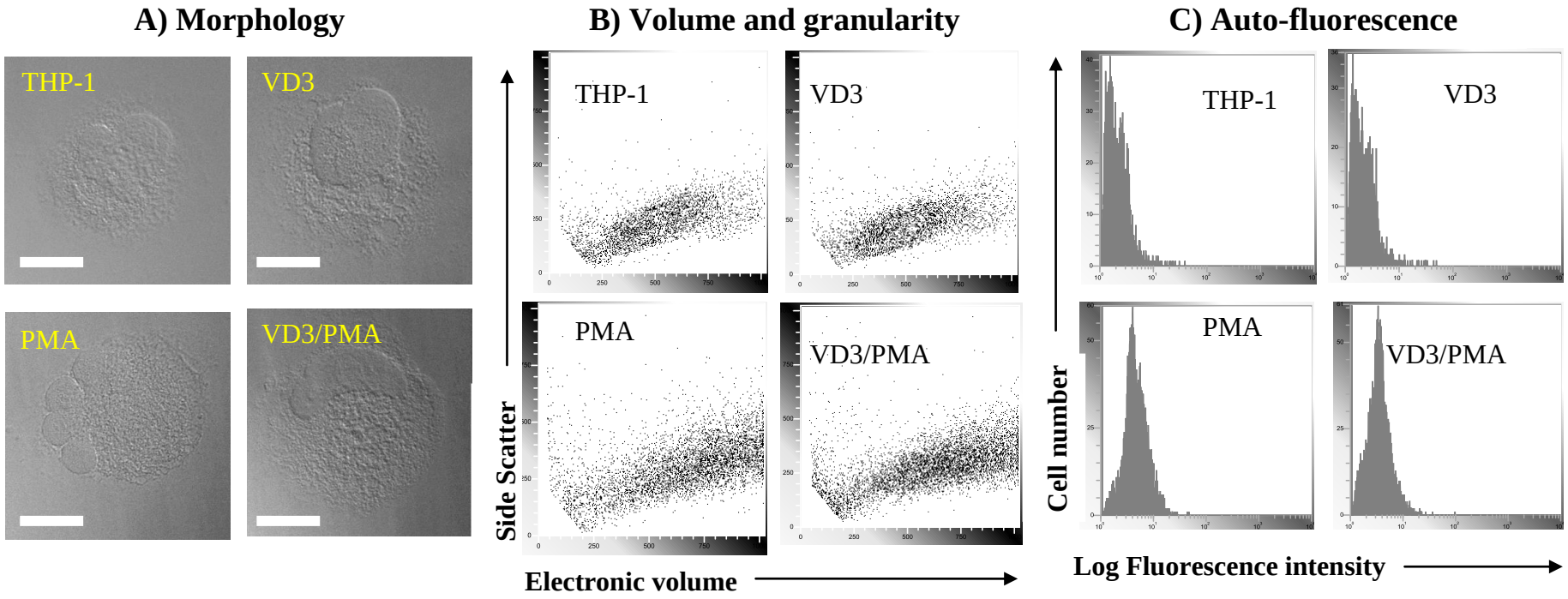

Figure 17: Microscopic and flow cytometric analysis of THP-1-derived macrophages
THP-1 were seeded to plastic tissue culture plates in the absence (THP-1) or presence of 100nM vitamin D3 (VD3), 250nM PMA (PMA) or both (vitamin D3 and PMA) for 72h. Post-stimulation microscopic analysis or flow cytometric analysis was conducted. **A.** (63x) DIC images of representative macrophages were taken (Scale bar =16µm). Post-stimulation cells were stripped up using 5mM EDTA and analysed by flow cytometry. **B**. Electronic volume against side scatter plots were generated from 10,000 events and **C**. histograms of auto-fluorescence FL1 channel (wavelength 530nm) generated using VenturiOne flow cytometric analysis software. (*The x-axis is a log scale and y axis event number*).

3.2.2 Cell surface CD14 expression

The function of CD14 in mediating response to LPS or apoptotic cells was central to this programme of work. Therefore the assessment of surface expression of CD14 in each macrophage model was vital. Cell surface expression of CD14 was assessed using indirect immunofluorescence staining of viable cells with established CD14 mAb 63D3 followed by flow cytometric analysis (Devitt *et al.*, 1998) (figure 18).

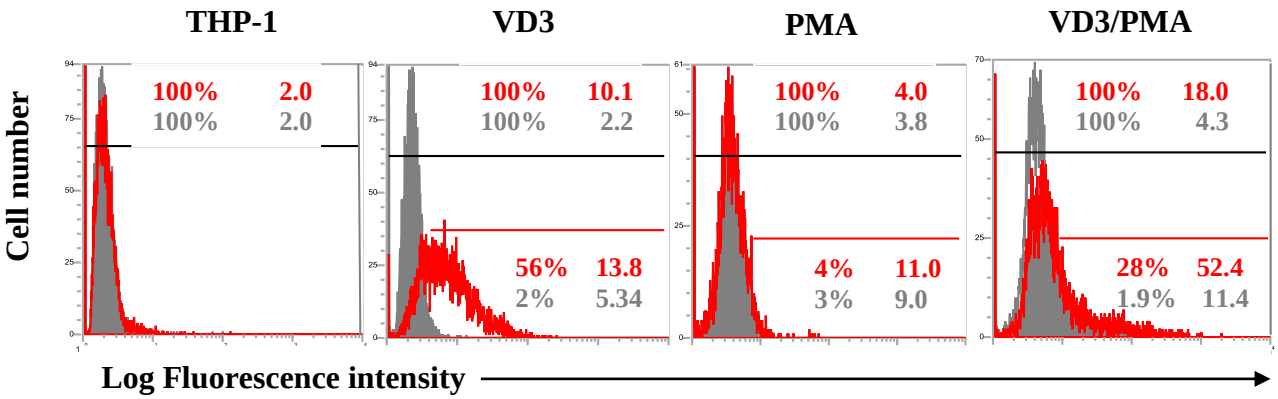

Figure 18: Cell surface expression of CD14 on THP-1-derived macrophages
THP-1 was differentiated with vitamin D3 (VD3), PMA or both (VD3/PMA) for 72 hours. Surface expression of CD14 on untreated THP-1 and resultant macrophages was detected with flow cytometric staining using indirect immunofluorescence with the monoclonal antibody 63D3 (red) or MOPC 21, isotype-control (filled grey). The percentage of cells gated and the mean fluorescence intensity is shown for both the total (black line) and positive cell regions (red line). The positive region was established by setting a 2-3% false-positive gate on MOPC 21 plot for each macrophage model using venturiOne analysis software. The results are representative of four independent experiments.

Constitutive expression of CD14 was not detected on the surface of THP-1 (THP-1 MFI 2.0 compared to MOPC 21 MFI=2.0), this is in agreement with previous studies (Schwende et al., 1996 & Hmama et al., 1999). Differentiation of THP-1 with vitamin D3 caused the population to increase in fluorescence, suggesting that CD14 expression on all cells had increased. The MFI was also considerably higher than the control, suggesting that both the number and level of CD14 expression on these cells had increased. These findings are in agreement with previous studies by Sadeghi et al. (2006) and Hmama et al.(1999) which also found that vitamin D3 treatment of THP-1 increased cell surface expression of CD14. CD14 expression on PMA macrophages was undetectable (PMA MFI=4.0 compared to MOPC 21 MFI= 3.8), this agrees with previous data published by Daigneault et al.(2010). Following double-stimulation at least 28% of the population of cells increased in fluorescence and the MFI of the cells also increased (VD3/PMA MFI= 52 compared to

MOPC 21 MFI=11.4). This suggests differentiation increases the number of cells expressing CD14 as well as increasing the level of expression on a per-cell basis.

3.3 Functional characterisation of THP-1-derived macrophages

Given the variation in CD14 expression data (figure 18), the ability of each THP-1-derived macrophage model to interact with LPS and apoptotic cells in a CD14-dependent manner was compared. Any variation in the interaction of these two ligands within a particular macrophage model may provide an understanding of the mechanistic difference underlying the role of CD14 in contrasting macrophage responses. To this end, preliminary work was necessary to establish a cell-based system that would permit this comparison.

3.3.1 LPS responsiveness

3.3.1.1 Establishing an LPS response

Preliminary experiments were conducted to examine macrophage responses to LPS by measuring TNF-α production in cell culture supernatants. Initial experiments involved seeding THP-1 at $1x10^5$ cells per well and inducing differentiation. Following differentiation for 72 hours, macrophages were stimulated with 10ng/ml of LPS, incubated for 24 hours and TNF-α levels analysed by capture ELISA (figure 19).

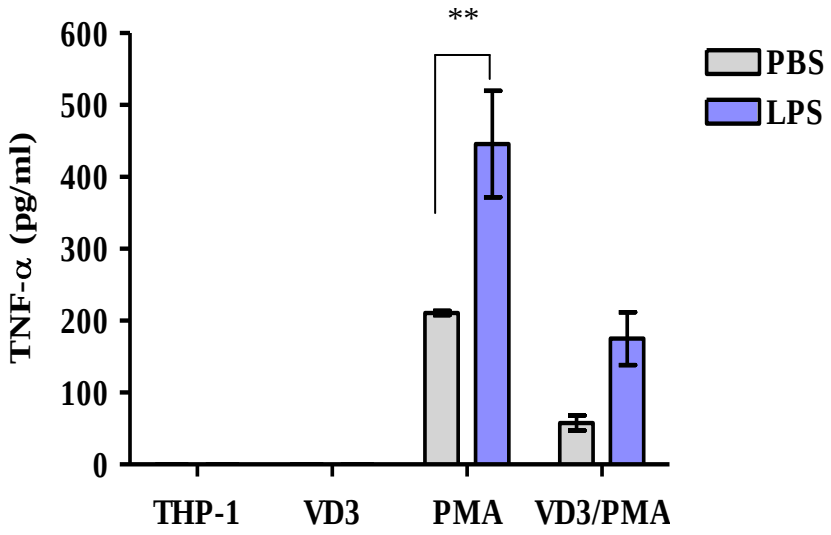

Figure 19: Establishing LPS responses for THP-1-derived macrophages
THP-1 were seeded in 24 well plates at a density of $1x10^5$ cells per well in cRPMI and induced to differentiate with vitamin D3 (VD3) PMA or both (VD3/PMA). Following differentiation for 72 hours, cells were stimulated with LPS at a final concentration of 5ng/well for 24 hours. Phagocyte response was assessed by measuring the production of TNF-α in the supernatants using capture ELISA. Mean ± SD for triplicate wells, each assayed by ELISA in triplicate are shown and results are representative of two independent experiments. *Statistical analysis was conducted using ANOVA followed by Bonferroni post test (**P<0.01).*

Following LPS stimulation, the results in figure 19 demonstrate that PMA and double-stimulated macrophages are LPS responsive, with PMA being the most sensitive to LPS treatment. Comparison of figure 19 with CD14 expression (figure 18) suggests expression of CD14 was not required for PMA macrophage to respond to LPS. CD14 is expressed on vitamin D3 macrophages however figure 19 suggests that these cells are not LPS responsive under these conditions. Therefore it was considered that the results (figure 19); particularly the vitamin D3 results, were possibly a reflection that the experiment was suboptimal. Therefore it was suggested that the experimental design be modified with regards to cell density and LPS response of each macrophage model titrated.

3.3.1.2 Optimising Cell density

Monolayer formation was assessed by seeding cells at a range of densities then following cell density over the differentiation period of 72 hours. The density of each macrophage model was assessed microscopically at 24h, 48h and 72h and scored qualitatively.

Seeding densities (cells/well)	24 hours post-stimulation				48 hours post-stimulation				72 hours post-stimulation			
	THP-1	VD3	PMA	VD3/PMA	THP-1	VD3	PMA	VD3/PMA	THP-1	VD3	PMA	VD3/PMA
1 x 10^5	+	+	+	+	++	++	+/++	+/++	+++	+++	++	++
2 x 10^5	++	++	+	+	+++	+++	++	++	+++++	+++	++	++
4 x 10^5	+++	+++	++	++	++++	+++	++	++	++++	+++	++	++
6 x 10^5	+++	+++	++	++	+++++	++++	+++	+++	+++++	+++++	+++	+++

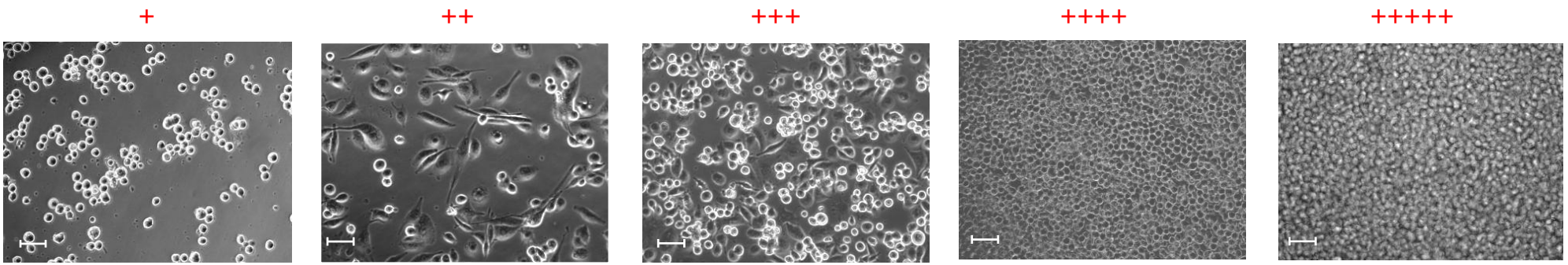

Figure 20: Microscopic analysis of THP-1-derived macrophages, for the formation of a monolayer
THP-1 were seeded at 1x10^5, 2x10^5, 4x10^5 and 6x10^5 cells per well in 24 well plates and stimulated to differentiate with vitamin D3, PMA or both (VD3/PMA). Cells were microscopically analysed for the formation of a monolayer and scored as follows: + very low density, ++ low density, +++ medium density, ++++ high density, +++++ very high density. Images of densities identified in red are also shown as examples of scores. (20x) DIC Images (scale bar = 20µm). Those scored as +++ (medium density) were considered to form a suitable monolayer**.**

The results in figure 20 generally suggest that the agents used to simulate differentiation generated cultures with profoundly different cell densities following 72 hours of differentiation. The profound differences in cell densities could be a result of varying cell divisions, differential loss or possibly fusion of cells. Untreated THP-1 and vitamin D3 macrophages increased in cell number over 72 hours causing overcrowding especially within the centre of the wells. Furthermore PMA and double-stimulated macrophages appeared not to change in cell density following 24 hours of differentiation. However PMA and double-stimulated macrophages produce a monolayer when initially seeded at $6x10^5$ cells per well. A flow cytometric-based assay of this seeding density was conducted to assess changes in cell number between macrophages models, post differentiation (figure 21).

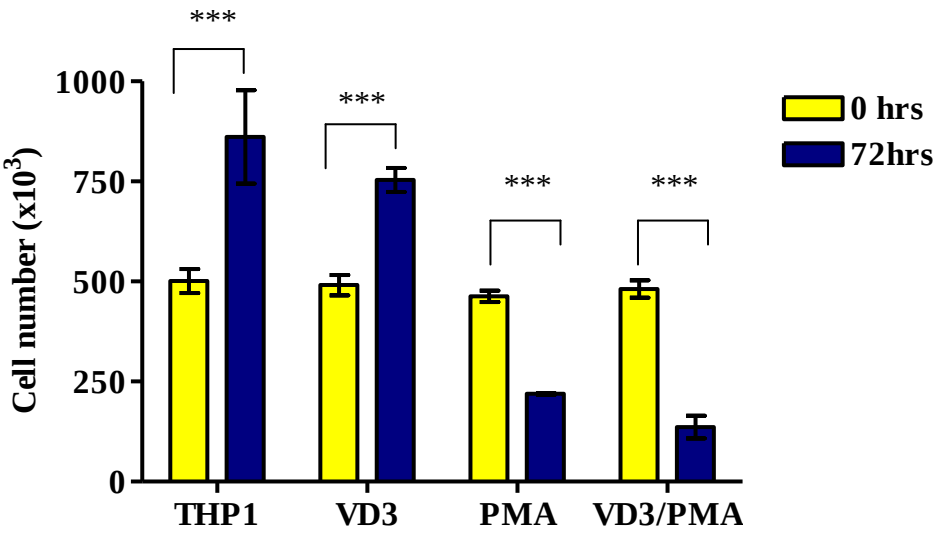

Figure 21: Flow-based analysis of cell number following differentiation
THP-1 was seeded at $6x10^5$ cells per well and stimulated to differentiate with vitamin D3 (VD3), PMA (PMA) or both (VD3/PMA). Prior to seeding, cells were counted using flow cytometry. Following incubation for 72 hours the cells were treated with trypsin: EDTA until detached and cell number was measured. Mean ± SD for triplicate wells, each assayed in duplicate are shown and results are representative of two independent experiments. *Statistical analysis conducted was T-test (***P<0.001).*

The flow-based analyses (figure 21) confirmed a significant increase in cell number within untreated THP-1 and vitamin D3 wells. Taken together this data suggests that untreated THP-1 and vitamin D3 macrophages demonstrate growth. While the data suggests that PMA and double-stimulated macrophages have decreased in cell number possible as a result of increased cell adhesion, cell fusion or even death. However cells were treated with 5mM EDTA and washed several time to remove all adherent cells and microscopic analysis did not suggest cell death. Therefore the data in figures 20 and 21 may suggest

that differentiation induced with PMA or double-stimulation caused a reduction in cell proliferation which is a characteristic feature of macrophage differentiation.

3.3.1.3 Seeding THP-1-derived macrophages

The results in figures 20 and 21 suggest that differentiation induced with vitamin D3 does not affect cell proliferation while treatment with PMA may have inhibited proliferation or potentially induce cell fusion. However in order to ensure cell density following 72 hours of differentiation is similar, a seeding protocol was established. THP-1 were seeded at $6x10^5$ cells per well in 24 well plates and induced to differentiate for 72 hours. Following differentiation, THP-1 and vitamin D3 macrophages were reseeded at original density $6x10^5$ to ensure all macrophages models had similar cell densities.

3.3.2 Titrating LPS responsiveness of macrophages

Having established a suitable seeding protocol, macrophage response to varying concentrations of LPS in the presence of LBP was assessed (figure 22).

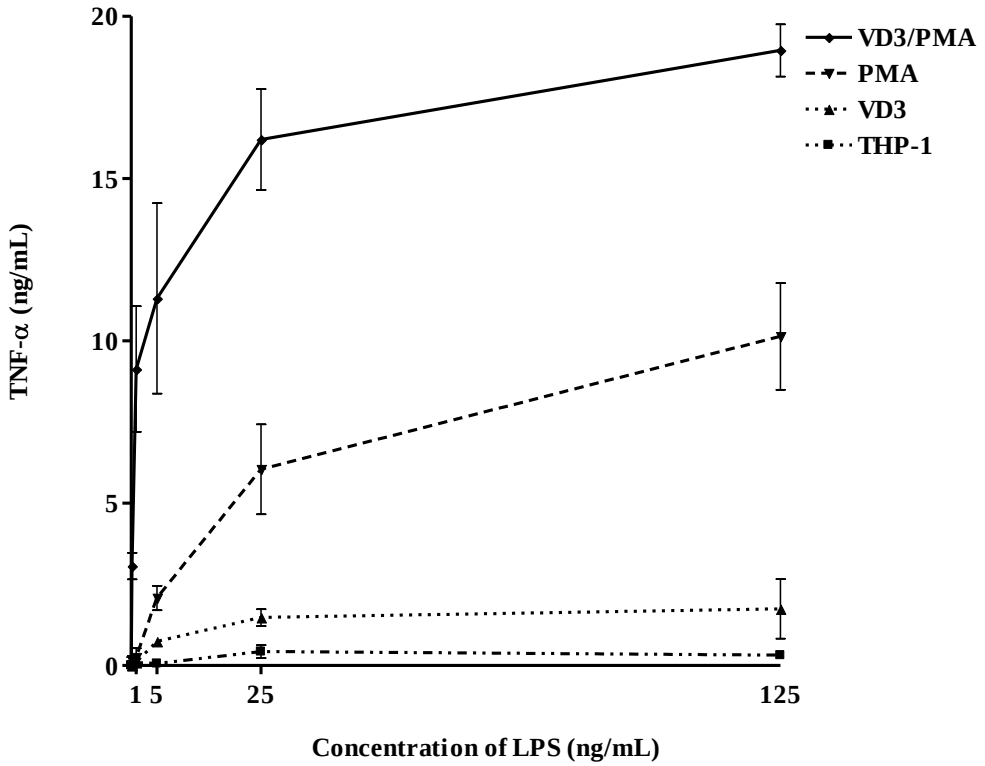

Figure 22: LPS-induced TNF-α response of THP-1-derived macrophages
Following differentiation THP-1-derived macrophages were challenged for 4h with increasing concentrations of LPS in 500µl of cRPMI containing 10% (v/v) NHS. TNF-α released was quantified using a capture ELISA. Mean ± SD for triplicate wells, each assayed in duplicate are shown. These results are representative of three independent experiments.

The results in figure 22 illustrate that PMA and double-stimulated macrophages are very LPS responsive and agrees with previous data by Park *et al.*(2007) and Daigneault *et al.*(2010). The results also suggest that THP-1 and vitamin D3 macrophages, when stimulated with LPS produced low levels of TNF-α compared to PMA macrophage and agrees with findings by Daigneault *et al.*(2010).

3.3.3 Cell surface expression of TLR4

TLR4 is required for LPS signalling as shown by Hoshino *et al.*, 1999) that demonstrated mice lacking TLR4 are hyporesponsive to LPS. Given the LPS response data (figure 22) cell surface expression of TLR4 on THP-1-derived macrophages was assessed using direct immunofluorescence staining followed by flow cytometric analysis (figure 23).

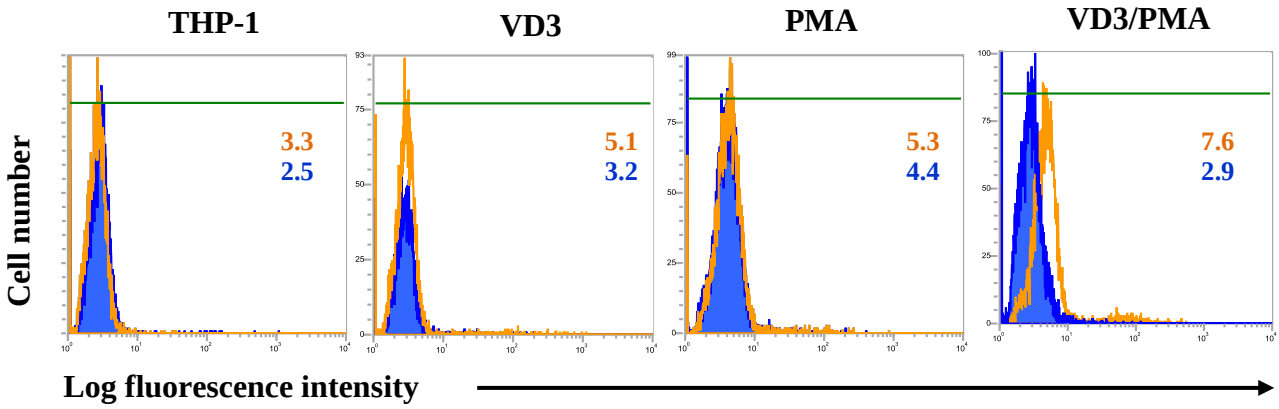

Figure 23: Cell surface expression of TLR4 on THP-1-derived macrophages
THP-1 was differentiated with vitamin D3 (VD3), PMA or both (VD3/PMA) for 72 h. Surface expression of TLR4 on resultant macrophages was detected with flow cytometric staining using direct immunofluorescence with the monoclonal antibody HTA125 (orange) or isotype-control (blue). The mean fluorescence intensity of 100% of the population of the cells region is also shown. The results are representative of four independent experiments.

Flow cytometric results in figure 23 suggest that only double-stimulated macrophages expressed detectable levels of cell surface TLR4. While cell surface expression of TLR4 was undetectable on THP-1 and VD3 macrophages, the flow cytometric data suggests that a small population of PMA macrophage express TLR4. These findings are supported by Sadeghi *et al.*(2006) that found treating monocytes with vitamin D3 causes an increase in CD14 expression and a decrease in TLR4 expression, therefore causing cells to become hyporesponsive to LPS stimulation.

3.3.4 CD14-dependent macrophage response to LPS

Having demonstrated the LPS-sensitivity of each THP-1-derived macrophage, the involvement of CD14 in cellular response to LPS was assessed using the mouse anti-

human CD14 mAb MEM18, a known blocker of LPS-induced inflammation (Juan *et al.*, 1995a) (figure 24).

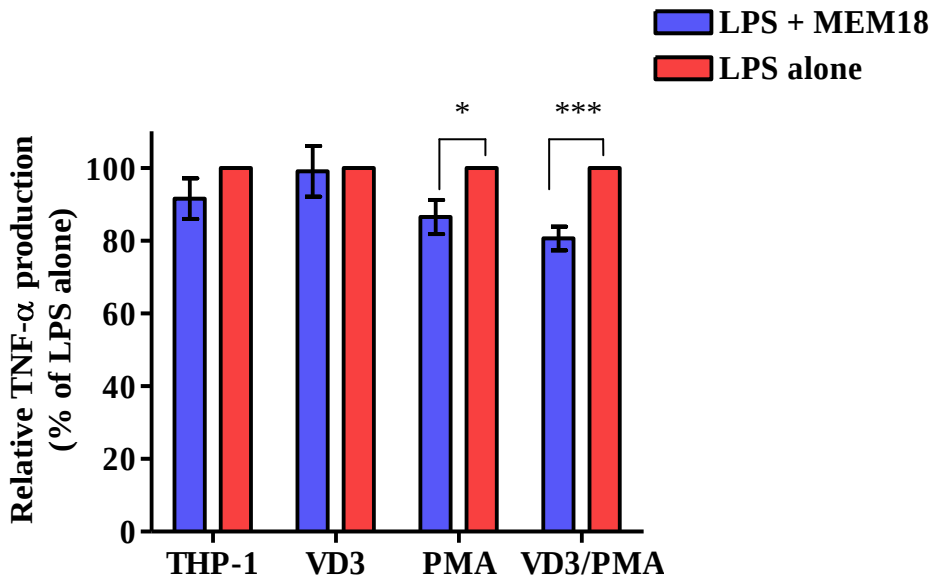

Figure 24: The role of CD14 in macrophage response to LPS
THP-1, vitamin D3, PMA and double-stimulated macrophages were incubated with 10% NHS and stimulated with LPS (THP-1 and VD3 25ng/ml, PMA 5ng/ml and VD3/PMA 1ng/ml) in the presence or absence of the established CD14-blocking mAb MEM18 (10µg/ml) in a final volume of 500µl/well. Following incubation at 37°C for 4 h plates were frozen at -20°C until TNF-α production was measured by capture ELISA. Each treatment was repeated in duplicate on 24 well plates and each well analysed in triplicate on 96 well ELISA plate. The results shown are from six separate experiments and normalised to LPS alone for each experiment. *Statistical analysis was conducted using AVOVA with Bonferroni post test. (*P<0.05, ***P<0.001).*

As shown in figure 24, MEM18 inhibits CD14-dependent LPS responses produced by both PMA and double-stimulated macrophages which suggests that CD14 is required for the LPS responsiveness of these cells. Notably flow cytometric data suggests that CD14 expression (figure 18) on PMA cell is low but cells are LPS responsive. Therefore collectively, these data support the theory that LPS responses produced by PMA macrophages are due to a potent, but low level of CD14 expression, undetectable by flow cytometry. CD14 expression on THP-1 was undetectable and these cells were not as LPS responsive as other macrophage-like cells. Furthermore vitamin D3 macrophages expressed relatively high levels of CD14 but were not as LPS responsive as expected.

3.3.4.1 LPS-FITC binding to vitamin D3 macrophages

Given the involvement of CD14 in LPS response by macrophages (figure 22) the ability of LPS to bind to THP-1-derived macrophages was assessed. Previous data found that vitamin D3 macrophages expressed the highest surface level of CD14 (figure 18) therefore

cells were incubated with increasing concentrations of LPS-FITC and binding detected using flow cytometry (figure 25).

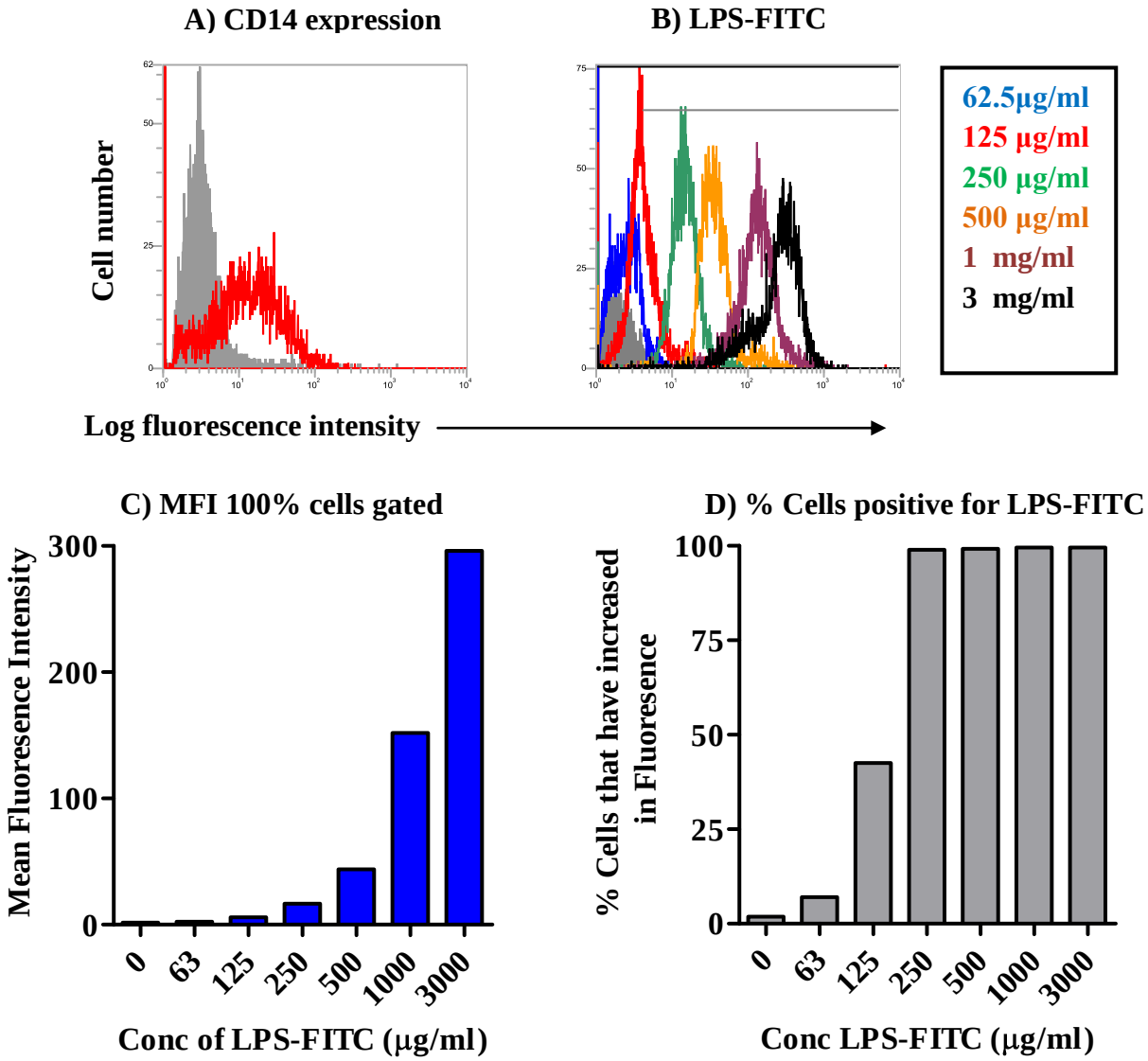

Figure 25: LPS-FITC binding to CD14 expressed on vitamin D3 macrophages
THP-1 was stimulated to differentiate with vitamin D3 for 72 hours. **A.** Cell surface expression of CD14 was assessed using indirect immunofluorescence staining with MEM18 (red) or MOPC 21 iostype negative control (grey). **B.** Vitamin D3 macrophages were incubated with indicated concentrations of LPS-FITC in 10% NHS in a final volume of 500µl for 15 min on ice. Following incubation cells were washed and LPS-bound detected using flow cytometry. **C.** The mean fluorescence intensity (MFI) of cells incubated with increasing concentrations of LPS-FITC. **D.** The % of cells that have increased from 1.9% false positive gate set against cells alone (grey). Representative of four independent experiments.

The data in figure 25 suggests that LPS binds to vitamin D3 macrophages in a dose-dependent manner. The flow cytometry results shows that as the percentage of cells binding LPS reach 100% the mean fluorescence intensity then increased, suggesting more LPS bound on a per/cell basis. A maximal MFI was not reached as concentration over

1mg/ml of LPS-FITC were toxic to the cells and resulted in lysis as denoted by a change in the electronic volume and side scatter of the population.

3.3.4.2 MEM18 inhibition of LPS-FITC binding

Having established that LPS-FITC binds to vitamin D3 macrophages, CD14-dependent binding of LPS was assessed using CD14-blocking mAb MEM18 (Juan *et al.,* 1995). Here vitamin D3 macrophages were incubated with LPS-FITC in the presence or absence of MEM18 and detected using flow cytometry (figure 26).

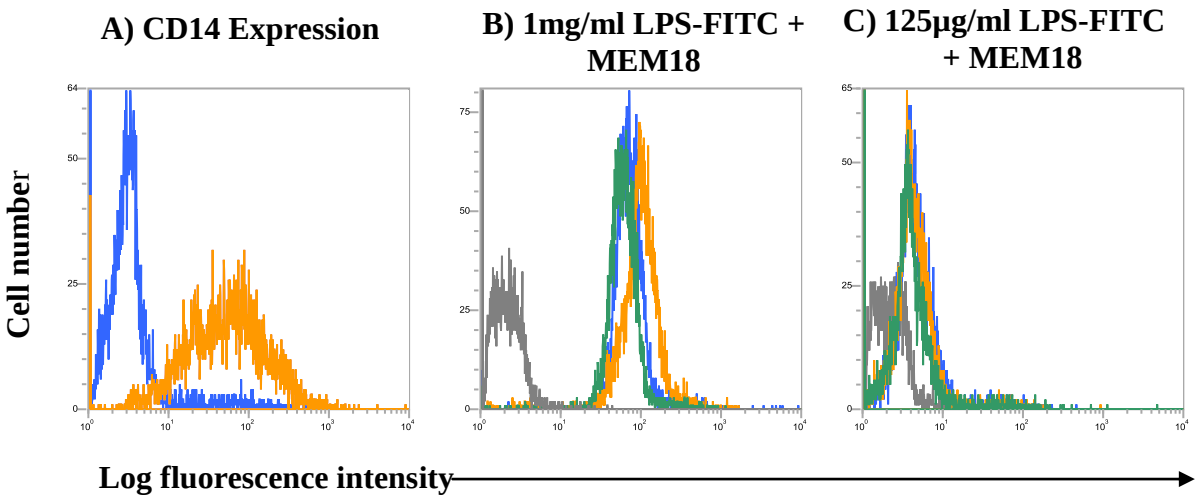

Figure 26: CD14-dependent binding of LPS to vitamin D3 macrophages
THP-1 were stimulated to differentiate with vitamin D3 and incubated for 72 h. **A** Following differentiation, cell surface expression of CD14 was analysed using indirect immunofluorescence staining with MEM18 (orange) or isotype-control MOPC 21 (blue). **B&C.** vitamin D3 macrophages were incubated in absence or presence of 10µg/ml of MEM18 (orange) or MOPC 21 (blue) for 15 min at 4°C. Prior to incubation with flow buffer containing 10%NHS and 1mg/ml **B.** or 125µg/ml **C.** of LPS-FITC (green) for 15 min on ice. Following incubation LPS-FITC binding was detected using flow cytometry. Vitamin D3 macrophages without LPS or mAb staining are shown in grey. The results shown are representative of two independent experiments.

The data here suggests that MEM18 does not inhibit 1mg/ml or 125µg/ml of LPS-FITC binding despite clear CD14 expression detected by flow cytometry (figure 26a). As previously shown (figure 25) both concentrations of LPS-FITC bind to CD14 expressed by vitamin D3 macrophages, with a significant increase in the percentage of cells binding 1mg/ml LPS-FITC. Interpretation of the data may suggest that the concentration of MEM18 used to block LPS binding was not saturating. However the concentration of MEM18 was titrated against vitamin D3 macrophages using flow cytometry to ensure a saturating concentration of antibody was used for all experiments. Therefore results here may suggest that MEM18 does not block LPS binding but blocks LPS response by inhibiting interaction with signalling molecule.

3.3.5 Apoptotic cell clearance

Having characterised the role of CD14 in LPS responses of macrophages, the THP-1-derived macrophages were used to assess the role of CD14 in apoptotic cell interaction.

3.3.5.1 THP-1-derived macrophages interact with apoptotic cells

Firstly the ability of the THP-1-derived macrophages to interact with apoptotic cells was established. This was conducted by co-culturing THP-1-derived macrophages with UV-induced apoptotic Mutu (figure 27). The ability of untreated THP-1 to interact with apoptotic cells was not considered due to poor adhesion to glass slides and removal during the washing stages of the protocol.

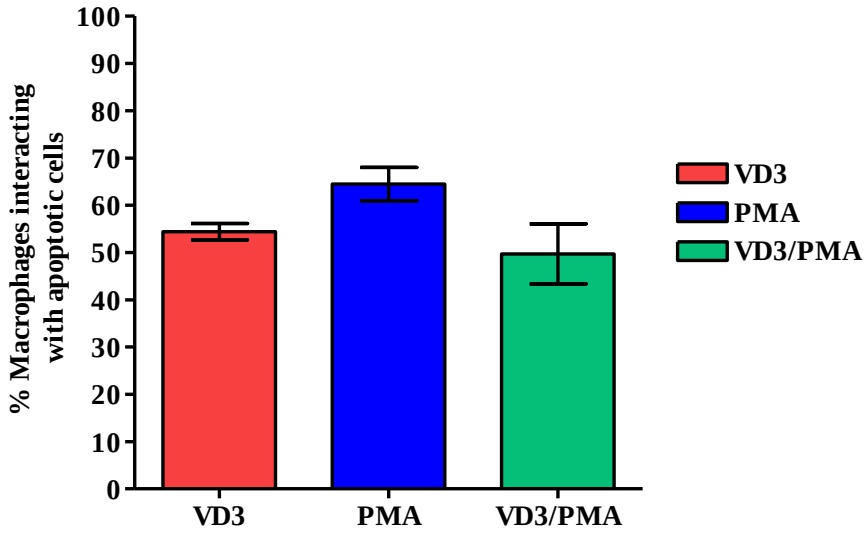

Figure 27: Interaction of apoptotic cells with THP-1-derived macrophages
Following differentiation THP-1-derived macrophages were co-cultured with apoptotic Mutu for 1 h at 37°C. Macrophages were washed in cold PBS, fixed in methanol and Jenna-Giemsa stained before being scored by light microscopy. The results shown are the mean ± SEM of seven independent experiments.

All THP-1-derived macrophage interact with apoptotic cells (figure 27). Furthermore there is no significant difference in the percentage of apoptotic cells interacting with each THP-1-derived macrophage. This may suggest that all THP-1-derived macrophage utilise the same receptors to interact with apoptotic cells. However as previously shown (figure 18) each THP-1-derived macrophage expresses different levels of surface CD14. Therefore the next logical step was to assess the role of CD14 in the clearance of apoptotic cells by THP-1-derived macrophages.

3.3.5.2 CD14-dependent clearance of apoptotic cells

To investigate the involvement of CD14 in the clearance of apoptotic cells by THP-1-derived macrophages, an interaction assay was conducted including the inhibitory monoclonal antibody 61D3 (Devitt *et al.*, 1998) (figure 28).

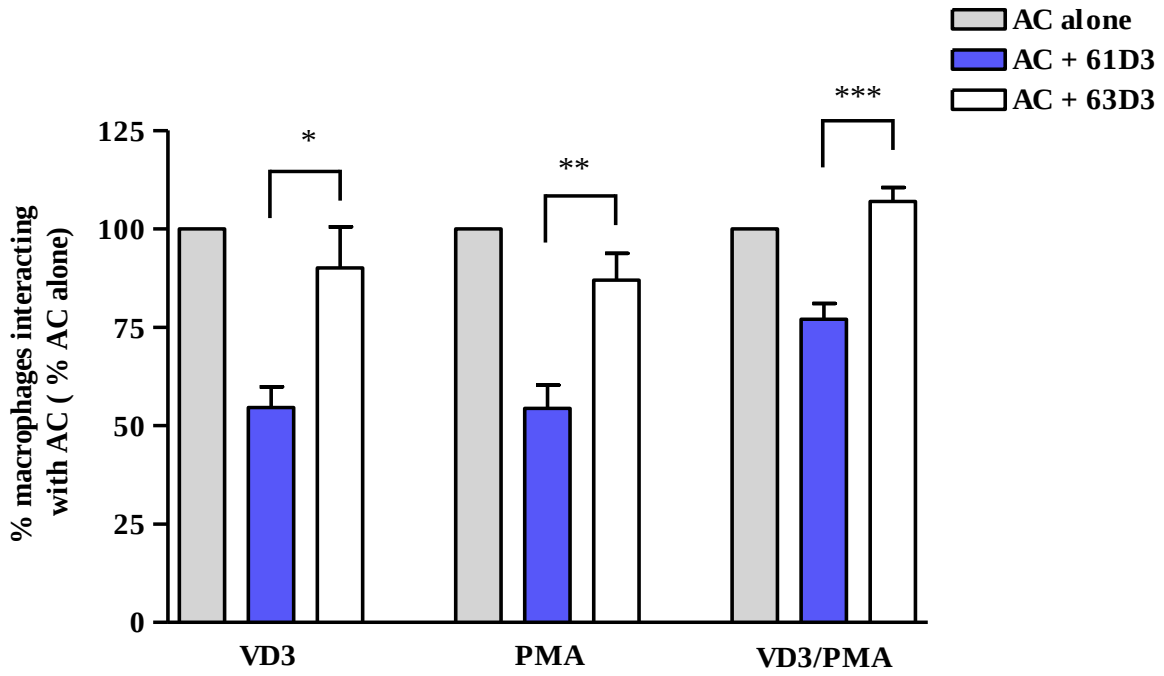

Figure 28:CD14-dependent apoptotic cell clearance
Following differentiation, macrophages were co-cultured with apoptotic Mutu for 1 h at 37°C alone or with CD14-blocking antibody 61D3 or isotype-control. Macrophages interacting with apoptotic cells were scored by light microscopy. The results shown are the mean ± SEM of seven separate experiments and normalised to apoptotic cells alone. *Statistical analysis used was unpaired T-test * P<0.05, ** P<0.01, P< 0.001.*

All THP-1-derived macrophages use CD14 to clear apoptotic cells, but the level of CD14-dependent clearance varies between the macrophages. The results in figure 28 show a 50% reduction in the clearance of apoptotic cells by vitamin D3 and PMA macrophages and a 25% reduction by double-stimulated macrophages. The results also suggest that all macrophages express other receptors also involved in the clearance of apoptotic cells.

3.3.5.3 Macrophage response to LPS in the presence of apoptotic cells

The clearance of apoptotic cells by macrophages has been reported to be a tolerogenic process that actively inhibits the production of proinflammatory cytokines following LPS stimulation (Voll *et al.*, 1997 & Fadok *et al.*, 1998b). This dominant anti-inflammatory response produced by macrophages is considered to be caused, at least in part by the secretion of anti-inflammatory cytokines released as a result of the interaction with apoptotic cells (Fadok *et al.*, 1998b). Here the ability of THP-1-derived macrophages to inhibit LPS induced inflammation following incubation with apoptotic cells was considered (figure 29).

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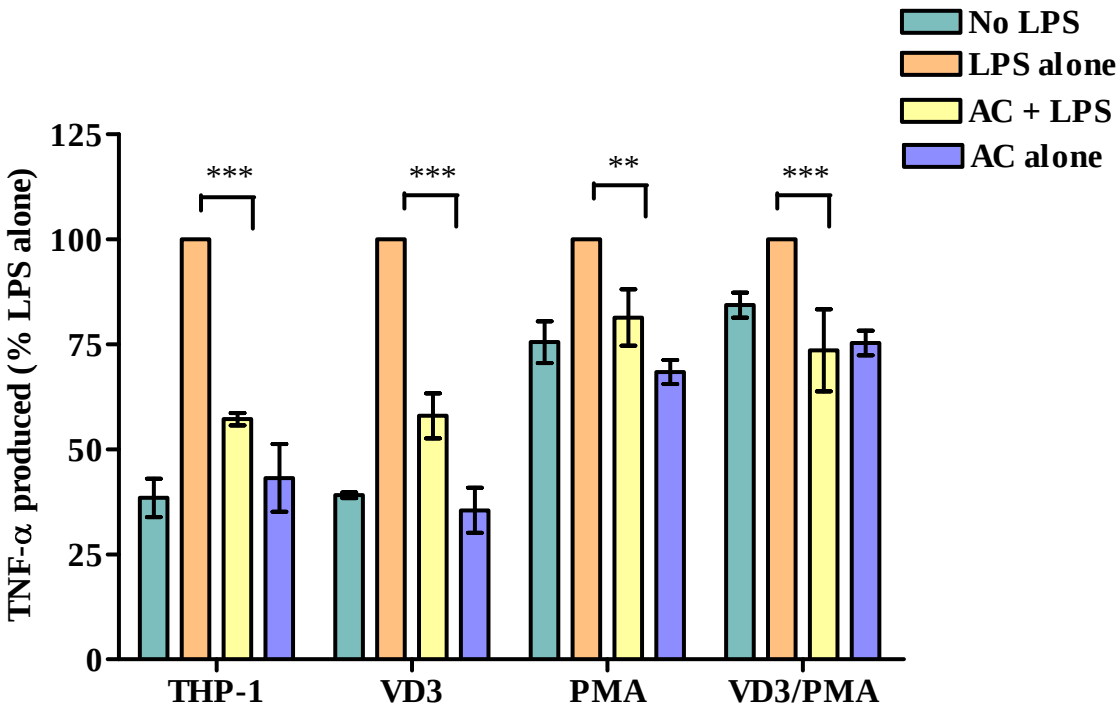


Figure 29: Apoptotic cell clearance inhibits LPS-induced TNF-α response
LPS-induced TNF-α released by THP-1-derived macrophages was assessed following 18h co-cultured with apoptotic Mutu. TNF-α released was quantified by ELISA. The results shown are the mean ± SEM or triplicate wells, each assayed in triplicate by ELISA and normalised to LPS alone for four independent experiments. *Statistical analysis used was ANOVA followed by Bonferroni post test (** P<0.01, ***P<0.001).*

THP-1-derived macrophages were incubated with apoptotic cells for 18 hours prior to LPS stimulation. Following incubation macrophages were stimulated with LPS for 4 h and TNF-α produced was measured by assaying cell supernatants using a TNF-α capture ELISA. The results (figure 29) suggest that the clearance of apoptotic cells by THP-1-derived macrophages did not stimulate the release of TNF-α. Furthermore incubation of apoptotic cells with PMA or double-stimulated macrophages inhibited TNF-α production to basal levels (No LPS), compared to THP-1 and vitamin D3 macrophages. However the basal expression of TNF-α produced was higher in PMA and double-stimulated possible as a consequence of PMA induced differentiation.

3.4 RAGE: another PRR involved in apoptotic cell clearance?

Another innate immune molecule that may be involved in the clearance of apoptotic cells is the receptor for advanced glycation end-products (RAGE). Given that RAGE, like CD14 can bind LPS (Yamamoto *et al.*, 2011) and previous findings here that suggest apoptotic cells expose LPS-like patterns on the surface (figure 50), the role of RAGE in the binding and subsequent clearance of apoptotic cells was assessed.

3.4.1 Cell surface expression of RAGE on THP-1-derived macrophages

To assesse a role for RAGE in apoptotic cell clearance, the expression of RAGE on THP-1-derived macrophage was assessed using indirect immunofluorescence and subsequent flow cytometry (figure 30).

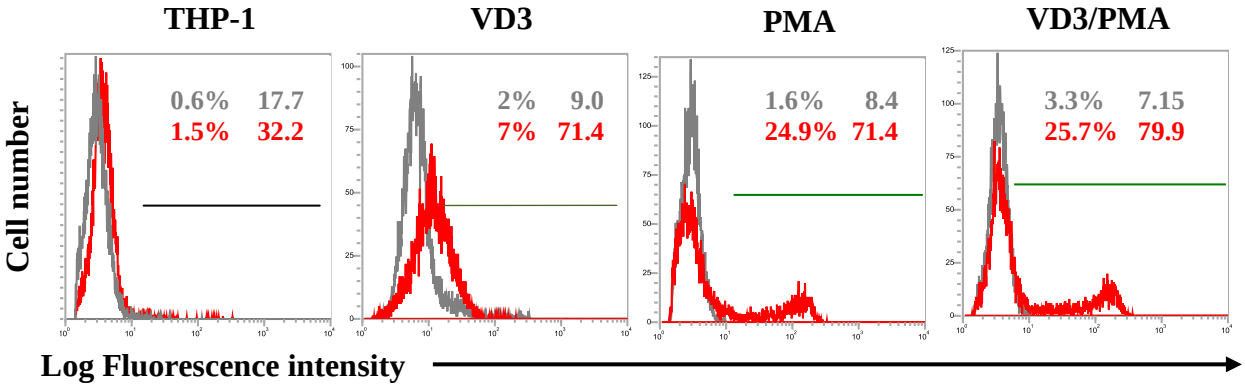


Figure 30: Cell surface expression of RAGE on THP-1-derived macrophages
THP-1 was differentiated with vitamin D3 (VD3), PMA or both (VD3/PMA) for 72 hours. Cell surface expression of RAGE on THP-1-derived macrophages was detected with flow cytometric staining using indirect immunofluorescence with the polyclonal anti-RAGE antibody (red) or pre-immune rabbit serum as negative control (grey). The percentage of positive cells gated and the mean fluorescence intensity are shown. The results are representative of three independent experiments.

Flow cytometric analysis in figure 30 show all THP-1-derived macrophages expressed surface RAGE to a small degree though differentiation induced with PMA or both agents (vitamin D3/PMA) caused a sub-population of cells to express high levels of RAGE.

3.4.2 Subcloning and expression of sRAGE

To identify a possible role for RAGE in the clearance of apoptotic cells, a mAb approach was considered where anti-RAGE mAbs would be screened for their ability to block apoptotic cell clearance. Such studies used for the identification of a number of apoptotic cell clearance receptors, including CD14, are potentially limited by the available mAbs and the presence of epitopes in crucial functional sites on the protein. However many studies into the function of membrane RAGE (mRAGE) use soluble RAGE (sRAGE) as a competitive, neutralising ligand along with polyclonal antibodies to block mRAGE (Foell *et al.*, 2006, Hofmann *et al.*, 1999, Schmidt *et al.*, 2001, Toure *et al.*, 2008, Lutterloh *et al.*, 2007). Therefore in order to assess the role of RAGE in the clearance of apoptotic cells, sRAGE was produced as a decoy for mRAGE (section 2.6). Following transfection and subsequent culturing, the expression of sRAGE into culture supernatant was confirmed by indirect ELISA (figure 11).

3.4.3 Binding of sRAGE to LPS

LPS is known to bind CD14 (Wright *et al.*, 1990 & Ziegler-Heitbrock and Ulevitch, 1993), however binding is facilitated by the serum protein LBP (Tobias *et al.*, 1986). A study by Wurfel *et al.*(1997) found that LBP-deficient mice retained their ability to respond to LPS, *in vivo*, suggesting an LBP-independent mechanism of cellular response to LPS existed. Furthermore, Youn *et al.*(2008) found that HMGB1 competed with LBP for binding to CD14 and also facilitated the transfer of LPS to CD14. RAGE is also reported to bind LPS therefore to establish if RAGE will bind LPS-like structure presented on the surface of the cells as previously found (figure 50), the ability of sRAGE to bind LPS was first assessed, using an ELISA based assay (figure 31).

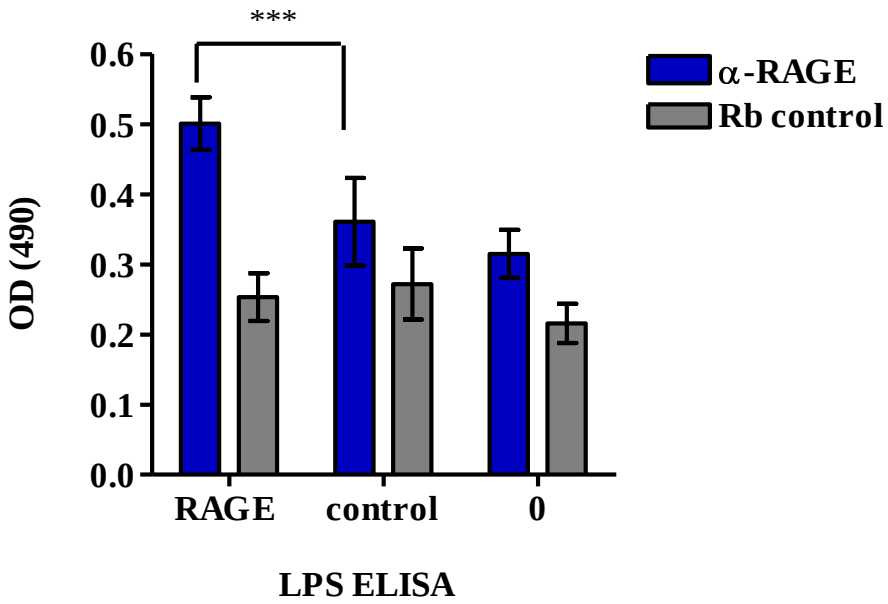


Figure 31: sRAGE binds to LPS
LPS was immobilised to an ELISA plate overnight and non-specific binding blocked with 0.005% (w/v) Tween (PBS) for 2h at 37°C. Following incubation, 100µl of supernatant taken from HEK293 transfected with sRAGE or control (non-RAGE expressing pSec Tag2A plasmid) was added to wells. The plate was washed and bound sRAGE was first detected with polyclonal anti-RAGE antibody or control (pre-immune) rabbit serum then subsequently bound-antibody was detected with anti-rabbit HRP. OPD substrate was added to plate and optical density was read at 490nm. The results shown are the mean ± SD of quadruplet wells from two independent experiments. *Statistical analysis was conducted using AVOVA followed by Bonferroni post-test. (*** P<0.001).*

Having immobilised LPS to an ELISA plate and established a suitable blocking agent (0.005% (w/v) Tween) to reduce non-specific binding, the ability of sRAGE to bind to LPS was assessed. The results in figure 31 suggest that supernatant taken from HEK293 transfected with pSec Tag 2B containing the sRAGE insert, bound LPS to a higher degree than supernatant taken from cells transfected with pSec Tag 2A, that contains the sRAGE insert out of frame. Given the limitations of the experiment, the results may suggest that

sRAGE binds LPS, in agreement with findings by Yamamoto *et al.* (2011). As supernatant from transfected HEK293 was used as a source of RAGE, it is possible that some of the RAGE was pre-bound to HMGB1, released from transfected cells.

3.4.4 Binding of sRAGE to apoptotic cells

Previous data in figure 50, has suggested that that apoptotic cells display LPS-like patterns on the cell surface and are most likely epitopes recognised by CD14. Given previous data (figure 31) that suggests sRAGE binds LPS, the ability of sRAGE to bind apoptotic cells is assessed (figure 32).

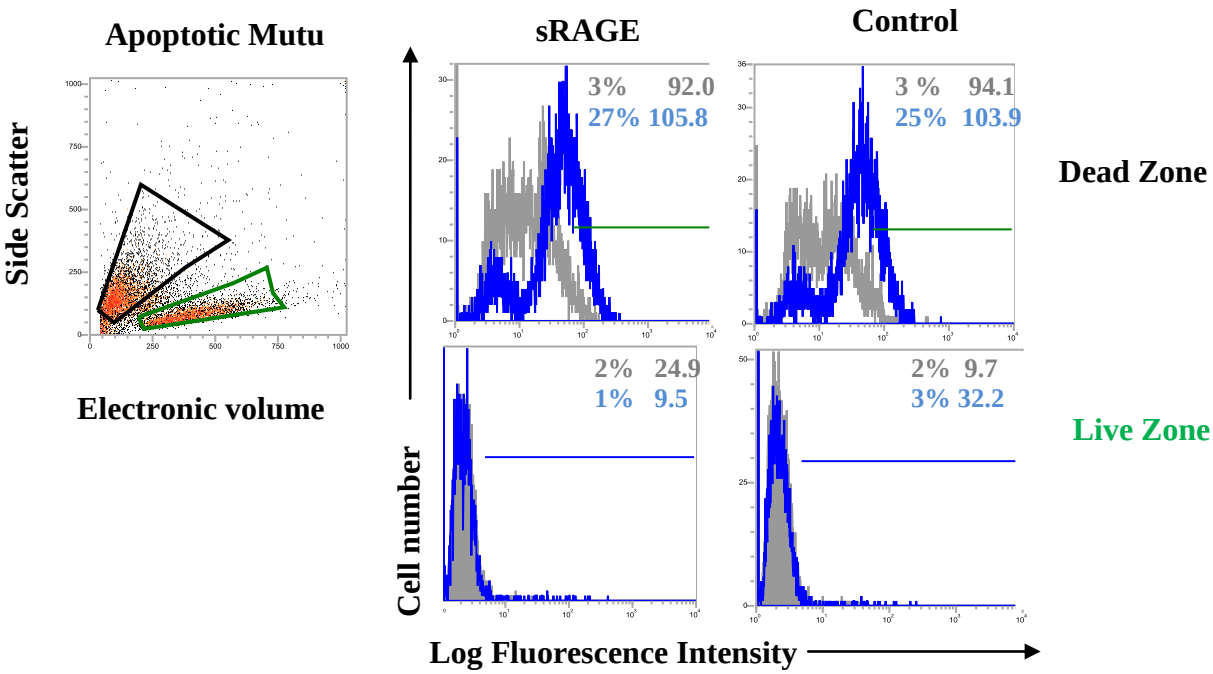


Figure 32: Flow cytometric analysis of apoptotic cells stained with sRAGE
Mutu were induced to undergo apoptosis following UV treatment and incubated overnight prior to assay. Mutu were classified as apoptotic following flow cytometric analysis of electronic volume and side scatter. Apoptotic Mutu were incubated with supernatant from HEK293 transfected with sRAGE or control (non-RAGE expressing pSec Tag 2A plasmid) for 30 min on ice. Following incubation, bound RAGE was detected using polyclonal anti-RAGE antibody (blue) or (negative control) pre-immune rabbit serum (grey) bound antibody was subsequently detected with anti-rabbit PE and cells were analysed by flow cytometry. The percentage of cells and the mean fluorescence intensity of the cells within the positive gated region are shown. The results are representative of two independent experiments.

The ability of sRAGE to bind apoptotic cells was examined by immunofluorescence staining of apoptotic Mutu with sRAGE or control (non-RAGE expressing pSec Tag 2A plasmid). The flow cytometric histograms, shown in figure 32 appear identical and suggest that the secondary antibody (anti-rabbit PE) bound non-specifically to the cells. However

regardless of the limitations of the assay the results suggest that sRAGE did not bind to apoptotic cells.

3.4.5 Role of RAGE in the clearance of apoptotic cells

Previously all THP-1-derived macrophages were shown to interact with apoptotic cells (figure 27) and express varying levels of mRAGE (figure 30). In previous studies sRAGE has been used as competitive, inhibitor of mRAGE (Foell *et al.*, 2006, Hofmann *et al.*, 1999) therefore to assess the ability of sRAGE to interact with apoptotic cells, THP-1-derived macrophages were co-cultured with apoptotic cells in the presence or absence of sRAGE (figure 33).

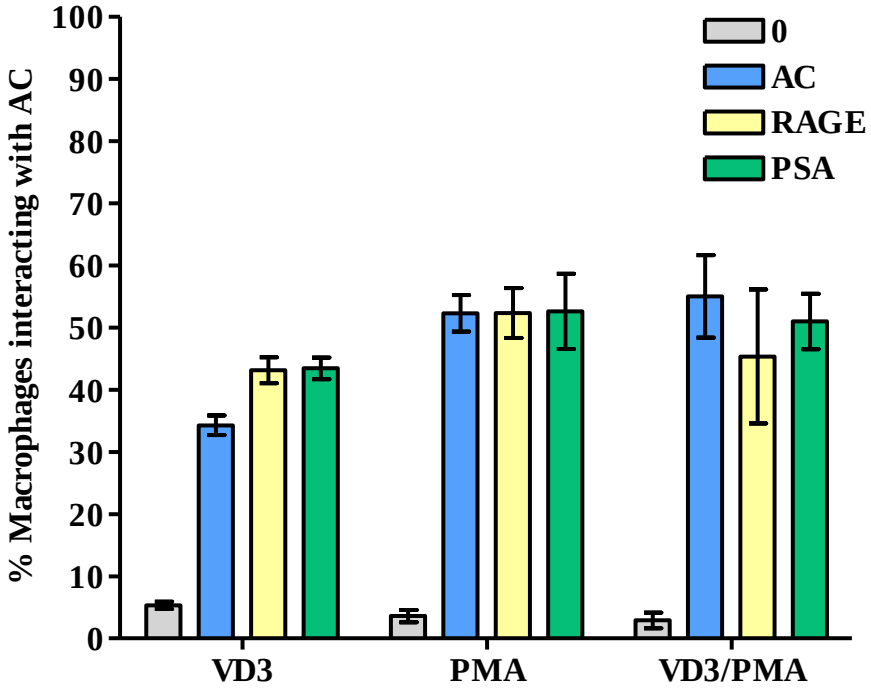


Figure 33: The role of sRAGE in the clearance of apoptotic cells
THP-1-derived macrophages were co-cultured with apoptotic Mutu for 1 h at 37°C in the presence of sRAGE or control (PSA). Macrophages were washed in cold PBS, fixed in methanol and Jenna-Giemsa stained before being scored by light microscopy. The results shown are the mean ± SEM of three independent experiments. *Statistical analysis was conducted using ANOVA followed by Bonferroni post-test*

As an initial assessment of the role of RAGE in the clearance of apoptotic cells supernatant taken from HEK 293 expressing PSA or sRAGE was incubated with apoptotic cells prior to co-culturing with THP-1-derived macrophages. The results shown in figure 33, support previous findings that all THP-1-derived macrophages interact with apoptotic cells though in this particular example PMA and double-stimulated macrophages were most efficient at interacting with apoptotic cells relative to vitamin D3 macrophages. Flow cytometric staining in figure 30 illustrates that THP-1-derived macrophages express different levels of

mRAGE. Analyses of the results in figure 33 suggest that incubation with sRAGE or PSA increased the ability of vitamin D3 macrophages to interact with apoptotic cells. Also sRAGE reduced the level of apoptotic cell interaction with double stimulated macrophages. However taken together the data suggests that incubation with sRAGE had no significant effect on the ability of THP-1-derived macrophages to interact with apoptotic cells. This may suggest that sRAGE is not involved in the clearance of apoptotic cells or that sRAGE may also participate in clearance of apoptotic cells. Therefore whilst the use of sRAGE may block mRAGE function, if sRAGE is also involved in apoptotic cell clearance the result would be a null effect.

3.4.6 sRAGE contains mutations

RAGE is a 45kDa cell surface transmembrane receptor that belongs to the immunoglobulin superfamily (Park *et al.,* 2010). It consists of an extracellular domain, a transmembrane domain and a C-terminus cytoplasmic tail required for signalling (Dattilo *et al.,* 2007,Matsumoto *et al.,* 2008 & Park *et al.,* 2010). The extracellular domain consists of three immunoglobulin-like domains of which the first and second domain (figure 34) function as one component responsible for ligand binding properties of RAGE (Dattilo *et al.,* 2007& Park *et al.,* 2010).

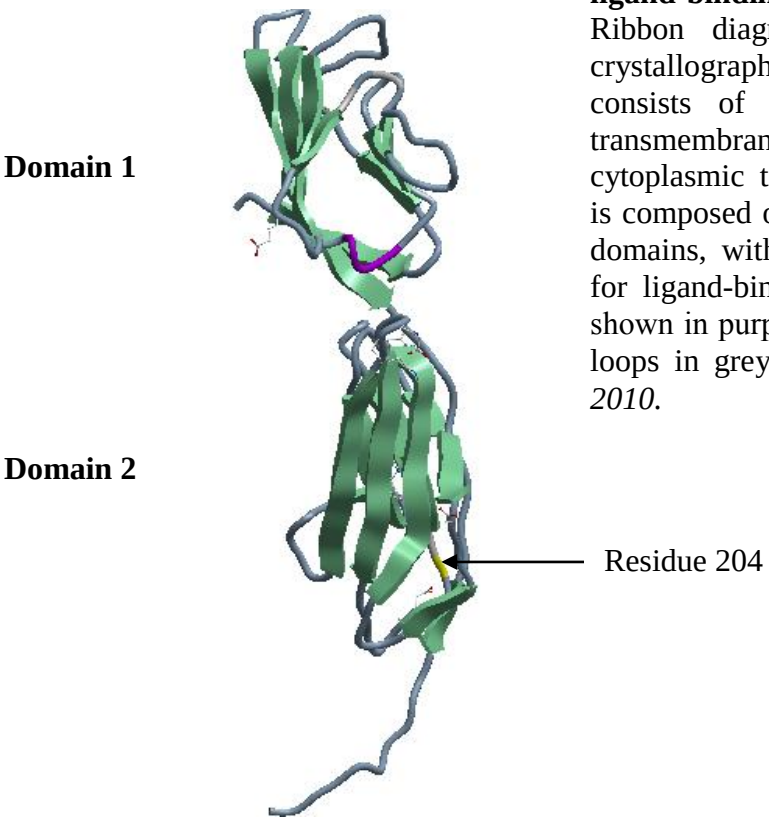


Figure 34: The crystal structure of the ligand-binding domains of RAGE
Ribbon diagram based on the X-ray crystallography of human RAGE. RAGE consists of an extracellular region, a transmembrane region and a C-terminus cytoplasmic tail. The extracellular region is composed of three immunoglobulin-like domains, with domains 1 and 2 required for ligand-binding as shown. Helices are shown in purple, β-strands are in cyan and loops in grey. *Adapted from Park et al., 2010.*

Sequence analysis of sRAGE produced revealed two single point mutations within the parental DNA sequence that codes for the extracellular ligand-binding domain. The first single point mutation occurs at nucleotide position 635 and is caused by a cytosine to thymine substitution, as a result the amino acid proline at position 204 is replaced with a leucine. The cyclic structure of the side chain on proline disrupts the secondary structure of proteins and therefore proline is often required to initiate the rolling required to form α-helixes. Loss or replacement of proline is likely to change the three-dimensional conformation of the local surrounding region. As shown in figure 34 (yellow residue highlighted) the mutation is within the second domain on a loop between β-stands. Furthermore the second single point mutation occurs at nucleotide position 915 and adenine is substituted for a guanine, however this mutation is silent. Both mutations were present in the parental DNA received and there is no record of these point mutations within NCBI library consequently the effects on ligand binding of RAGE are unknown.

3.5 Discussion

The rapid removal of apoptotic cells by professional phagocytes involves an array of macrophage receptors, apoptotic cell associated-molecules and soluble bridging molecules (Savill *et al.,* 2002). Of particular interest is CD14, a PRR expressed on monocytes and macrophages and well characterised for its ability to recognise the PAMP, LPS and promote inflammation (Wright *et al.,* 1990, Ziegler-Heitbrock and Ulevitch, 1993 & Park *et al.,* 2007b). However Devitt *et al.* (1998) transiently expressed CD14 in non-myeloid cells and found that CD14 was also involved in the clearance of apoptotic cells, a process known to be immunosuppressive and anti-inflammatory (Voll *et al.,* 1997& Fadok *et al.,* 1998b). This led to the suggestion that CD14 was involved in the assembly of two distinct ligand-dependent macrophage responses. In order to understand the binding patterns underlying the molecular mechanisms employed by CD14 in innate immune responses, several THP-1-derived macrophages were used to characterise the responses of CD14 to LPS or apoptotic cells.

3.5.1 Characterisation of THP-1-derieved macrophages

THP-1 are a human monocytic leukaemia cell line, which following differentiation produced macrophage-like cells that are commonly used to model macrophage functions (Schwende *et al.,* 1996, Park *et al.,* 2007b & Daigneault *et al.,* 2010). Here monocyte differentiation was induced by treatment of the cells with 1,25-dihydroxyvitamin D3

(vitamin D3), phorbol-13-acetate (PMA) or both (vitamin D3/PMA) for 72 hours. Resulting macrophages were characterised phenotypically and functionally with respect to morphology, CD14 expression, LPS responsiveness and ability to interact with apoptotic cells. PMA and double-stimulation induced profound changes in morphology, cells increased in size, granularity, began spreading and induced lamellipodia formation, characteristic traits of macrophages. However no morphological differences between vitamin D3 and untreated THP-1 were observed, furthermore an increase in cell adhesion was noted with all treatments.

CD14 is not expressed by early progenitor stem cells but expressed by mature blood monocytes and macrophages suggesting that monocyte differentiation causes an upregulation in CD14 expression (Zhang *et al.,* 1994b, Ziegler-Heitbrock and Ulevitch, 1993, Hmama *et al.,* 1999). CD14 is also up-regulated following LPS stimulation without the need for protein synthesis (Marchant *et al.,* 1992). Studies have found that CD14 expression is up-regulated at an mRNA level on THP-1 following differentiation with the steroid hormone, vitamin D3 (Schwende *et al.,* 1996). Treatment results in a complex with nuclear vitamin D3 receptor that binds to specific response elements that promoter the expression of monocytic genes and up-regulates monocytic markers including CD11b and CD14 (Hmama *et al.,* 1999). THP-1 differentiation can also be induced with PMA treatment (Schwende *et al.,* 1996), phorbol esters are analogues of diacylglycerol and therefore activates protein kinase C and influences various cellular process including maturation, differentiation and proliferation (Monick *et al.,* 1998 & Lin *et al.,* 2007). Here differentiation induced with vitamin D3 or double-stimulation caused an increase in cell surface expression of CD14, while expression was undetected on PMA macrophages. These profound changes in morphology along with flow cytometric analysis of CD14 expression are supported by Schwende *et al.*(1996) and Daigneault *et al.*(2010). However Park *et al.*(2007) report that PMA macrophages do not express surface CD14, but RT-PCR found that PMA upregulated CD14 expression at an mRNA level. Furthermore Bazil and Strominger (1991) found that differentiation of human monocytes with LPS or PMA induced shedding of CD14 and therefore may be responsible for undetectable cell surface levels of CD14 on PMA macrophages. PMA treatment also had a profound effect on cell density following incubation for 72 hours. THP-1 and vitamin D3 macrophages increased in cell number over the incubation period while the number of PMA and double-stimulated macrophages decreased. A decrease in cell number was not caused by an increase in adherence of macrophages to flasks, as cells were treated with EDTA until detached.

Previously studies have reported that PMA treatment induced cell fusion resulting in multi-nucleated, giant cells. Mystkowska and Sawicki (1987) found that embryonic cells treated with PMA produced binuclear cells, possibly by inhibition of cytokinesis or causing structural changes in the phospholipid bilayer permitting fusion. The fusion of monocyte-derived macrophages was also described by Hassan *et al.*(1989) that observed cell fusion in blood-derived macrophages following PMA treatment for 5 to 7 days.

3.5.2 Role of CD14 in LPS responses of THP-1-derived macrophages

Having characterised the expression of CD14 on each THP-1-derived macrophage the ability of these cells to respond to the PAMPs LPS, was assessed. LPS response data (figure 22) showed that vitamin D3 macrophages, with high surface expression of CD14, were not as LPS responsive as PMA or double-simulated macrophages that expressed low levels of CD14. However TLR4 expression data (figure 23) revealed that although CD14 expression on vitamin D3 macrophages is high, the signalling partner TLR4; required for propagation of LPS response (Hoshino *et al.,* 1999), was not detected. These finding were supported by Sadeghi *et al.*(2006) that used RT-PCR and found human monocytes, following vitamin D3 treatment increased CD14 expression but diminished TLR4 and TLR2 expression at mRNA level. Sadeghi *et al.*(2006) suggests that both CD14 up-regulation and TLR4 down-regulation are induced following vitamin D3 receptor complex assembly, suggesting that vitamin D3 treatment has immunosuppressive effects. These immunosuppressive effects were also reported by Rosenblatt *et al.*(2010) that found vitamin D3 treatment of dendritic cells impaired maturation and reduced the expression of co-stimulatory molecules subsequently reducing T cell-mediated alloreactive responses. Therefore the hyporesponsiveness of vitamin D3 macrophages to LPS is most likely due to down-regulation of TLR4 caused by vitamin D3 treatment. These data support previous findings that TLR4 is required for CD14 to propagate an inflammatory response (Hoshino *et al.,* 1999).

Double-stimulated macrophages expressed TLR4 and relatively low levels of CD14 and were very LPS responsive (compared to other THP-1-derived macrophages), demonstrating the potency of CD14 to generating an LPS response. PMA macrophages present an anomaly, as CD14 and TLR4 expression was undetectable by flow cytometry but macrophages were more LPS responsive than vitamin D3 macrophages. This led to the suggestion that CD14 and TLR4 may be expressed at levels undetectable by flow cytometry and therefore the LPS response produced could be the potent effects of CD14

and TLR4 expressed at very low levels. Further analysis with CD14-blocking mAb MEM18 revealed that the LPS response produced by PMA macrophages was CD14-dependent. This supports the suggestion that PMA macrophages express undetectable levels of surface CD14. Further analysis of the mRNA levels of CD14 and TLR4 would be required to detect true changes in receptor expression. Park *et al.*(2007) found that treatment of THP-1 cells with 5ng/ml of PMA was sufficient in induce an increase in expression of CD14 and cells became responsive to LPS at concentrations ranging from 10-100ng/ml. Park *et al.*(2007) also found that differentiation induced with concentrations of PMA higher than 5ng/ml caused a reduction in CD14 mRNA expression although cells were still LPS responsive. However a reduction in mRNA expression may not reduce cell membrane expression but in combination with previous findings that PMA causes shedding of membrane CD14 (Bazil and Strominger, 1991) would suggest that CD14 expression is undetectable on PMA-induced macrophages. However epithelial and endothelial cells do not express CD14 but are reported to use sCD14 to produce an LPS response (Frey *et al.,* 1992, Pugin *et al.,* 1993). This could suggest that following PMA-induced shedding macrophages use sCD14 to facilitate an LPS response though a rational for shedding to then use sCD14 to promote an LPS response would be questionable. Haziot *et al.*(1996) found that CD14-deficient macrophages could use sCD14 to produce an LPS response, when stimulated with concentrations over 100ng/ml. Although PMA macrophages here were sensitive to LPS at concentrations as low as 5ng/ml suggesting that sCD14 may not be used by these macrophages to generate an LPS response.

Another interpretation of these findings is that PMA macrophages induce inflammation in a CD14-independent manner. Lynn *et al.*(1993) suggested that CD14-independent LPS signaling pathways exist in monocytes and macrophages with receptors including scavenger receptor, β2-integrins and L-selectins reported to bind LPS (Fenton and Golenbock, 1998 & Peppelenbosch *et al.,* 1999). Though β2 integrins and L-selectins are considered to have a low-affinity for LPS with high concentrations required for activation (Peppelenbosch *et al.,* 1999). Alternatively LPS binding is also reported to induce CD14-independent phagocytosis and pinocytosis (Peppelenbosch *et al.,* 1999, Latz, 2002, 2003 & Pei *et al.,* 2007). Pinocytosis of macrophage cell membranes is a constrictive process which is enhanced by phobol esters, including PMA (Peppelenbosch *et al.,* 1999). Therefore cellular-pinocytosis may resulting in LPS loaded vesicles within monocytes and macrophages which are rapidly trafficked to the golgi and could activate intracellular TLR4 (Peppelenbosch *et al.,* 1999, Latz, 2002, 2003 & Pei *et al.,* 2007).

Due to the hyporesponsive nature of vitamin D3 macrophages, the ability of LPS to bind these cells was assessed. Vitamin D3 induces a large increase in the surface expression of CD14 compared to PMA or double-stimulation of THP-1. The flow cytometric results shown in figure 25, suggests that LPS-FITC bound to vitamin D3 macrophages in a dose-dependent manner. Therefore the role of CD14 in LPS binding was assessed using CD14-blocking mAb MEM18 (Juan *et al.*, 1995). Blockade of CD14 on vitamin D3 macrophages had no effect on LPS-FITC binding (figure 26) which may suggest that LPS binding to cells is CD14-independent. Alternatively this data may suggest that the concentration of MEM18 used to inhibit LPS binding was not saturating. However prior to this assay, MEM18 binding was titrated using vitamin D3 macrophages and a saturating concentration of MEM18 established. Furthermore as illustrated in figure 25, incubation with concentrations of LPS-FITC below 62.5µg/ml were not detected by flow cytometry but data in figure 22 suggests that concentrations above 25ng/ml induced a small but measurable TNF-α response. This suggests that the binding of lower concentrations of LPS-FITC is undetectable by flow cytometry, possibly due to inefficient FITC labelling of LPS. Previous attempts to saturate CD14-dependent binding of fluorescein-labelled LPS is described by Troelstra *et al.*(1997) as poor due to inefficient conjugation of FITC. Therefore incubation with LPS at concentrations of 125µg/ml may induce LPS to integrate into cellular membranes due to the amphiphilic nature of the molecule and induce cell lysis. This is supported by the inability of high concentrations of LPS to induce receptor saturation due to cell lysis, as assessed by changes in electronic volume and side scatter. An alternative method of examining LPS binding to CD14 expressed by THP-1-derived macrophages may be to use radioactively-labelled LPS.

3.5.3 Role of CD14 in apoptotic cell clearance by THP-1-derived macrophages

Having characterised the role of CD14 in the LPS responses of THP-1-derived macrophages the ability of these cells to interact with apoptotic cells was assessed. All THP-1-derived macrophages interacted with apoptotic cells in a similar manner (figure 27). The role of CD14 in the clearance of apoptotic cells by THP-1-derived macrophages was assessed with CD14-blocking mAb 61D3 (Devitt *et al.*, 1998). The data (figure 28) illustrates that while all macrophages use CD14 to interact with apoptotic cells, the level of CD14-dependent interaction varied between macrophages. Incubation of apoptotic cells with vitamin D3 or PMA macrophages, in the presence of 61D3 resulted in 50% reduction

in interaction. Furthermore CD14 expression data (figure 18) shows that vitamin D3 induced a large increase in surface expression of CD14 compared to PMA and double-stimulated macrophages. Therefore the result may suggest that CD14 expressed on PMA macrophages is more efficient at clearing apoptotic cells compared to vitamin D3 and double-stimulated macrophages. This may suggests that PMA treatment enhances phagocytosis of apoptotic cells compared to vitamin D3 macrophages with CD14 recycled to the cell surface quicker. This theory is supported by Schwende *et al.*(1996) and Daigneault *et al.*(2010) that reported differentiation of THP-1, with PMA for 72 hours enhanced the phagocytic activity of cells compared to vitamin D3 macrophages. Phaire-Washington *et al.*(1980) also reported that PMA enhanced pinocytosis along with the turn over and recirculation of membrane constituents. Double-stimulated macrophages, due to treatment with both agents may have a membrane turnover rate similar to vitamin D3 macrophages and therefore a low expression of CD14 on the surface will translate into less CD14-dependedent apoptotic cell interaction and uptake. However due to the expression of other molecules, double-stimulated macrophages are relatively efficient at interacting with apoptotic cells compared to PMA and vitamin D3 macrophages.

3.5.4 Immunosuppressive properties of apoptotic cells

Voll *et al.*(1997) found that monocytes stimulated with LPS in the presence of apoptotic cells secreted anti-inflammatory and immunosuppressive cytokines and reduced pro-inflammatory cytokine secretion. Fadok *et al.*(1998) proposed that it was the release of TGF-β following apoptotic cell uptake that acted in an autocrine or paracrine fashion to suppress LPS-induced inflammation. While CD14 is known and previously found to interact with apoptotic cells, the involvement of CD14 in the anti-inflammatory effects promoted by the uptake of apoptotic cells by macrophages is unknown. Therefore THP-1-derived macrophages were stimulated with LPS, following incubation with apoptotic cells and the release of the pro-inflammatory cytokine, TNF-α measured. The results (figure 29) show that the interaction of apoptotic cells with THP-1-derived macrophages prior to LPS stimulation has an immunosuppressive effect. PMA and double-stimulated macrophages express relatively little CD14 at the cell surface compared to vitamin D3 cell. This suggests that the immunosuppressive effects of apoptotic cell uptake, by THP-1-derived macrophages is independent to CD14 expression. With respect to TLR4 expression, THP-1 and vitamin D3 macrophages are hyporesponsive to LPS in comparison to PMA and double-stimulated macrophages due to a lack of TLR4. CD14 and TLR4 are required for LPS signalling and Tapping *et al.*(2000) showed that by transfecting TLR4 into THP-1

cells expressing CD14, increased LPS induced inflammation. The immunosuppressive effects of apoptotic cell interaction inhibited the small but measurable TNF-α response produced by THP-1 and vitamin D3 macrophages. Therefore these data may suggest that TLR4 is not required for the immunosuppressive effects of apoptotic cells clearance.

3.5.5 The role of CD14 in innate immune responses

Hoffmann *et al.*(2001) found that the binding and phagocytic uptake of apoptotic cells by macrophages could be separated into two separate events and suggested that receptors for tethering and engulfment were required. Hoffmann *et al.*(2001) proposed the "tether and tickle" model to describe the binding and uptake of apoptotic cells required to generate an anti-inflammatory and immunosuppressive response. This was supported by Lucas *et al.* (2006) that found that TGF-β alone was insufficient to inhibit LPS-induced inflammation and contact with the dying cell was also required for the anti-inflammatory response. Lucas *et al.* (2006) speculated that the first signal is generated following direct contact of the macrophage with the dying cells denoted as 'tether'. This event stimulates the phagocytosis and TGF-β mediated priming events required for the second signal. The second signal, known as "tickle", is generated following the phagocytosis of apoptotic cells and results in the production and response to TGF-β (Lucas *et al.*, 2006).

Hoffmann *et al.*(2001) suggested several receptors were involved in tethering apoptotic cells to phagocytes but found that uptake was inhibited in the absence of PS. Fadok *et al.* (1992) illustrated the important of PS exposure following activation of apoptotic programme and how failure to expose PS resulted in defective clearance. However Hoffmann *et al.*(2001) found that PS alone was not sufficient to induce uptake but in combination with known tethering receptor, engulfment was enhanced. The clearance of apoptotic cells is now considered to involve four phases: 1. Recognition of apoptotic cells possible due to a loss of 'don't eat me' signals and an increase in 'eat me' signals (Grimsley, 2003), 2 Tethering of the receptor to the phagocyte, 3. Signalling and 4. Phagocytic uptake of apoptotic cell (Gregory and Devitt, 2004). Results illustrated by Hoffmann *et al.*(2001) and conclusions made by Devitt *et al.*(2004) suggest that CD14 functions as a tethering receptor which facilitates the phagocytic uptake of apoptotic cells.

Furthermore CD14; located within micro-domains in the plasma membrane, upon ligation with LPS induces several molecules including TLR4 to cluster and propagate a signal (Triantafilou *et al.*, 2002). These molecules include heat shock proteins 70 and 90,

chemokine receptor 4 and growth differentiation factor 5 (Triantafilou *et al.,* 2001 and Triantafilou and Triantafilou, 2002). It was suggested (Triantafilou *et al.,* 2001 and Triantafilou and Triantafilou, 2002) that LPS is released from CD14 and diffuses laterally within the micro-domain to newly recruited core receptor cluster and activate several pro-inflammatory pathways including NF-κB, and ERK1/2. Therefore ligand interaction with CD14 may dictate receptor clustering and therefore signalling pathways activated.

Previous findings (Devitt *et al.,* 1998) and data shown within this thesis also suggest that CD14 functions to tether apoptotic cells to the phagocyte. However the exact role of CD14 in the anti-inflammatory consequences of apoptotic cell clearance is unknown. Several models are possible (figure 5): 1) Apoptotic cells and LPS bind to different regions of CD14 that, upon ligation, interact with proinflammatory or anti-inflammatory receptor clusters. 2) Alternatively LPS and apoptotic cells interact with the same region of CD14 which indiscriminately activates pro-inflammatory receptor clustering. However interaction of additional ACAMPs with other phagocyte receptors causes immunosuppressive and anti-inflammatory signalling. 3) CD14 functions simply as a tethering receptor for apoptotic cells and surrounding receptors within the micro-domain of CD14 are responsible for phagocytic uptake and anti-inflammatory signalling events. Hoffmann *et al.*(2001) suggests that the PS-dependent uptake of apoptotic cells requires a tethering receptor.

3.5.6 RAGE

RAGE, another PRR expressed by monocytes and macrophages, shares many similarities with CD14, RAGE too is known to bind LPS and activate TLR4 (Bianchi, 2009, Park *et al.,* 2004, Youn *et al.,* 2008, Hofmann *et al.,* 1999 & Ziegler-Heitbrock and Ulevitch, 1993). Furthermore, HMGB1, a ligand for RAGE, was found to compete with LBP for binding to CD14 and also facilitated the transfer of LPS to CD14 (Youn *et al.,* 2008). However little is reported about the ability of RAGE to bind apoptotic cells. While previous data (figure 50) has led to the suggestion that apoptotic cells display LPS-like structures which function as the epitope recognised by CD14. Here RAGE, another LPS-binding molecule is assessed for its ability to bind apoptotic cells and facilitate clearance. Prior to this study research by several groups has led to the suggestion that RAGE may potentially facilitate apoptotic cells clearance. A study by Liu *et al.*(2008) reported that HMGB1 binds to PS on the surface of apoptotic neutrophils and inhibits the phagocytic uptake by mouse peritoneal macrophages *in vitro* and *in vivo*. Moreover Banerjee *et*

al.(2010) found that loss of acidic C-terminus of HMGB1 diminished both the inhibitory effects on apoptotic cell clearance and ability to bind RAGE. Taken together this data may suggest that HMGB1 and apoptotic cells compete for binding to RAGE. Therefore this could suggest that ligand-binding to RAGE potentially has a role in modulating macrophage responses.

3.5.7 Role of RAGE in LPS and apoptotic cell binding

Previously THP-1-derived macrophages models have been a useful tool to facilitate the study of CD14 and therefore were also used to characterise the role of RAGE in the clearance of apoptotic cells. Little is documented about the expression of RAGE during monocyte maturation but generally RAGE expression is described as low on mononuclear phagocytes (Raucci *et al.,* 2008). All THP-1-derived macrophages expressed RAGE while differentiation induced with PMA or double-stimulation produced a sub-population with a high surface expression of RAGE. Raucci *et al.*(2008) also reported that THP-1 expressed low levels of membrane and sRAGE. However PMA treatment is known to activate protein kinase C which causes phosphorylation of inhibitor, IκB leading to NF-κB activation (Bomsztyk *et al.,* 1991). Due to the presence of a positive feedback loop, RAGE expression is regulated by NF-κB activation (Li and Schmidt, 1997). This positive feedback loop is important for host defence as HMGB1 released from damaged cells binds RAGE and triggers an immune response which sustains inflammation and therefore stems invasion of pathogens and reduces tissue damage (Raucci *et al.,* 2008). Previously Bazil and Strominger (1991) found that PMA induced shedding of membrane-bound proteins. Raucci *et al.*(2008) also found that PMA treatment induced proteolytic shedding of mRAGE, therefore this data suggested that PMA treatment increased both mRAGE and most likely led to shedding.

Previous studies have used sRAGE as a decoy for the membrane form (Foell *et al.,* 2006, Hofmann *et al.,* 1999) therefore sRAGE was produced by sub-cloning the extracellular domain, containing the ligand-binding regions, into the expression vector pSec Tag2. Having expressed sRAGE, its ability to bind LPS was assessed as previous results (section 5.7) have suggested that apoptotic cells express LPS-like structures at the cell surface (figure 50). The results presented here suggested that sRAGE may bind to immobilised LPS and is supported by Yamamoto *et al.*(2011) who used surface plasmon resonance and found that LPS bound directly to RAGE. Furthermore following LPS-induced stimulation, mouse-macrophages taken from wildtype mice (expressing mRAGE) produced

significantly more TNF-α compared to RAGE-deficient mice (Yamamoto *et al.,* 2011). However having looked at this study, a possible concern was the level of HMGB1 in these mice following LPS stimulation, serum levels of HMGB1 increased in wildtype but not RAGE deficient mice therefore it is possible that HMGB1 facilitated the transfer of LPS to CD14 which enhanced cellular responses in wildtype.

Having expressed sRAGE the ability of RAGE to bind apoptotic cells was assessed. Immunofluorescence staining suggested that sRAGE did not bind apoptotic cells. Therefore having established that THP-1-derived macrophages express different levels of mRAGE the ability of each macrophage model to clear apoptotic cells in a RAGE-dependent manner was assessed. Prior to co-culturing with macrophages, apoptotic cells were incubated with sRAGE as a competitive inhibitor of mRAGE and the results illustrated that sRAGE did not block apoptotic cell clearance. This led to the suggestion that either RAGE is not involved in clearance or alternatively that sRAGE also facilitates the clearance of apoptotic cells.

Sequence analysis of sRAGE cloned revealed two single point mutations within the parental DNA sequence that codes for the extracellular ligand-binding domain and consequently the effects on ligand binding of RAGE is unknown. Previous results suggested that sRAGE bound LPS therefore suggesting that the ligand-binding site is possibly unaffected. This would support the suggestion that sRAGE does not bind apoptotic cells. However studies conducted following this research by Banerjee *et al.*(2010) and Friggeri *et al.*(2011) implicated RAGE in the phagocytic uptake of apoptotic cells. Friggeri *et al.*(2011) found that macrophages taken from mice deficient in RAGE showed a decreased ability to phagocytose apoptotic cells. Furthermore over-expressing RAGE in amateur phagocytes increased their ability to take up apoptotic cells. With respect to the soluble form, Friggeri *et al.*(2011) also illustrates that incubation of macrophages with sRAGE increased phagocytosis of apoptotic cells. It was suggested that RAGE participates in clearance by binding to PS in a dose-dependent manner that could be blocked by incubation with annexin V, this along with i*n vitro* studies reported that RAGE is involved in the phagocytosis of apoptotic cells (Friggeri *et al.,* 2011). Ligation of RAGE is well characterized for producing inflammation therefore causing upregulation of receptor expression (Schmidt *et al.,* 2000). Friggeri *et al.*(2011) speculates that RAGE is involved in the uptake of apoptotic cells in order to reduce further inflammation and facilitate resolution.

Here the ability of RAGE to bind to apoptotic cell was assessed using flow cytometry and findings suggested that sRAGE did not bind to apoptotic cells. This may suggest that the affinity for apoptotic cells is low and subsequent washing removed bound sRAGE. However the role of RAGE in the clearance of apoptotic cells was assessed using sRAGE as a competitive inhibitor of mRAGE and the findings contrast with published data by Friggeri *et al.*(2011). Friggeri *et al.*(2011) found that sRAGE bound to PS and facilitated clearance most likely by a different manner to the membrane form. Therefore suggesting that sRAGE is not a suitable decoy to examine the role of mRAGE in the clearance of apoptotic cells. An alternative method to examine the role of RAGE in the clearance of apoptotic cells may be to use small interfering RNA (siRNA) to silence wildtype mRAGE. This would avoid the use of knock-out mice which may develop very differently in the absence of mRAGE.

The lack of sRAGE binding to apoptotic cells could be a result of the effects of the single-point mutations within the ligand-binding domain of sRAGE used. This mutation is likely to effect the three dimensional shape of the surrounding regions. It is possible that sRAGE used within this study did not bind to the apoptotic cells due to disruption of the three-dimensional shape of the ligand-binding domain. However sRAGE used within this study bound to LPS immobilised to an ELISA plate suggesting the ligand-binding domain of RAGE retained some of its ability to bind ligands. This may suggest that different ligands interact with different residues within RAGE. Another factor to consider is the size of the ligands, LPS is a small molecule compared to an apoptotic cell, and although RAGE recognises PS on the surface of an apoptotic cell the ligand-binding domain may have to accommodate part of the apoptotic cells. Therefore any mutation within the ligand binding domain that affects the three-dimensional shape may sterically hinder the docking of an apoptotic cell. One way to examine the effects of the mutation on the ligand-binding of RAGE would be to conduct and ELISA with HMGB1.

Results 2: Clearance of apoptotic cells by non-myeloid cells

4.1 Introduction

Macrophages are considered the professional scavengers of apoptotic cells as they are recruited to sites of apoptosis when amateur phagocytes are overwhelmed (Gregory and Devitt, 2004). These cells, unlike amateur phagocytes are highly motile, phagocytic and can uptake apoptotic cells upon first contact (Parnaik *et al.,* 2000 & Grimsley, 2003). Macrophages also have a large repertoire of PRR that are involved in the uptake of apoptotic cells as well as host defence, permitting adaptation within any internal environment (Savill *et al.,* 2002 & Monks *et al.,* 2005). However at normal rates of apoptosis, neighbouring cells (i.e. amateur phagocytes) are capable of clearing apoptotic cells (Parnaik *et al.,* 2000, Gregory and Devitt, 2004).

The receptors used by amateur phagocytes to clear apoptotic cells is largely unknown however previous findings (Wood *et al.,* 2000, Parnaik *et al.,* 2000 & Monks *et al.,* 2005) may suggest that the repertoire of receptors available are limited and saturable. Monks *et al.*(2005) showed that mammary epithelial cells clear apoptotic cells in a PS-dependent manner, using receptors also used by macrophages. Blocking-antibodies to calreticulin, CD91 and the vitronectin receptor were used, which reduced phagocytosis of apoptotic cells by mammary epithelial cells. Therefore given these findings and previous observations within our laboratory group; that found respiratory epithelial cells were LPS responsive, **it is hypothesised that CD14 is expressed by epithelial cells, within specific sites with an intended role in modulating immune responses.**

4.2 Cell surface expression of CD14 on amateur phagocytes

Here the role of CD14 in innate immune responses by non-myeloid, amateur phagocytes is considered. Therefore the assessment of cell surface CD14 on various tissue epithelial cells and human pulmonary fibroblasts was assessed by indirect immunofluorescence staining of viable cells with established mAb 63D3 followed by flow cytometric analysis (figure 35).

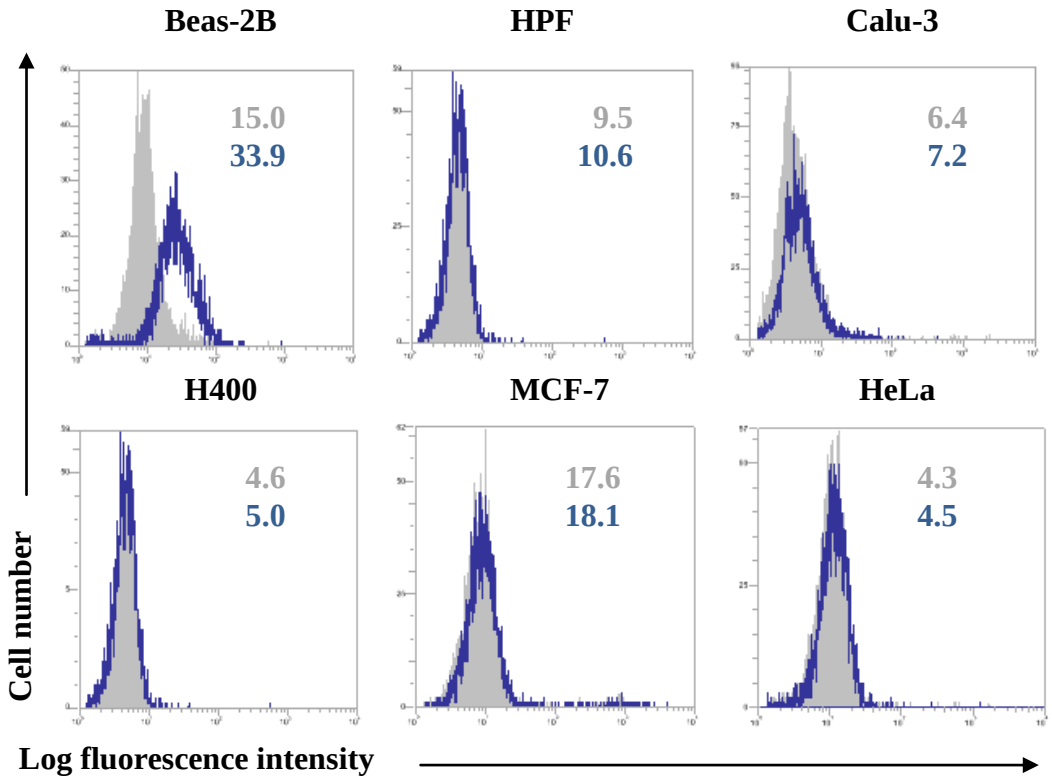


Figure 35: Surface expression of CD14 on epithelial cells
Surface expression of CD14 on pulmonary (Beas 2B and Calu-3), oral (H400), mammary (MCF-7) and cervical (HeLa) epithelial cell lines, along with human pulmonary fibroblast (HPF), was detected with flow cytometric staining using indirect immunofluorescence with the mAb 63D3 (blue) or isotype-matched control MOPC 21 (grey). The mean fluorescence intensity of the population is shown the top corner of each histogram. The results are representative of three independent experiments.

Flow cytometric analysis suggests that CD14 is only expressed on pulmonary epithelial cells, Beas-2B and Calu-3. Surface expression of membrane CD14 was undetectable on cervical (HeLa), oral (H400), mammary (MCF-7) epithelial cells and HPF.

4.2.1 LPS-responsiveness of CD14-expressing epithelial cells

CD14 is well characterised for its ability to bind LPS released during Gram negative bacterial infections and promote inflammation (Wright *et al.*, 1990 and Ulevitch and Tobias, 1999). Given previous flow cytometric findings that suggest Beas-2B and Calu-3 pulmonary epithelial cells express significant levels of membrane CD14, the ability of these cells to response to LPS-stimulation was assessed (figure 36).

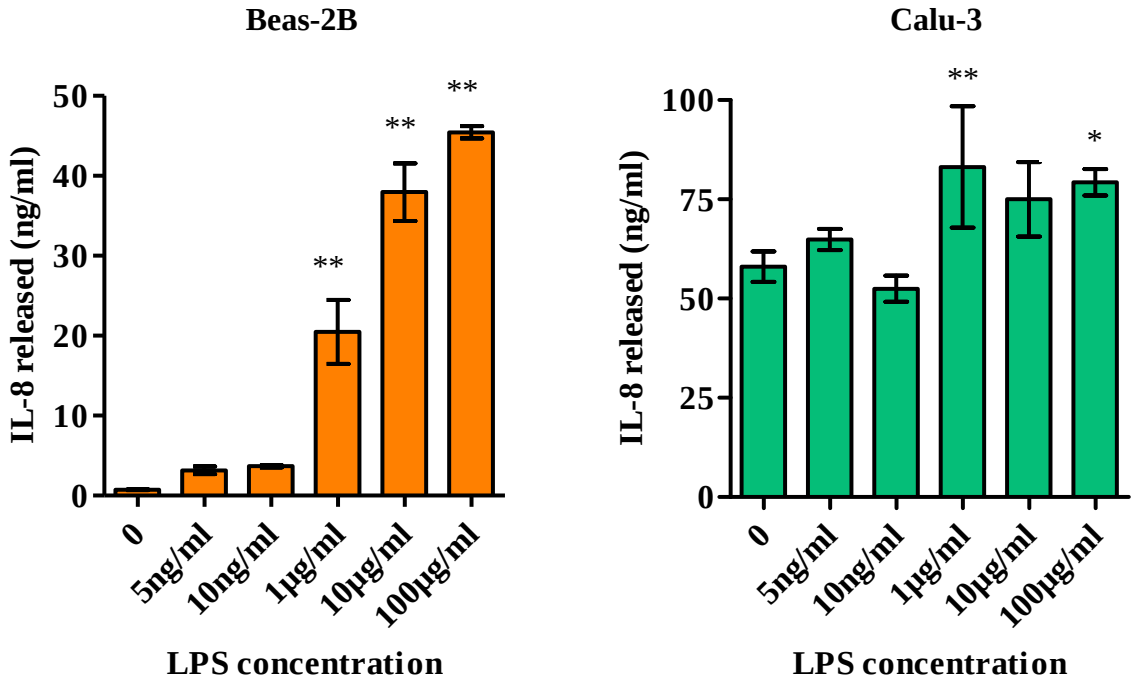


Figure 36: LPS-stimulation of pulmonary epithelial cells
Cells were seeded at $1x10^5$cells per well in 24 well plates and incubated overnight. Following incubation cells were stimulated with increasing concentrations of LPS in the presence of 10% NHS for 24 h prior to analysis for IL-8 by ELISA. The results shown are the mean ± SD of quadruplet wells. *Statistical analysis conducted was compared to no LPS using ANOVA followed by Dunnetts post test and result (*P<0.05, **P<0.01)*

The results in figure 36 suggest that LPS-induced stimulation of Beas-2B resulted in a dose-dependent increase in IL8-released following stimulation with 1µg/ml of LPS. LPS-induced stimulation of Calu-3 (above of 1µg/ml) also resulted in IL-8 response but, unlike Beas-2B, did not appear dose-dependent and basal levels were much higher. This may suggest that the level of mCD14 expressed by Calu-3 but not Beas-2B was a limiting factor. CD14 expression along with LPS response data may suggest that pulmonary epithelial cells respond to LPS in a CD14-dependent manner.

4.2.2 TLR4 expression on pulmonary epithelial cells

Previous data has suggested that Beas-2B and Calu-3 express cell surface CD14 (figure 35) and are LPS-responsive (figure 36) possibly in a CD14-dependent manner. However mCD14 does not contain a cytosolic signalling domain therefore a signalling partner is required for cellular response. Typically TLR4 is required to confer an LPS-dependent response (Hoshino *et al.*, 1999,Miyake, 2004) therefore the expression of TLR4 on Beas-2B and Calu-3 was assessed with direct immunofluorescence staining (figure 37).

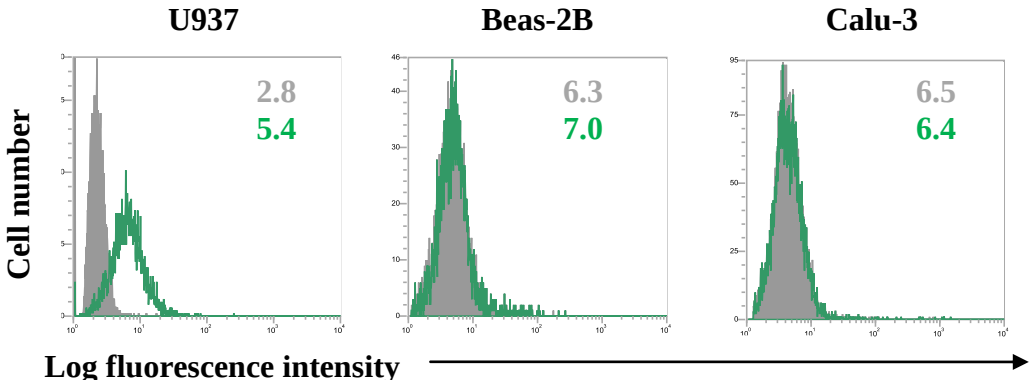


Figure 37: Surface expression of TLR4 on Beas-2B and Calu-3
Surface expression of TLR4 on a variety of cells was detected with flow cytometric staining using direct immunofluorescence with the monoclonal antibody HTA125-PE (green) or IgG2a-PE (Isotype-matched control, (grey)). U937 cells were used as a positive control. The results are representative of three separate experiments.

Cell surface expression of TLR4 was undetectable on Beas-2B and Calu-3 following flow cytometric analysis. This data is supported by Guillot *et al.*(2004) that used RT-PCR and found that in Beas-2B, TLR4 is expressed at mRNA level but was not found at the cell surface.

4.3 The clearance of apoptotic cells by amateur phagocytes

Little is known about the receptors utilised by amateur phagocytes to bind and engulf apoptotic cells. Monks *et al.*(2005) found that mammary epithelial cells used the same receptors as macrophages to clear apoptotic cells. Furthermore Devitt *et al.*(2004) found that in CD14-deficient mice, the clearance of apoptotic cells at specific sites was reduced suggesting that CD14 may be involved in the removal of apoptotic cells by amateur phagocytes. Therefore here the ability of amateur phagocytes to interact with apoptotic cells was assessed along with the role of CD14 in clearance (figure 38).

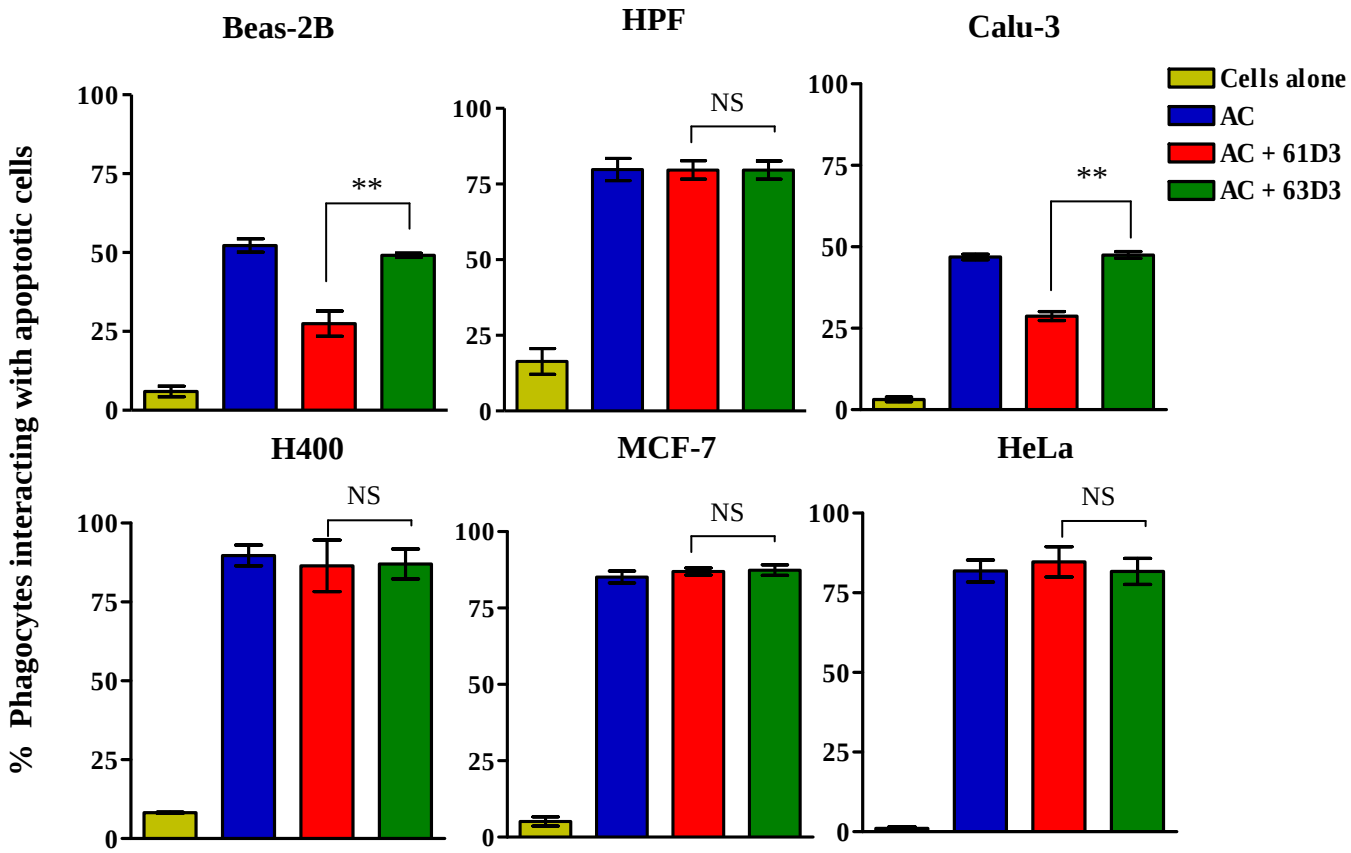


Figure 38: CD14-dependent apoptotic cell clearance by amateur phagocytes
All epithelial cell and fibroblasts were seeded on four-well glass slides and incubated overnight to allow for adhesion. Following incubation cells were incubated with medium or co-cultured with apoptotic Mutu for 1 h at 37°C alone and with CD14-blocking mAb 61D3 or non-blocking mAb 63D3. Slides were then washed in ice-cold PBS and fixed in 1% (v/v) formaldehyde prior to Jenner-Giemsa staining. Phagocyte interaction with apoptotic cells was assessed by light microscopy and values scored as a percentage. The results shown are the mean ± SEM of three independent experiments. *Statistical analysis conducted was ANOVA followed by Bonferroni post-test (** $P<0.01$).*

The results in figure 38 suggest that all amateur phagocytes analysed interacted with apoptotic cells. The percentage of H400, HPF, MCF-7 and HeLa cells interacting with apoptotic cells is 75% (approximately the same), which may suggest that the receptors or mechanisms to remove apoptotic cells is the same. While the percentage of Beas-2B and Calu-3 interacting with apoptotic cells is 50%, the level of interaction is lower than other amateur phagocytes shown (HeLa, MCF-7, H400 and HPF). The ratio of apoptotic cells to phagocytes remained constant therefore any variation in the level of interaction is dependent on the receptors or mechanisms used by each cell type to clear apoptotic cells. Previous data showed that pulmonary epithelial cells expressed cell surface CD14 and as shown in figure 35, CD14-blocking mAb 61D3 inhibited apoptotic cell clearance by both Beas-2B and Calu-3 cells by around 50%. This suggests that pulmonary epithelial cells utilised CD14 to clear apoptotic cells unlike fibroblast, oral, mammary and cervical epithelial cells.

4.4 Conclusion

4.4.1 LPS responses in non-myeloid cells

The expression of membrane CD14 was originally considered to be limited to myeloid cells, B cells and mammary cells (Ziegler-Heitbrock and Ulevitch, 1993). However Fearns *et al.*(1995) found that CD14 RNA was expressed in murine tissues within various organs, including, the lung, spleen, heart and thymus. Within these tissues, CD14 RNA was detected within epithelial cells, suggesting these cells may function as more than simply a physical barrier. Therefore recent findings within our research laboratory group, that suggested pulmonary epithelial cells were LPS-responsive was of great interest, as the role of CD14 in the innate immune response of non-myeloid cell was unclear. Consequently a range of human epithelial cells from different tissue origins was obtained and cell surface CD14 expression assessed. Flow cytometric analysis suggests that pulmonary epithelial cells express mCD14 while expression was not detected on oral, mammary and cervical epithelial cells as well as pulmonary fibroblasts tested. Given previous findings that CD14 is required for LPS responsiveness of cells, the ability of pulmonary epithelial cells to respond to LPS was assessed and shown to induce an IL-8 response. The role of CD14 in LPS-responses within the lungs is supported by Brass *et al.*(2007) who observed that CD14-deficient mice were hyporesponsive to LPS inhalation but intratracheal treatment with sCD14 restored inflammation to that of wild-type. Furthermore findings by Jeyaseelan *et al.*(2005) suggest that CD14 and TLR4 are required for LPS responses within the lungs. However here cell surface expression of TLR4 was not detected on pulmonary epithelial cells but may suggest that cell surface levels were below the limits detectable by flow cytometry. These findings are supported by Guillot *et al.*(2004) who suggested that TLR4 was not expressed at the surface of Beas-2B cells but using RT-PCR found that TLR4 was expressed at an mRNA level. Furthermore immunoblotting of cellular lysates from Beas-2B cells suggested that TLR4 was present in intracellular compartments (Guillot *et al.*2004). Findings by Triantafilou and Triantafilou (2002) suggested that TLR4 is not present at the cell surface, unlike CD14, but is recruited to lipid rafts following LPS stimulation. However Guillot *et al.*(2004) found that LPS-stimulation did not induce cell surface expression of TLR4 at any point during stimulation but found that cellular response to LPS was TLR4-dependent. Guillot *et al.*(2004) suggested that the lack of cell surface TLR4 and failure of LPS to up-regulate CD14 expression on pulmonary epithelial cells may provide a protective mechanism against small doses of LPS contained with air inhaled. This may suggest that LPS-induced activation of pulmonary epithelial cells occurs

following internalisation of LPS caused by a receptor mediated event, which then encounters intracellular TLR4. A similar mechanism of LPS-induced TLR4 activation is reported within the gastrointestinal tract in the absence of cell surface TLR4. The absence of TLR4 has been linked to hyporesponsive of intestinal epithelium to resident enteric flora and therefore prevents unnecessary inflammation and local tissue damage (Hornef *et al.*, 2002). Hornef *et al.*(2002) identified that in intestinal epithelial cells, TLR4 is located within the golgi but furthermore found that LPS is also associated with TLR4 in this location too. Although findings by Latz *et al.*(2002) identified that in HEK293 and most likely all epithelial cells, TLR4 and CD14 are recycled from the golgi to the cell membrane. Latz *et al.*(2002) found that cross linking membrane TLR4 resulted in activation and concluded that TLR4 found in the golgi is not required for LPS-induced stimulation of these cells. However this study does not really address the role of intracellular TLR4 in LPS-mediated signalling in the absence of cell surface TLR4. The results by Latz *et al.*(2002) may suggest that if TLR4 and CD14 are present at the cell surface signalling occurs from the surface but in the absence of TLR4, LPS is internalized possible using CD14 and may result in TLR4 intracellular signalling. Some experiments to consider, as future work might be to establish if the LPS responsiveness of pulmonary epithelial cells is CD14-dependent. This could be established by stimulating Beas-2B and Calu-3 with LPS in the presence of CD14-blocking mAb MEM18. If the data suggests that CD14 is required for LPS responses then the role of TLR4 in LPS signalling could be assessed using small hairpin RNA to silence TLR4 expression. Subsequently if cells are hyporesponsive to LPS, in the absence of TLR4 this would suggest that both CD14 and TLR4 are required for the LPS responsiveness of pulmonary epithelial cells. Given the findings shown here, this would support the suggestion that LPS activates intracellular TLR4, following the CD14-dependent uptake of LPS. A possible method of assessing the role of LPS uptake in TLR4 activation may be to prevent membrane turnover then examine LPS-induced responses.

4.4.2 Role of non-myeloid CD14 in apoptotic cell clearance

While little is known about the receptors utilised by amateur phagocytes to mediate clearance, mammary epithelial cells were found to express and utilise CD36, the vitronectin receptor, calreticulin/CD91 and the PS receptor in the clearance of apoptotic cells (Monks *et al.,* 2005). Other receptors analysed within the study included CD14, but flow cytometric analysis suggested that mammary epithelial cells did not express cell surface CD14 (Monks *et al.,* 2005). Monks *et al.* (2005) reported that the anti-

inflammatory effects of apoptotic cell clearance occurs in amateur phagocytes, as stimulation with LPS in presence of apoptotic cells, inhibited TNF-α production. Given these findings, here a series of amateur phagocytes were screened for their ability to interact with apoptotic cells in a CD14-dependent manner. The data suggested that oral, mammary and cervical epithelial along with pulmonary fibroblasts, interacted with apoptotic cells to the same level (75%). This may suggest that the mechanisms of clearance used by these phagocytes are the same and lack of inhibition with CD14-blocking mAb 61D3, suggests that clearance is not CD14-dependent. The percentage of pulmonary epithelial cells (Beas-2B and Calu-3) interacting with apoptotic cells was lower than other phagocytes analysed; suggesting that the clearance of apoptotic cells by pulmonary epithelial cells was not as efficient. This suggests that the mechanisms used by pulmonary epithelial cells to remove apoptotic cells from the lungs differ from other sites within the body. However apoptotic cells are rarely observed in healthy human lungs (Vandivier *et al.*, 2006), suggesting that the level of cell death is either lower *in vivo* or that apoptotic cells are rapidly removed. The assay used here to measure apoptotic cell interaction is a snap shot at a one hour time point, with pulmonary epithelial cells appearing to have less apoptotic cells bound. Therefore this data may indicate that interaction of pulmonary epithelial cells with apoptotic cells is lower due to more efficient uptake and digestion, indicated by less surface-bound apoptotic cells. However Parnaik *et al.*(2000) reported that while both professional and amateur phagocytes bound apoptotic cells it took several hours for amateur phagocytes to engulf apoptotic cells but digestion time was the same. Parnaik *et al.* (2000) suggested that the uptake of apoptotic cells was not mediated until additional cell surface changes had occurred. Therefore the events within the later stages of apoptosis such as PS exposure may be required for uptake of apoptotic cells by amateur phagocytes. However the clearance of apoptotic cells by phagocytes can be separated into two distinct phases, tethering and uptake (Hoffmann *et al.*, 2001 and Lucas *et al.*, 2006). Parnaik *et al.*(2000) findings suggests that amateur phagocytes have the receptors for tethering apoptotic cells but either the receptors that mediate uptake are limited or only recognise late apoptotic cells. Alternatively rearrangement of cytoskeleton within amateur phagocytes may take several hours to allow uptake. However the persistence of apoptotic cells is considered to lead to secondary necrosis and possibly inflammation (Erwig and Henson, 2007). In the lung particularly, failed clearance of cells such as, neutrophils would be massively detrimental with proinflammatory consequences. While innate immune components within the lungs provide defence against invading pathogens, many have been implicated in the clearance of apoptotic cells, including, the mucociliary escalator,

immunoglobulins, complement factors, surfactant proteins A and D along and alveolar macrophages. Therefore pulmonary epithelial cells may maintain mucosal integrity by modulating immune responses. Pulmonary epithelial cells express a variety of innate immune receptors to mediate host defence (Diamond *et al.,* 2000). Several innate immune receptors are known also to be involved in the clearance of apoptotic cells, therefore it is likely that pulmonary epithelial cells utilise multiple receptors to interact with and mediate uptake of apoptotic cells. This may suggest that pulmonary epithelial cells either express receptor also found on macrophages to mediate uptake upon contact or can facilitate uptake quicker than other amateur epithelial cells.

4.4.3 Role of CD14 in innate immune responses of pulmonary epithelial cells

LPS data presented here suggests that the pulmonary epithelial cells, Beas-2B and Calu-3 express mCD14 and are responsive to LPS stimulation. CD14 is also involved in the clearance of apoptotic cells (Devitt *et al.,* 1998), findings by Devitt *et al.*(2004) suggested that CD14-deficient mice have persistent apoptotic cells within specific tissue, including, the lungs. Taken together, these findings may suggest that the expression of CD14 by alveolar macrophages and pulmonary epithelial cells is required for the efficient clearance of apoptotic cells within the lungs. Furthermore the binding of apoptotic cells to CD14 was uncoupled from the anti-inflammatory consequences of apoptotic cell clearance, as persistence of apoptotic cells did not induce inflammation (Devitt *et al.,* 2004). This led to the suggestion that CD14 functioned as a tethering receptor in apoptotic cell clearance. Here the requirement of CD14 in the clearance of apoptotic cells by pulmonary epithelial cells has been shown in the absence of cell surface TLR4. This data therefore supports previous findings within this thesis that have suggested that TLR4 is not required for the clearance of apoptotic cells by CD14.

In conclusion, the data presented here may suggest that the presence of CD14 on pulmonary epithelial cells facilitated efficient and rapid removal of apoptotic cells. The proposed theoretical mechanism is that apoptotic cells are rapidly tethered to mCD14, which enhances apoptotic cell interaction with engulfment receptors such at TIM-4 or Mer, subsequently promoting rapid uptake. Given findings here that suggest pulmonary epithelial cells express receptors also utilised by macrophages to bind apoptotic cells and that epithelial cells efficiently clear apoptotic cells, what dictates the level of professionalism of a phagocyte? If non-resident macrophages are only recruited to sites site

of apoptosis, when neighbouring phagocytes are overwhelmed this may suggest that non-resident macrophages are the ‘amateurs’?

Results 3: The mapping of soluble CD14 point-mutants

5.1 Introduction

As previously mentioned CD14 is also expressed in a soluble form lacking the phosphatidylinositol tail which anchors the receptor to the membrane. Soluble CD14 can be produced following secretion of the protein, prior to addition of the phosphatidylinositol tail during post-translational modifications in the golgi, or by enzymatic digestion of membrane CD14 (Bufler *et al.,* 1995). While little is currently known about the role of sCD14 in the clearance of apoptotic cells, several groups (Frey *et al.,* 1992 &Pugin *et al.,* 1993) have reported that sCD14 enables endothelial and epithelial cells that do not express mCD14, to respond to LPS. Frey *et al.*(1992) reported that human umbilical vein endothelial cells (HUVEC) and U393 astrocytoma cells require sCD14 along with LBP to respond to LPS. Frey *et al.*(1992) also suggested that following LPS binding, sCD14 propagated a stimulatory signal by interacting with a cell surface bound receptor; this was prior to the discovery of TLR4. The principles of this work were then used to underpin the studies used by several groups (Darveau *et al.,* 1998, Cunningham *et al.,* 1999,Shapiro *et al.,* 1997 & Juan *et al.,* 1995c) along with mutagenesis studies to map the regions of CD14 required for LPS binding. Devitt *et al.*(1998) reported that CD14 is also involved in the clearance of apoptotic cells and shows that mAbs 61D3 and MEM18 blocked both LPS responses and apoptotic cell binding to CD14. This lead to the suggestion that the same region of CD14 bound LPS and apoptotic cells and was able to facilitate contrasting ligand-dependent responses (Devitt *et al.,* 1998 & Gregory and Devitt, 2004). **However apoptotic cell binding to CD14 has not been mapped therefore the divergence in macrophage response to apoptotic cells and LPS could be caused by a variation in the residues involved in ligand (LPS and AC) binding.** If apoptotic cells interact with the same regions of CD14 as LPS, then any differences in response would most likely be caused by the activation of contrasting signalling partners. Therefore the aim of this chapter is to test the hypothesis that residues within CD14 required for LPS binding, are also involved in apoptotic cell binding. This will establish an understanding of the molecular mechanism employed by CD14 to generate two distinct ligand-dependent macrophages responses. Devitt *et al.* (2004) also found that sCD14 bound apoptotic cells but not live cells. Therefore here a series of sCD14, point-mutants are used to consider the key residues involved in the binding of LPS and apoptotic cells.

5.2 Introducing the soluble CD14 mutants

sCD14 point-mutants were used in studies to map *E.coli, P.gingivalis* and *H.pylori* LPS binding to CD14 and kindly received from Richard Darveau (Washington University, USA) (Darveau *et al.*, 1995, Shapiro *et al.*, 1997, Cunningham *et al.*, 1999, 2000). The plasmids encode for human CD14 containing single residue mutation within the N-terminal 60 amino acids of the protein upstream of cDNA encoding for hinge, CH_2 and CH_3 domains of human IgG1 in the mammalian expression plasmid pcDNA3.1. All mutations are within the N-terminal region of CD14, (figure 39) as this region is required for the binding of LPS (Viriyakosol and Kirkland, 1995). More specifically the mutations are within three of the four LPS binding regions previously identified for E.*coli* LPS binding (Juan *et al.*, 1995a, Stelter *et al.*, 1997, Shapiro *et al.*, 1997 & Cunningham *et al.*, 2000).

Figure 39: The LPS binding regions of CD14 are located within the N-terminal
All four LPS binding regions of CD14 as shown in red (labelled R1-R4) are located within the first 66 amino acids of CD14 Previous work with the CD14-blocking antibody 61D3 and MEM18 have suggested that apoptotic cells also bind within the N-terminus of CD14(Juan *et al.*, 1995a, Stelter *et al.*, 1997, Shapiro *et al.*, 1997 & Cunningham *et al.*, 2000). Arrows indicate where the point-mutations received are located, in relation to-LPS binding regions of CD14.

The ten soluble CD14-point mutants received (table 2) are alanine replacement and same charge mutations with the exception of E11R which is a charge reversal mutation (Table 2).

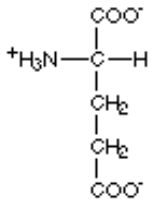
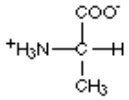
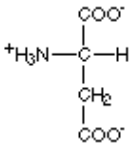
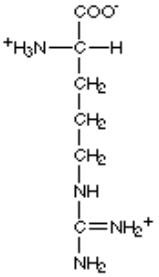
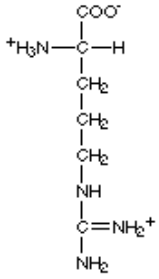
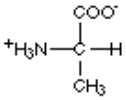
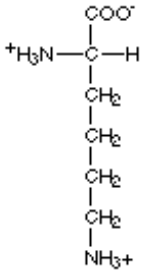
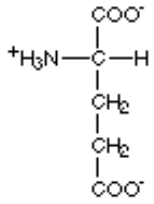
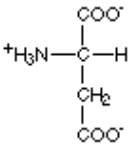
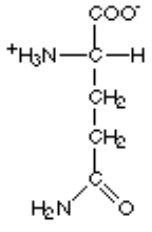
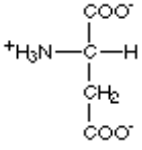
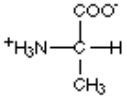
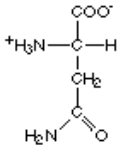
Native amino acid	Position	Substituted Amino acids/ Name mutation		
(E)Glutamic Acid  Hydrophilic Negative	11	(A)Alanine  Hydrophobic Neutral E11A	(D)Aspartic Acid  Hydrophilic Negative E11D	(R)Arginine  Hydrophilic Positive E11R
(R)Arginine  Hydrophilic Positive	14	(A)Alanine  Hydrophobic Neutral R14A		(K)Lysine  Hydrophilic Positive R14K
(E)Glutamic Acid  Hydrophilic Negative	37	(D)Aspartic Acid  Hydrophilic Negative E37D		(Q)Glutamine  Hydrophilic Neutral E37Q
(D)Aspartic Acid  Hydrophilic Negative	59	(A)Alanine  Hydrophobic Neutral D59A		(N) Asparagine  Hydrophilic Neutral D59N

Table 2: The structure and charge details of each mutated sCD14 received

5.3 Establishing a method of transfection

Having acquired the glycerol stocks of wildtype and mutant sCD14, the first task was to produce all sCD14 mutant plasmids suitable for transfection into mammalian cells. Following culturing, plasmid DNA was recovered and sequenced to confirm the presence of the mutation.

HEK293 are routinely used for the transient transfection of plasmids expressing soluble protein. HEK293 are easy to culture and transfect, especially with calcium phosphate-mediated transfection, one of the first methods explored due to its simplicity and cost-effectiveness. The efficiency of calcium phosphate-mediated transfection of DNA was assessed (figure 40).

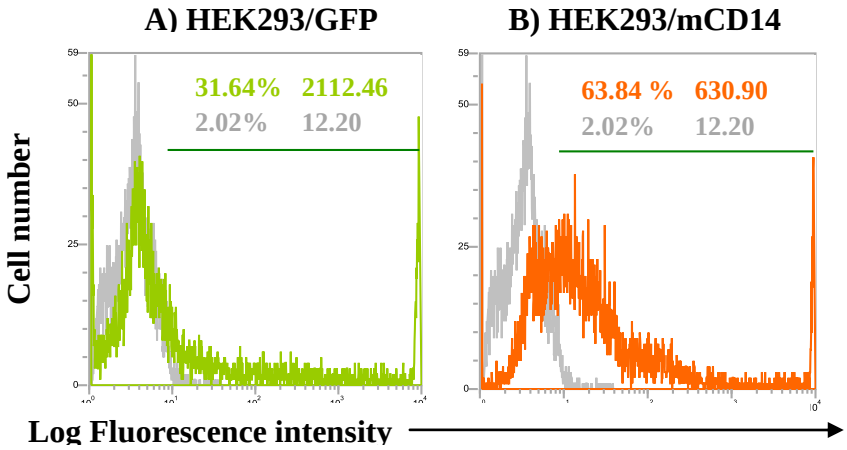


Figure 40: Calcium phosphate-mediated transfection of HEK293
HEK293 were transfected using calcium phosphate with 2µg of mCD14 or plasmid containing GFP and incubated for 24h post-transfection. Following incubation **A.** CD14 expression was analysed using indirect immunofluorescence with mAb 63D3 or control mAb BU65 (anti-ICAM-3 was used as isotype-matched control) (grey) and analysed using flow cytometry. **B.** HEK293 transfected with a plasmid expressing GFP (green) or mock transfection were also analysed using flow cytometry. The percentage of cells and the mean fluorescence intensity of the population within the positive gated region are shown.

The results in figure 40 suggest calcium phosphate-mediated transfection of plasmid DNA was successful. Both CD14 expression (as detected by indirect immunofluorescence) and GFP expression show an increase in the mean fluorescence intensity compared to the control. However the results suggested that the transfection efficiency was lower with GFP compared to mCD14 as the percentage of cells having increased in fluorescence was lower. Any differences in transfection efficiency may cause a variation in the yield of sCD14 mutants produced, while attempts were made to optimise calcium phosphate-mediated

transfection method, each experiment varied in the efficiency. Therefore a more robust and consistent method of transfection was required.

LT1-mediated transfection was previously used within the laboratory and found to produce reproducibly high transfection efficiencies. Therefore LT1-mediated transfection was used to transfect HEK293 with a plasmid expressing GFP at different ratios of reagent to DNA to optimise expression of antigen (figure 41).

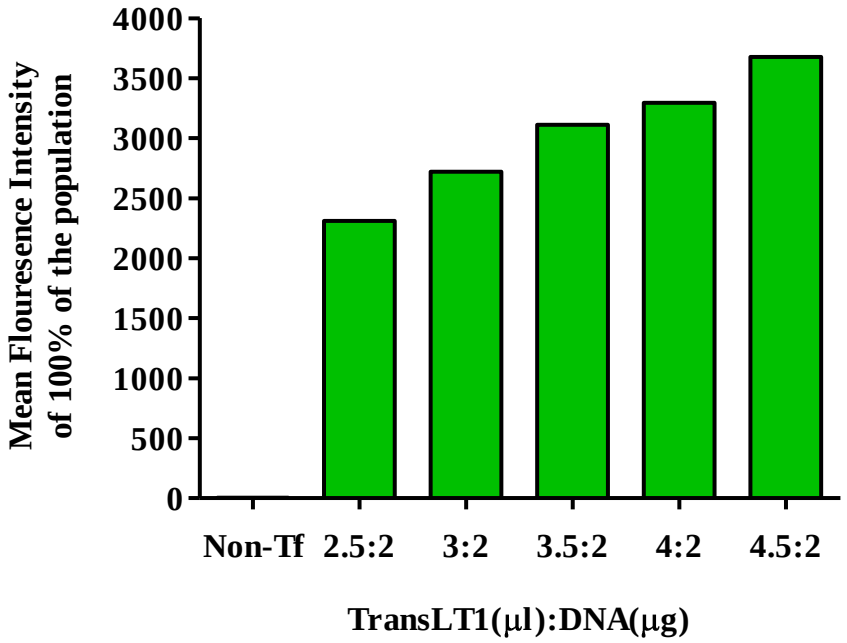


Figure 41: Optimisation of LT1-mediated transfection of HEK293
HEK293 were seeded at $3x10^5$per well in 6 well plates and incubated overnight. Different ratios of LT1 was mixed with 2µg of plasmid DNA within 250µl of sfRPMI and incubated for 15-30min to allow for the formation of DNA-complexes. Following incubation, the transfection mixture was added drop-wise to the HEK293 and incubated for 24-72 h. Cells were then analysed using flow cytometry for an increase in the mean fluorescence intensity for 100% of the population. The results are representative of two independent experiments.

The results in figure 41 suggest that as the ratio of LT1 to DNA increased, the mean fluorescence intensity of HEK293 also increased. The increase in the mean fluorescence intensity of the population suggests a greater uptake of cDNA per cell. This method was found to be more efficient and reproducible than calcium phosphate-mediated transfection, as the mean fluorescence intensity of the whole population always increased. The ratio, 3.5µl:2µg ratio of LT1 reagent to DNA was chosen and mutant sCD14-Fc plasmids were transfected into HEK293. 24 hours following transfection, the medium was changed to sfDMEM and the cells are incubated for a further 72 hours to maximise protein production. The transfection supernatant was harvested, centrifuged to remove cells then a capture ELISA conducted to verify the expression of wtCD14 and mutants (figure 42).

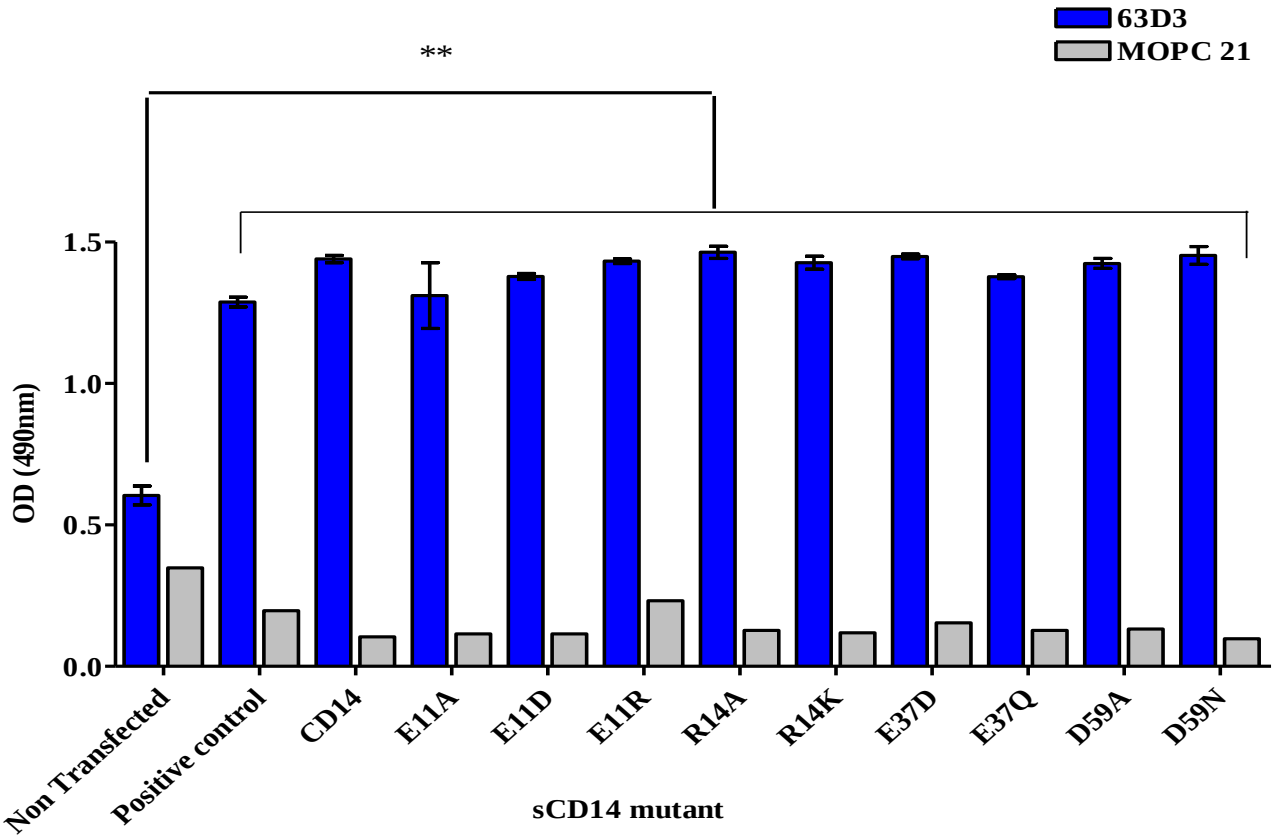


Figure 42: ELISA of sCD14-Fc production
Following transfection, sCD14 expression and release was verified by capture ELISA. sCD14-Fc mutants were immobilised from supernatants, by capture with 5µg/ml of sheep anti-human Fc thus capturing the mutants by the Fc tags. Bound sCD14 was detected with mAb 63D3 using MOPC 21 as isotype negative control. Purified sCD14-Fc previously used within our laboratory was used as a positive control while supernatant taken from cells transfected with no DNA (non-transfected) was used as a further negative control. The data shown is the mean ± SD of duplicate wells and representative of three independent experiments. *Statistical analysis used was ANOVA followed by Dunnetts post-test (**P< 0.01)*

The ELISA results in figure 42 suggest that wtCD14 and mutants were successfully expressed in HEK293. 63D3 binds towards the C-terminus of the protein (Viriyakosol *et al.*, 2000) away from the suggested LPS or apoptotic cell binding regions of CD14. As the results show, 63D3 binds all of the mutants tested, suggesting that no mutant induced a profound conformational change that altered the epitope of 63D3 binding to the C-terminus of CD14. Any variation in 63D3 binding was therefore likely to be a result of varying concentrations of protein.

5.4 Mapping LPS binding

The highly conserved lipid A region of LPS (figure 3) provides the molecular motif recognised by the innate immune system and is responsible for the inflammatory properties of LPS (Rietschel *et al.*, 1994). CD14 is well characterised for its ability to bind the lipid A

region of LPS (Fenton and Golenbock, 1998), however at concentration above 11µg/ml LPS forms micelles preventing binding to CD14 (Aurell and Wistrom, 1998). The lipid transfer protein, LBP is required for shuttling LPS monomers from micelles to CD14 (Fenton and Golenbock, 1998 &Viriyakosol and Kirkland, 1995). The resulting complex is recognised by TLR4 and causes the generation of a proinflammatory event (Hoshino *et al.*, 1999 & Lien *et al.*, 2000).

Previous research (Juan *et al.*, 1995a, Stelter *et al.*, 1997, Shapiro *et al.*, 1997 & Cunningham *et al.*, 2000) using LPS blocking mAbs, truncated and mutated versions of CD14 has identified four regions involved in LPS binding. All four LPS binding regions of CD14 are located within the first 65 amino acids of the amino-terminal of the protein (Viriyakosol and Kirkland, 1995). The crystal structure proposed by Kim *et al.*(2005) identified this region of CD14 as a hydrophobic pocket that contains all four LPS binding regions (figure 43). Juan *et al.*(1995) reported that mAb MEM18 competed with LPS for binding to residues 57-64 of CD14. Furthermore Devitt *et al.*(1998) found that both 61D3 and MEM18 blocked apoptotic cell binding and competed with each other for binding to CD14. Devitt *et al.*(1998) also found that 61D3 blocked LPS responses and taken together these data suggest that the binding sites of LPS, MEM18, 61D3 and apoptotic cells are closely associated or possibly the same. This raises the suggestion that the N-terminal pocket of CD14, which contains the LPS binding sites (figure 43) also contains the apoptotic cell binding sites. **Therefore the aim of this chapter is to compare and contract LPS and apoptotic cell binding to sCD14 mutants.**

A) Sequence alignments

```
Human   PEPCELDDEDFRCVCNFSEPQPDWSEAFQCVSAVEVEIHAGGLNLEPFLKRVDADADPRQYA
Mouse   PEPCELDEE--SCSCNFSDPKPDWSSAFNCLGAADVELYGGGRSLEYLLKRVDTEADLGQFT
        *******:*   * ****:*:****.**:*:.*.:**::.** .** :******::**  *::
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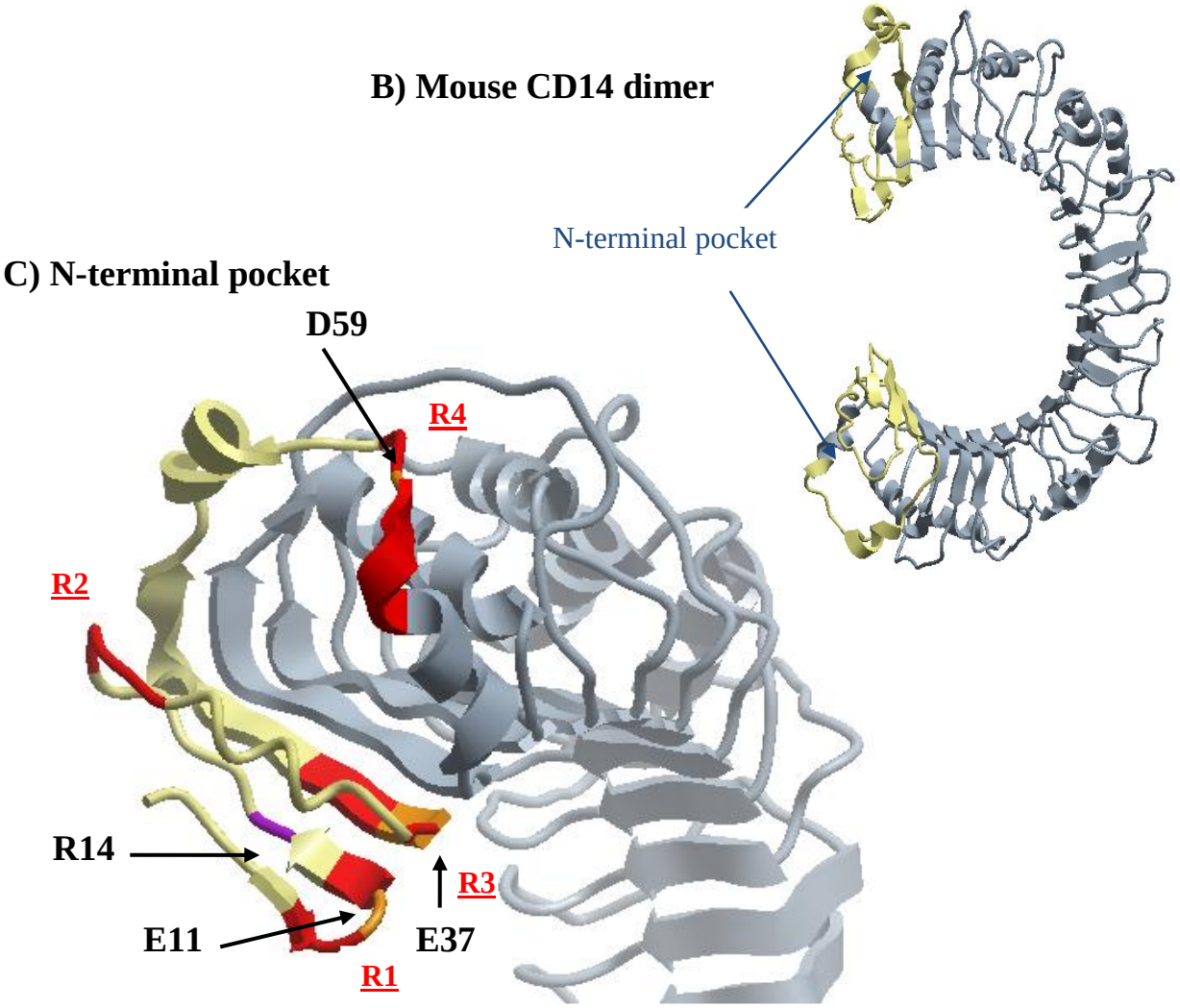

Figure 43: The sequence alignment of human and mouse CD14 and the crystal structure of mouse CD14

A. Sequence alignment of the NH_2-terminal region of CD14 reveals 67% homology between human and mouse. The nucleotides within the LPS binding regions, outlined in red are identical (*) or highly conserved (:). **B**. The crystal structure of mouse CD14 shown suggests CD14 forms a horse-shoe shaped dimer in solution, with each monomeric unit composed of thirteen β-strands containing a similar number of LRRs similar to TLR4. **C**. The N-terminus (yellow) of each monomeric unit (grey) contains a hydrophobic pocket (labelled with arrows) responsible for LPS binding. Closer analysis of the pocket revealed four regions (shown in red and labelled R1-R4) that are required for LPS binding. The location the residues E11, E37 and D59 are shown in orange as these residues are located within the first, third and fourth LPS binding regions of CD14. R14 is shown in purple as this residue is outside the first LPS binding region. *Adapted using Mol ICM browser from Kim et al., 2005.*

Also shown in figure 43 is the location of each residue that has been mutated in generating the sCD14 point-mutants used within this study. Previously Cunningham *et al.* (2000) used capture ELISA to measure and block LPS binding to wildtype sCD14 and mutants. Therefore the ability of each sCD14 mutant to bind LPS was examined in a capture ELISA format (figure 44).

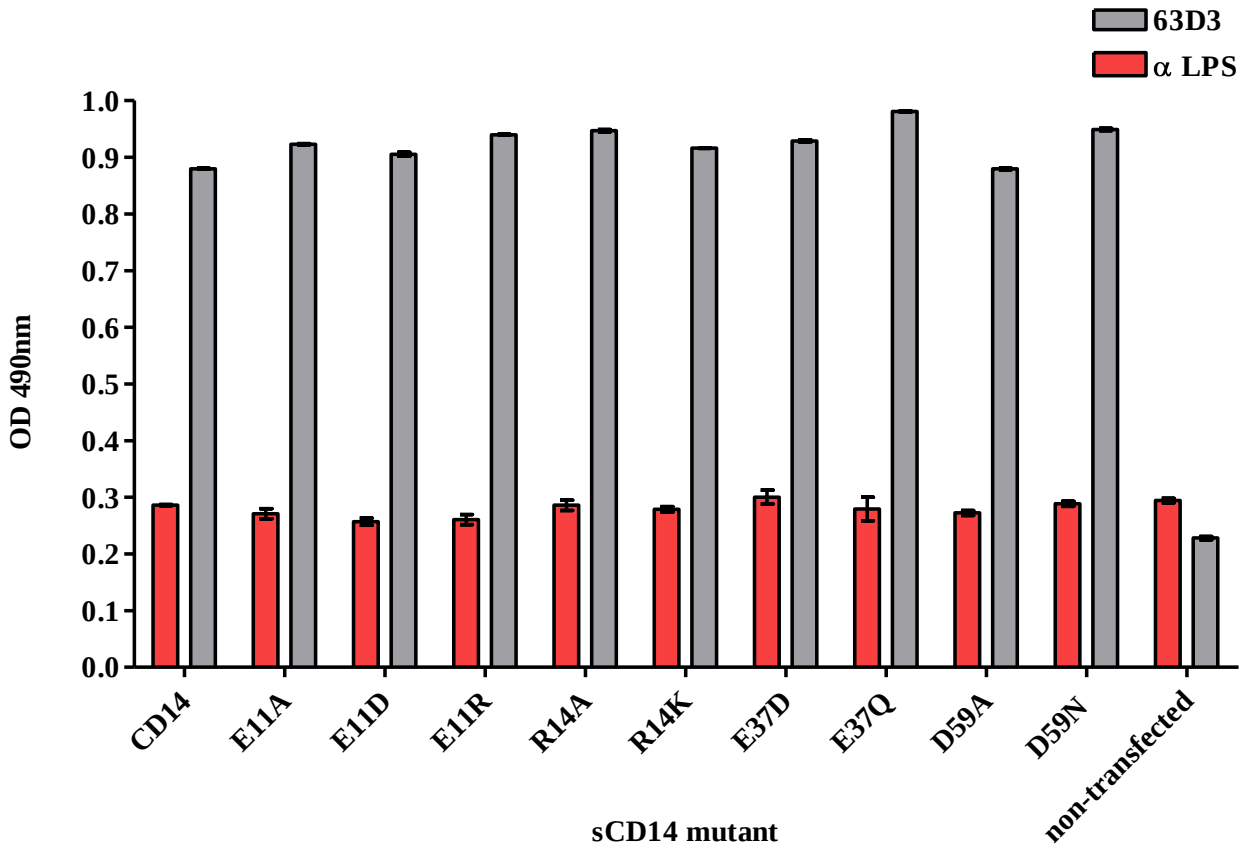

Figure 44: LPS binding ELISA required a blocking agent
Soluble CD14-Fc mutants or mock (non-transfected) were immobilised from supernatants by capture with 5µg/ml of sheep anti-human Fc for 1 h at 37°C. Following incubation 10µg/ml of LPS in 10% NHS or 63D3 was added to wells in triplicate and incubated at 37°C for 1 h. The plate was washed and LPS bound was detected first with polyclonal anti-LPS antibody then with anti-Rabbit HRP or anti-mouse HRP for 63D3 binding. OPD substrate was added to the plate and the optical density read at 490nm.

As shown in figure 44, detectable binding of LPS in all wells, including the control supernatant (containing no CD14) was equivalent, suggesting a lack of specific interaction with CD14, under the conditions used. Due to the sticky nature of LPS, previous experiments conducted and studies have required a blocking agent when conducting an LPS ELISA. Cunningham *et al.*(2000) used 0.5% BSA in PBS. However a study by Péterfi and Kocsis (2000) compared various blocking agents for use in LPS binding ELISA format. The results of the study suggested normal goat serum (NGS) was the most effective agent at reducing non-specific binding of LPS. Taking this into consideration the

LPS binding ELISA was repeated and following incubation of LPS for 1 h at 37°C the ELISA plate was washed three times and incubated with NGS at room temperature overnight (figure 45).

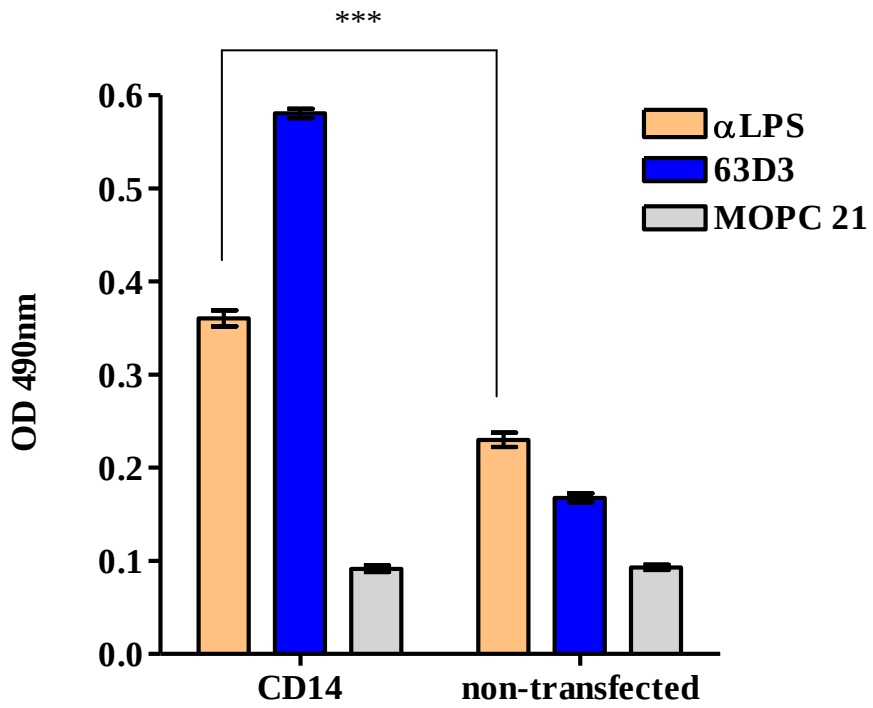

Figure 45: LPS-binding ELISA, goat serum was used as blocking agent
Wildtype sCD14-Fc or mock transfection (control containing no CD14) were immobilised from supernatants by capture with 5µg/ml of sheep anti-human Fc for 1 h at 37°C. Following incubation the plate was blocked with 300µl of NGS at 20°C. The next day 63D3 or 10µg/ml of LPS in 10% NHS was added to wells in triplicate and incubated at 37°C for 1h. Any LPS bound was detected first with polyclonal anti-LPS antibody then with anti-Rabbit HRP or anti-mouse HRP for 63D3 binding. OPD substrate was added to the plate and the optical density read at 490nm. The data shown is the mean ± SD of triplicate well and is representative of four independent experiments. *Statistical analysis was conducted using T-test. (*** $P<0.001$).*

As shown in figure 45, goat serum reduced non-specific binding of LPS to the control supernatant coated wells. Comparison of figures 44 and 45 suggest that incubation with goat serum caused an increase in LPS binding to sCD14 that was reproducible. Initially it was considered that the increase in signal in CD14 following the blocking stage due to the goat serum containing endotoxin. However following contact with Sigma Aldrich, the concentration of endotoxin in goat serum supplied was described as less than 5EU/ml, equivalent to 0.5ng/ml of LPS. This concentration of endotoxin is negligible, as it is 1:20000 of the total of LPS added (10µg/ml). Another possible explanation considered was that goat serum was a second source of LBP that may have first bound CD14 then LPS

following incubation. As shown in figure 45, 63D3 binding to CD14 were used as a positive control.

Given the benefits of goat serum as a blocking agent, the panel of sCD14 mutants were immobilised to ELISA plates and LPS binding measured (figure 46). In order to rule out any variation in binding as a consequence of the concentrations of each mutant, LPS binding results were normalised to 63D3 then wildtype sCD14. Therefore 63D3 binding was used as a surrogate for estimating concentration as it binds towards the C-terminus of CD14, away from LPS binding regions (Viriyakosol *et al.*, 2000). However this makes the assumption that the confirmation of the C-terminus of each mutation remains unchanged.

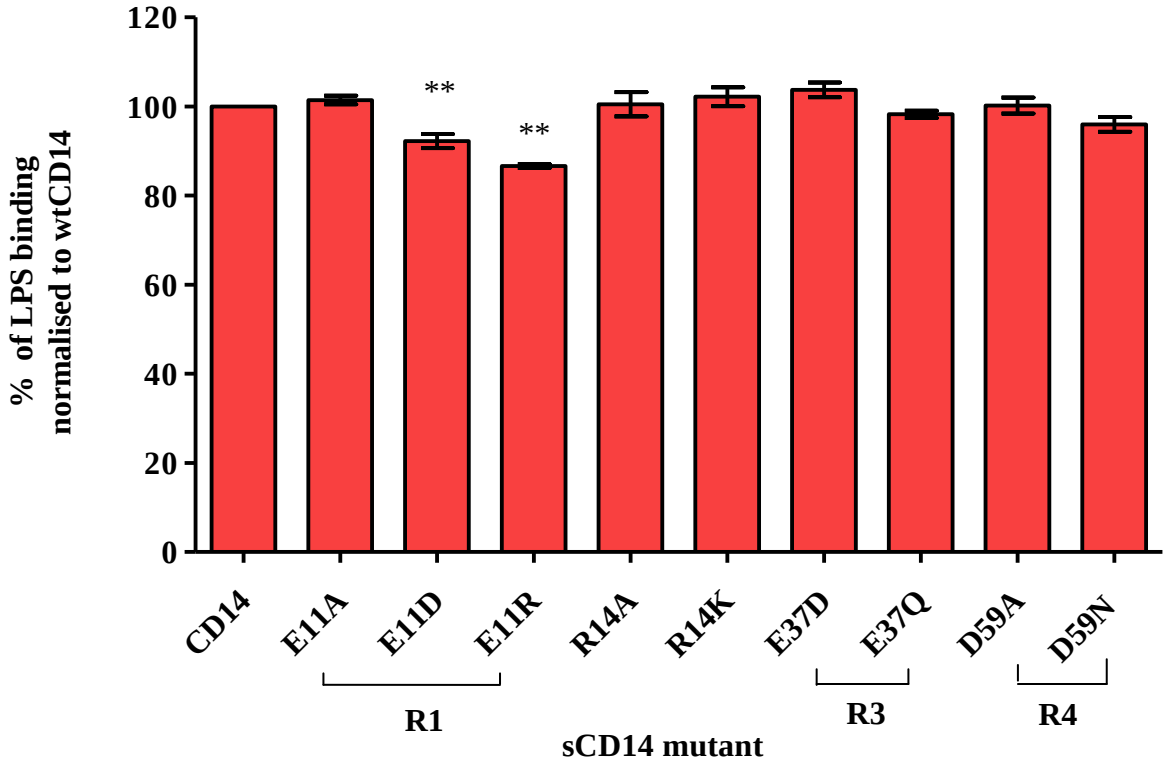

Figure 46: LPS-binding to sCD14 mutants
Soluble CD14-Fc mutants were immobilised from supernatants by capture with 5µg/ml of sheep anti-human Fc for 1 h at 37°C. Non-specific binding was blocked with 300µl of NGS and the plates were incubated with 10µg/ml of LPS in 10% NHS. Bound LPS was detected with polyclonal anti-LPS. The location of the single mutated residue with respect to the LPS binding regions of CD14 is identified as R1-4. The data shown is the mean ± SEM of seven independent experiments, with the OD of each mutant was first normalised to 63D3 binding then normalised to wildtype. *Statistical analysis conducted was ANOVA followed by Dunnetts post test (**P<0.01 comparison to wtCD14).*

The results in figure 46 demonstrate that single residue mutations within the first LPS binding regions of CD14, significantly reduce LPS binding. Alanine replacement of glutamic acid (E11A) has no significant effect on LPS binding to sCD14 despite the loss of

charge. However a same charge mutation (E11D) and a charge reversal mutation (E11R) caused a significant reduction in LPS binding to E11 when compared to wtCD14. R14, E37 or D59 mutants analysed had no significant effect on LPS binding compared to wtCD14.

5.5 MEM18 and 61D3 compete for binding to wildtype sCD14

Devitt *et al*.(1998) found that 61D3 and MEM18 were CD14 mAb that competed for binding to CD14 and both blocked LPS responses and apoptotic cell interaction. Furthermore Juan *et al*. (1995) mapped the epitope of the MEM18 to the fourth LPS binding region of CD14 and reported that MEM18 and LPS compete for binding to residues 57-64. This suggests that a region of CD14 defined for its ability to bind LPS may also bind apoptotic cells. The binding of mAb 61D3 has not been mapped to CD14 using a point mutant approach. Here we examine that possibility that, like LPS, 61D3 also competes with MEM18 for binding suggesting that apoptotic cells and LPS share common binding sites within CD14 (figure 47).

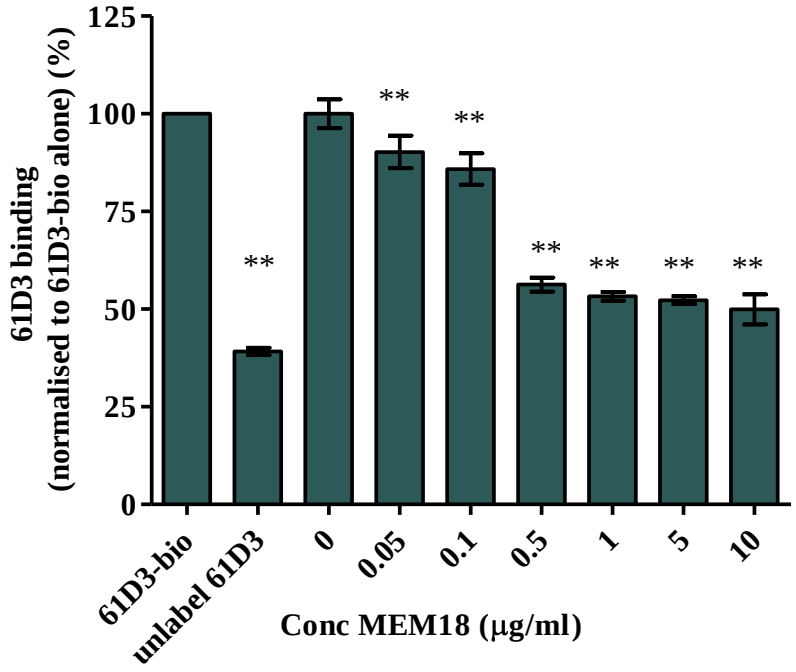

Figure 47: MEM18 and biotinylated-61D3 compete for binding to wildtype sCD14. Wildtype sCD14–Fc was immobilised from supernatant by capture with 5µg/ml of sheep anti-human Fc for 1 h at 37°C. Following incubation 100ng/ml of 61D3 (biotin) was added to every well then varying concentrations of unlabelled MEM18 was added and incubated for an h at 37°C. Biotinylated 61D3 bound to CD14 was detected with streptavidin-HRP for 1 h. Following incubation OPD substrate was added to the plate and the optical density read at 490nm. The data shown is the mean ± SD of triplicate wells and is representative of three independent experiments. *Statistical analysis conducted was unpaired T-test (**P<0.01 comparison to 61D3-bio).*

The results in figure 47 suggest that as the concentration of MEM18 increase there is a loss in the binding of the biotinylated mAb 61D3. This data along with previous findings by Juan *et al.* (1995) suggest that the epitopes of 61D3, MEM18 and LPS within CD14 are closely associated or the same. However previous data shown here has suggested that 61D3 and MEM18 block apoptotic cell interaction therefore these data suggest that the epitopes of LPS and apoptotic cells are closely associated or the same. Therefore if both ligands (LPS and apoptotic cells) interact with the same regions of CD14 any variation in ligand-dependent response may be due to variation in signalling.

5.6 61D3 and MEM18: Surrogate markers for apoptotic cell binding

5.6.1 Mapping 61D3 binding to sCD14 mutants

The N-terminal pocket of CD14 is important for LPS binding as all four LPS binding regions are within or clustered around the pocket (Kim *et al.*, 2005, Cunningham *et al.*, 2000and Viriyakosol & Kirkand). Previously MEM18 and 61D3 have been shown to block both LPS responses and apoptotic cell interaction (Devitt *et al.*, 1998), suggesting that the N-terminal pocket of CD14 is involved in the binding of apoptotic cells. Therefore here sCD14 point mutants were used to map the binding of 61D3 and MEM18 as surrogate markers of apoptotic cell binding (figure 48).

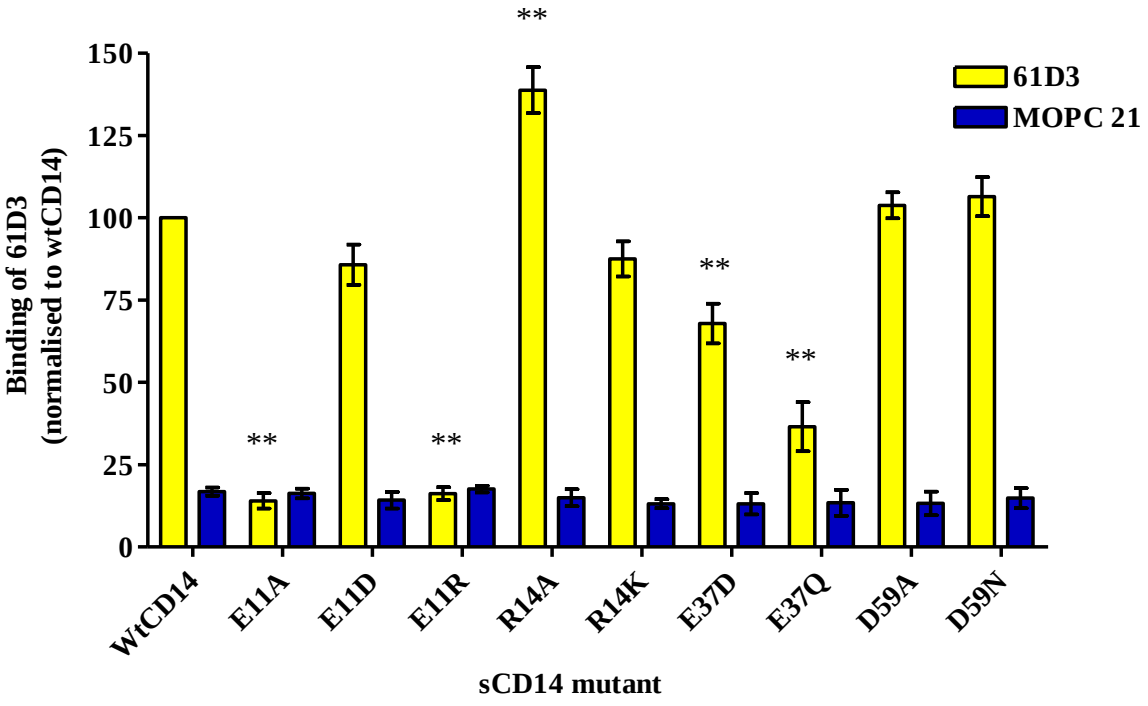

Figure 48: Mapping 61D3 binding to sCD14 mutants
Soluble CD14–Fc mutants were immobilised from supernatants by capture with 5µg/ml of sheep anti-human Fc for 1 h at 37°C. Bound sCD14 was detected with mAbs 61D3, 63D3 as a positive control or MOPC 21 as isotype-matched control. Binding was detected with anti-mouse-HRP for 1h at 37°C. Following incubation OPD substrate was added to the

plate and the optical density read at 490nm. The data shown is mean ± SEM of three independent experiments and 61D3 binding has been normalised to 63D3 then wtCD14 binding. *Statistical analysis conducted was AVOVA followed by Dunnetts post test (**P<0.01 comparison to wtCD14)*

The epitope of 61D3 is unknown but is suggested as a region of CD14 important for LPS and apoptotic cell binding. As previously stated 63D3 binding is considered to be unaffected by mutations within the C-terminus of CD14 therefore is used as a positive control and to standardise protein concentration. The results in figure 48 show a loss of 61D3 binding to CD14 upon substitution of residue 11. Replacement with alanine (E11A) or a charge reversal mutation (E11R) results in a loss of 61D3 binding, while replacement with aspartic acid (E11D), another acidic residue had no effect on 61D3 binding. This suggests that residue 11 is a contact residue required for binding of 61D3. The results also show an increase in 61D3 binding to CD14 upon alanine replacement at residue 14 but not upon lysine replacement. This suggests that a loss of the positive charge at residue 14 increases 61D3 binding. The data also shows replacement of glutamic acid at residue 37 with aspartic acid or glutamine causes a loss in 61D3 binding. The results suggest that residue 11, 14 and 37 may be required for apoptotic cell binding however it was noted that a loss is 61D3 binding is caused by a change in the charge of these key residues.

5.6.2 Mapping MEM18 binding to sCD14

MEM18 is reported to bind to CD14 between residue 57 and 64, defined as the fourth LPS binding region of CD14 (Juan *et al.,* 1995a). Previous result here have shown that MEM18 and 61D3 compete for binding to CD14 therefore suggesting that the epitopes are within close proximity. Here MEM18 binding was mapped to sCD14 mutants as a surrogate marker of apoptotic cell binding to CD14 (figure 49).

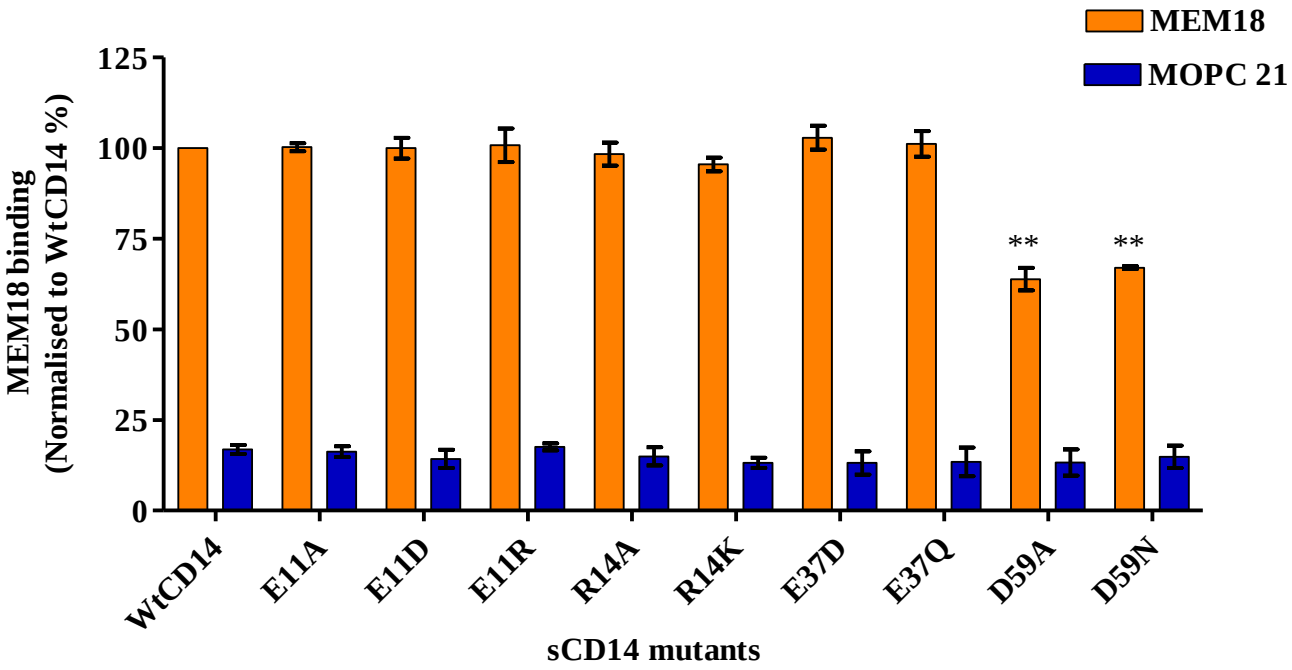

Figure 49: Mapping MEM18 binding to sCD14 mutants
Soluble CD14–Fc mutants were immobilised from supernatants by capture ELISA with 5µg/ml of sheep anti-human Fc for 1 h at 37°C. Bound sCD14 was detected with mAbs MEM18, 63D3 as a positive control or MOPC 21 as negative control. Binding was detected with anti-mouse-HRP for 1 h at 37°C. Following incubation OPD substrate was added to the plate and the optical density read at 490nm. The data shown is mean ± SEM of three independent experiments where the OD of MEM18 binding has been normalised to 63D3 and then wtCD14. *Statistical analysis conducted was ANOVA followed by Dunnetts post test (**P<0.01 comparison to wtCD14)*

MEM18 bound all E11, R14 and E37 mutations suggesting that none of the mutations cause a detrimental conformation change within the protein to prevent binding (unlike 61D3). A loss of MEM18 binding was observed upon mutation of residue 59 as previously reported (Juan *et al.*, 1995a) and suggests this residue is required for apoptotic cell binding to CD14. However both alanine and asparagine have no overall charge, unlike aspartic acid, emphasising the importance of the negative charge at position 59 required for MEM18 binding.

5.7 LPS-like structures on the surface of apoptotic cells

The first, third and fourth LPS binding regions of CD14 are located on the same surface of N-terminal pocket. This region is rich in electrostatic charges that are considered to facilitate the capture of CD14 ligands (Cunningham *et al.*, 2000). The data above supports the suggestion that CD14 employs charged residues to interact with apoptotic cells and LPS. Due to similarities in binding patterns identified it was considered that apoptotic cells may have LPS-like structures on the surface that are recognised by CD14 (Gregory and

Devitt, 2004). Therefore induced and non-induced cells were examined for LPS-like structures using indirect immunofluorescence staining with polyclonal anti-LPS antibodies and analysed by flow cytometry (figure 50).

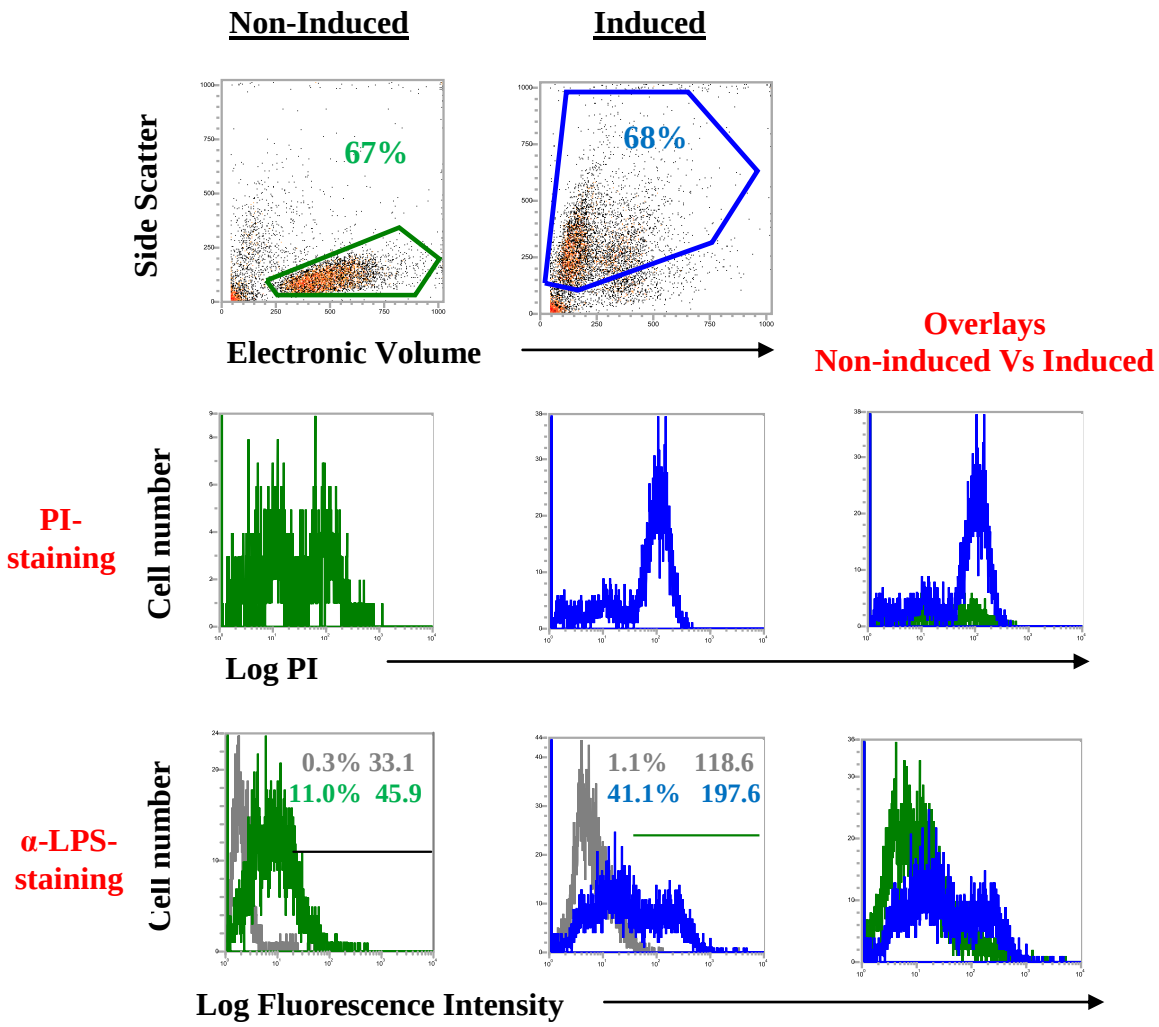

Figure 50: Analysis of LPS-like structures on the surface of apoptotic cells
Mutu were induced to undergo apoptosis by exposure to UV radiation and incubated overnight (induced). Plasma membrane integrity of both non-induced (green) and induced (blue) cells was assessed with propidium iodide staining (PI-staining) and histograms overlaid. Both non-induced and induced cells were analysed for LPS-like patterns on the surface (α-LPS-staining), with indirect immunofluorescence using anti-LPS polyclonal antibody (green/blue) or normal Rabbit serum (grey). Following staining of induced cells, a second peak was observed and a region denoted by a single green line was drawn to calculate the % gated and mean fluorescence intensity of the population shown in the top right of each histogram. This data is representative of four independent experiments.

Following UV treatment and incubation overnight, apoptosis was assessed by flow cytometry and the results suggest that 68% of the cells induced to undergo apoptosis, have increased in side scatter and decreased in electronic volume. Apoptosis was also assessed using the vital dye, propidium iodide (PI), which upon entry into cells, intercalates with DNA and was therefore used to assess plasma membrane integrity. Live cells and early

apoptotic cells have an intact plasma membrane and therefore are considered PI negative, however later stages of apoptosis causes loss of plasma membrane integrity and therefore the cells become PI positive. The PI staining results in figure 50 suggest that non-induced cells did not stain with PI indicating an intact plasma membrane. While induced cells stained with PI produced a peak suggesting that the plasma membrane of these cells was not intact therefore confirming that these cells were within the later stages of apoptosis. Both induced and non-induced cells were then stained with anti-LPS antibodies or rabbit serum as a negative control. The non-induced cells increased in fluorescence when stained with anti-LPS antibodies compared to rabbit serum suggesting binding. However two populations of cells were identified following staining of apoptotic cells with anti-LPS antibodies. The first population corresponded to the population seen when non-induced cells were stained with anti-LPS antibodies but the second population were more fluorescent. The results support findings by Tennant. (2005) that following apoptosis, cells demonstrated an increased ability to bind anti-LPS antibodies and therefore those apoptotic cells may share common epitopes with LPS.

5.8 Mapping apoptotic cell binding to soluble CD14 mutants

Devitt *et al.*(2004) found that sCD14 bound to apoptotic cells, therefore sCD14 point mutants was considered to map the binding of apoptotic cells. Here apoptotic Mutu were stained with soluble wtCD14-Fc and binding detected by flow cytometry (figure 51).

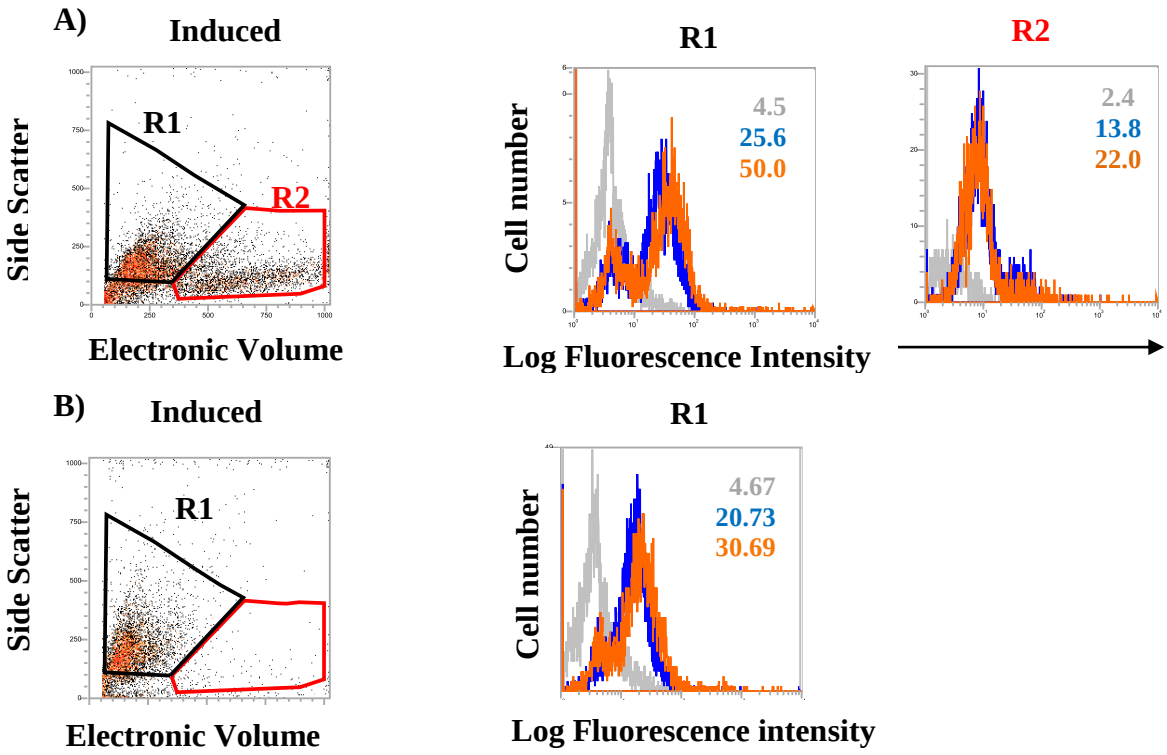

Figure 51: Analysis of apoptotic cells stained with wildtype sCD14-Fc
Mutu were induced to undergo apoptosis with UV treatment and incubated overnight prior to immunofluorescence staining. Cells were gated based on side scatter and electronic volume as shown above (R1/R2). Induced cells were incubated with supernatant from HEK293 transfected with soluble WtCD14-Fc (orange) or mock (no DNA) (blue) or wash buffer (grey) for a minimum of 30 min on ice. A) Cell bound-Fc was detected with anti-human FITC for 30 min on ice. B) Prior to the addition of (goat) anti-human FITC cells were washed twice with 5% NGS in PBS to reduce non specific binding. 5000 events within the gated region R1 were analysed for surface bound sCD14-Fc and the mean fluorescence intensity of each peak is shown on top right of the overlays.

This was first attempted by simply incubating apoptotic cells with supernatant harvested from HEK293 transfected with sCD14-Fc plasmid or no DNA (mock) (figure 51A). The flow cytometric results suggest that there was limited sCD14 bound to apoptotic cells, however there was also a significant amount of non-specific binding of anti-human FITC. Therefore following staining of apoptotic cells with sCD14 or mutants, cells were washed in 5% NGS prior to addition of anti-human Fc FITC in an attempt to reduce non-specific binding (figure 51B). Comparison of the flow cytometric results (figure 51) shows that the log fluorescence intensity of induced cells stained with sCD14-Fc was reduced following washing with 5% goat serum. Therefore it was decided that incubation with 20% goat serum for 15 minutes at 4 °C prior to incubation with sCD14 may be required to further reduce non-specific binding of (goat) anti-human-FITC (figure 52).

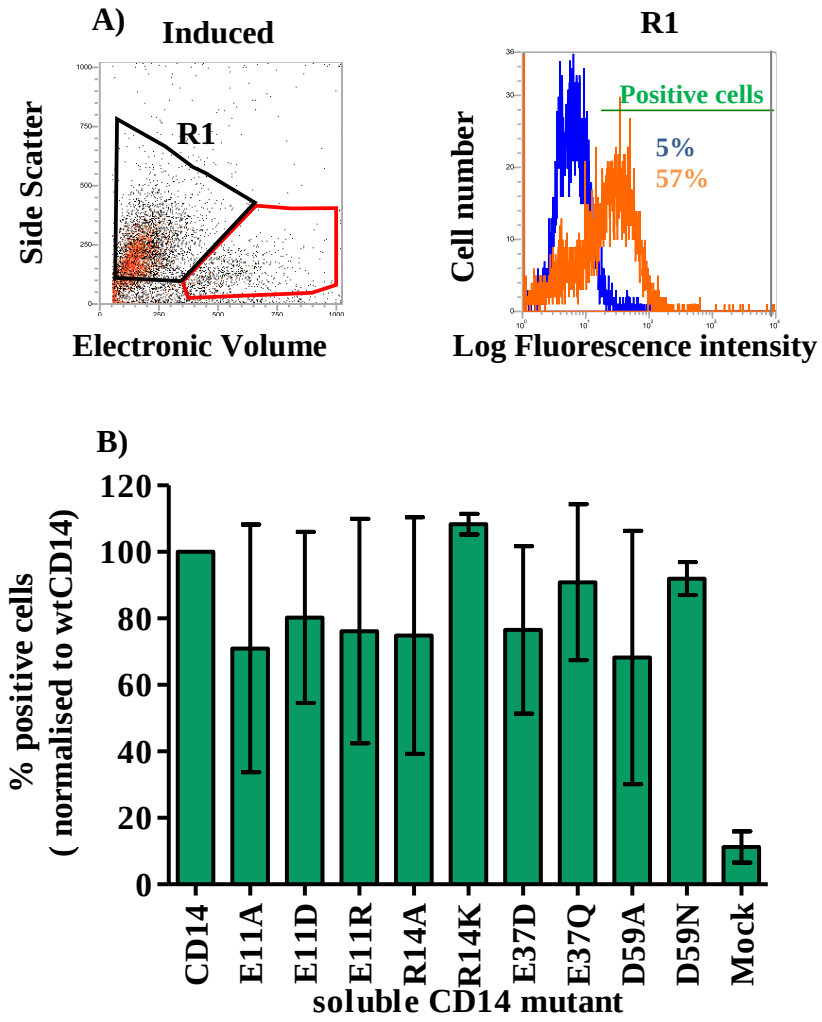

Figure 52: Mapping apoptotic cell binding with soluble CD14 mutants
A. Mutu was induced to undergo apoptosis with UV treatment and incubated overnight prior to immunofluoresence staining. Cells were gated based on side scatter and electronic volume as shown above (R1). Induced cells were incubated with 20% goat serum prior to incubation with sCD14 mutants (orange) or mock supernatant (blue)(taken from HEK293 transfected with no DNA) for 30 min on ice. Following incubation cells were washed twice with 5% goat serum then incubated with (goat) anti-human FITC. 5000 events within R1 were analysed for surface bound sCD14-Fc, only wildtype sCD14-Fc shown above and the mean fluorescence intensity of wtCD14-Fc is shown in top right of the overlay. **B.** Having successfully shown that wildtype sCD14-Fc bound to apoptotic cells, the sCD14 point mutants were used to map apoptotic cell binding. In order to compare the level of binding of each sCD14 mutants to apoptotic cells, the percentage of positive cells were normalised to wildtype sCD14-Fc. The results shown are the mean ± SD of two independent experiments.

Devitt *et al.*(2004) found that sCD14-Fc bound to apoptotic cells, therefore sCD14 mutants were considered, to map apoptotic cell binding to CD14. However due to the level of non-specific binding of (goat) anti-human Fc-FITC to apoptotic cells, binding of sCD14-Fc was obscured. Previously apoptotic cells were washed with 5% goat serum prior to incubation

with anti-human Fc-FITC to reduce non-specific binding. The results suggest that this treatment alone was insufficient to reduce non-specific binding therefore prior to incubation with sCD14-Fc the cells were pre-incubated with 20% goat serum and all washes were conducted with 5% goat serum. The flow cytometric results shown in figure 52 suggested that pre-incubating the cells with 20% goat serum reduced non-specific therefore binding of sCD14-Fc to cells was shown. Having established that wildtype sCD14-Fc bound to apoptotic cells, the level of binding of each CD14 point mutant was compared in an attempt to map apoptotic cell binding to CD14 (figure 52B). As shown in figure 52B the binding of wildtype and mutant sCD14-Fc to apoptotic cells varied each time the experiment was conducted. Therefore this could suggest that all sCD14 point mutations analysed, with the exception of R14K affected apoptotic cell binding to sCD14. However the level of R14K binding to apoptotic cells seemed persistently high suggesting that this mutation had no effect on apoptotic cell binding to sCD14.

5.9 Discussion

CD14 is well characterised for its ability to bind LPS and promote inflammation (Wright *et al.,* 1990, Ulevitch and Tobias, 1999). Mutagenesis studies and mAb mapping have identified four regions within the first 65 amino acids of CD14 that are required for LPS binding and responses (Viriyakosol and Kirkland, 1995, Juan *et al.*, 1995a, Stelter *et al.*, 1997, Shapiro *et al.*, 1997 & Cunningham *et al.*, 2000). The sequence alignment of human and mouse CD14 shared 67% homology and function the same in terms of LPS response (Haziot *et al.,* 1996) and apoptotic cell clearance (Devitt *et al.,* 2004). While the crystal structure of human CD14 is unknown, the crystal structure of mouse CD14 has identified a large hydrophobic pocket within the N-terminus which contains all four LPS binding regions. The crystal structure of CD14 developed by Kim *et al.*(2005) identifies the first, third and fourth LPS binding region of CD14 as being located on the same side of the pocket, within close proximity. Furthermore each of the LPS binding regions is predominantly composed of hydrophilic residues exerting an overall negative charge. Therefore the pocket is considered to display several charged residues to non-specifically capture LPS (Cunningham *et al.*, 2000).

While apoptotic cell binding to CD14 has not been previously mapped, previous work by Devitt *et al.*(1998), found that mAb 61D3 and MEM18 blocked both LPS responses and apoptotic cells interaction. This implies that the same regions of CD14 may be involved in both LPS and apoptotic cell binding and are capable of producing contrasting macrophage

responses. Therefore here a series of sCD14 point mutants were used to map LPS and apoptotic cell binding, in order to understand the mechanistic differences underlying the response of CD14 to PAMPs and ACAMPs.

5.9.1 Mapping LPS binding to sCD14

The sCD14 point mutants were first used to map LPS binding and the results were supported by previous findings that mutations within the four LPS binding regions of CD14 reduced LPS binding (Cunningham *et al.*, 2000, Juan *et al.*, 1995b and Stelter *et al.*, 1997). The first LPS binding region of CD14 spans residues 9-12 with the amino acid sequence DDED. Due to the acidic nature of aspartic and glutamic acid, this region is hydrophilic and predominantly negatively charged. Alanine replacement of glutamic acid (DDAD) has no significant effect on LPS binding to sCD14 despite the loss of charge. This is probably due to the small, uncharged nature of alanine being masked by the surrounding acidic residues exerting a dominant negative charge. However the results suggest that glutamic acid within this position is significant, as a same-charge replacement with aspartic acid; (DDDD) causes a reduction in LPS binding. Glutamic acid and aspartic acid are amino acids with acidic side chains (table 2) that vary in length by one carbonyl group. Glutamic acid has a slightly longer side chain than aspartic acid, suggesting that this residue may protrude from this highly negative region of CD14, possibly to capture LPS. Replacement with arginine (DDRD) is a charge-reversal mutation and this has the greatest effect on LPS binding causing a significant loss in binding. Arginine is a basic residue but is of the same length as glutamic acid. It is likely that arginine has disrupted the negative charge and possibly the conformation of this region of CD14. The data shown here is consistent with previous data by Juan *et al.* (1995) that reported mutations within this region of CD14 impair the ability of LPS to mediate cellular responses suggestion that residues, 9-12 are required for LPS signalling. As with this study, most of the previous work done by other groups used soluble CD14 therefore this region may simply be important for the transfer of LPS to cell membrane CD14.

Residue 14 lies in close proximity to the first LPS binding region of CD14 therefore any mutation of this residue may affect the surrounding LPS binding site. However the results demonstrate that alanine or lysine replacement had no significant effect on LPS binding. Both alanine and lysine have side groups that are shorter than arginine suggesting that a loss of charge or a reduction in dimension of this residue has no effect on the surrounding LPS binding regions. After considering the crystal structure of CD14 (figure 43) it is likely

that due to the location of residue 14, away from the surface of CD14 that contains most of the LPS binding regions, that it has no effect on LPS binding. This theory could be further examined by using a charge reversal mutation within this region, as charge reversal mutations are known to have the most significant effects.

The third proposed LPS binding region (AV**E**VE), spans residue 35-39, is located on the same surface of the pocket as the first and fourth LPS binding regions. These regions share similar properties with region one, as both are hydrophilic with an overall negative charge. Residue E37 upon replacement with aspartic acid, another acidic residue with a slightly shorter side chain causes a slight (but not significant) increase in LPS binding. However replacement with glutamine, an uncharged residue of the same length at native residue, causes a slight reduction in LPS binding. This may suggest that the charge at residue 37 is required for LPS binding and a loss of a carbonyl group protruding into this region facilitates LPS binding.

The fourth LPS binding region of CD14 spans residues 57-63 and is located on the same side of the N-terminal pocket as regions one and three. However residue 59 is located on the loop of the pocket considered as the rim of the pocket. The amino acid sequence DA**D**PROY in this region is predominantly amphipathic like LPS (Juan *et al.*, 1995). The data suggests that alanine replacement at residue 59 has no effect on LPS binding while asparagine replacement caused a slight (but not significant) reduction in LPS binding. Both alanine and asparagine are neutrally charged amino acids therefore a loss of the charge at residue 59 does not prevent LPS binding. Asparagine and glutamic acid are of similar length, however glutamic acid is negatively charged, suggesting that the negatively charged side group may be required to capture LPS. To further this study a charge reversal mutation at position 59 may assist in determining the importance of this residue within this LPS binding region of CD14.

Collectively these data support previous work by Cunningham *et al.*(2000) and suggests that it is the tertiary structure of the N-terminal pocket of CD14 that is important for LPS binding, with many hydrophilic resides within these regions interacting with LPS. The crystal structure suggests that the first, third and fourth LPS binding region of CD14 are located on the same surface of the pocket also suggested by Cunningham *et al.* (2000). Therefore it is possible that due to the close proximity of these negatively charged regions; CD14 displays several charged regions on one surface of protein to bind LPS.

Lipid A is a highly conserved portion of LPS that is responsible for the biological activity of LPS. Lipid A is an amphipathic molecule that consists of six hydrophobic C12-C14 fatty acids chains linked to a hydrophilic phosphorylated D-glucosamine disaccharide head group. Considering the hydrophobic nature of lipid A it would be predicted that a positively charged hydrophobic region of CD14 would be used to capture LPS. However a study by Rietschel *et al.*(1994) examined the relationship between the molecular structure and function of LPS and found that changing the hydrophilic backbone of lipid A reduced the ability of LPS to bind to monocytes and macrophages. The results suggested that while the hydrophobic region of lipid A was central to the activation of cells, it was the hydrophilic backbone of lipid A that was required for binding. Rietschel *et al.* (1994) found that deacylated LPS was inactive and removal of 1 or 4 phosphate groups on diglucosamine head group also reduced LPS binding. Darveau *et al.*(1998) along with Cunningham *et al.*(1999,2000) described the low biological activity of LPS from *P.gingivalis* and *H.pylori.* Cunningham *et al.*(2000) described how the binding characteristic of LPS varied depending on the structure of LPS, suggesting that LPS from *P.gingivalis* is less potent than from *E.coli* due to the absence of a ester-linked phosphate group at position 4 on diglucosamine head group of lipid A. Furthermore Coats *et al.* (2011) found that phosphate position of lipid A determined TLR4 response to LPS. Taken together these findings suggest that the LPS binding site of CD14 is a conformational epitope composed of several hydrophilic regions though the N-terminal pocket. This is consistent with previous findings by others (Cunningham *et al.,* 2000, Viriyakosol and Kirkland, 1995 & Stelter *et al.,* 1997) therefore suggesting that LPS interacts with several charged residues in a non-specific manner as found with other PRR such as the scavenger receptor (Krieger *et al.,* 1993).

5.9.2 MEM18 and 61D3, surrogate markers of apoptotic cell binding

Given the suggestion that MEM18 and 61D3 compete for binding to the same regions of CD14 (figure 47) and block both LPS responses and apoptotic cell interaction here we sought to map the binding of 61D3 and MEM18 to sCD14 mutants. 61D3 mapping data suggests that residue 11, within the first LPS binding region of CD14 is a contact residue required for 61D3 binding. Comparison of 61D3 binding with LPS binding data may suggest that a loss of the charge at residue 11 cause a reduction in 61D3 binding but not LPS binding. Whist the charge of residue 11 predetermines 61D3 binding and possibly

apoptotic cell binding, it is the effects of the three dimensional changes induced by the side groups that may affect LPS binding.

Analysis of the crystal structure (figure 43) suggests that residue 14 is away from the surface of the protein used to capture ligands and therefore mutation of this residue did not affect LPS binding. However 61D3 mapping suggests that a loss of the positive charge at residue 14 increases 61D3 binding, while LPS binding data suggests that alanine or lysine replacement of residue 14, has no effect on LPS binding. Taken together this may suggest that while R14 is not required for LPS binding it may facilitate 61D3 binding and possibly apoptotic cell binding to CD14.

Glutamic acid at residue 37 is within the third LPS binding region of CD14 and replacement with aspartic acid or glutamine causes a loss in 61D3 binding. Glutamine has a similar structure to glutamic acid however it contains a carboxylic acid group, while glutamine contains an amine group giving the residue an overall neutral charge. Aspartic acid causes a loss in 61D3 binding but an increase in LPS binding which may suggest that the protruding negative charge is required for 61D3 binding and possibly apoptotic cells but not LPS. However these data may suggest that it is predominantly the charge that effects 61D3 binding, while the three dimensional shape of these region predetermines the effects on LPS binding. Furthermore the loss of the negative charge at residue 59 also caused a loss in MEM18 binding. While comparison with LPS binding suggested that the change is charge at residue 59 was not sufficient to inhibit LPS binding. Collectively this may simply suggested that mAb are more sensitive to changes in charge of the epitope rather than a change in three-dimensional shape of the LPS binding regions.

61D3 mapping data suggests that the epitope of mAb 61D3 is within the first LPS binding region of CD14. While MEM18 binding was previously mapped to the fourth LPS binding region of CD14 (Juan *et al.,* 1995). With respect to competition for binding to CD14, antibodies have a molecular weight of approximately 150kDa in comparison to CD14 with a molecular weight of 55kDa. Therefore it may be that the binding of 61D3 to the first LPS binding region of CD14, sterically hinders the binding of MEM18 to the fourth LPS binding regions, due to the close proximity of these regions within the N-terminal pocket of CD14. This suggests that 61D3 and MEM18 inhibit LPS interaction by blocking the first and fourth LPS binding region of CD14. As surrogate markers of apoptotic cell binding, these data suggests that residues 11 and 59 may be required for apoptotic cell

binding. Therefore if apoptotic cells and LPS-binding sites have a common conformational epitope within CD14, any variation in macrophages response may be due to interaction with differing residues within that conformational epitope. However if both ligands (LPS and apoptotic cells) are only tethered by CD14 it may be the patterns displayed, once bound to CD14 that employ the signalling partners that are responsible for the ligand-dependent response produced.

5.9.3 Mapping apoptotic cell binding with sCD14

Considering findings that suggest both LPS and apoptotic cells share common binding site within CD14 it was suggested that apoptotic cells expose surface patterns similar in structure to LPS. This suggestion was supported by Gregory & Devitt (2004) that suggested apoptotic cells have patterns on the surface that resemble the three dimensional shape of LPS. Apoptotic cells, like pathogens are considered to present three dimensional motifs at the surface, termed apoptotic-cell-associated molecular patterns (ACAMPS) (Franc *et al.,* 1999 & Gregory *et al.,* 2000). ACAMPs are considered to share structural homologies with PAMPs and DAMPs and are recognised by PRR (Savill *et al.,* 2002 & Gregory and Devitt, 2004). In the case of CD14, Gregory and Devitt (2004) speculate that ACAMPs recognised by CD14 are LPS-like. Following immunofluorescence staining of apoptotic cells with LPS-polyclonal antibodies the data here suggested that the three-dimensional patterns displayed by LPS and apoptotic cells are the same. This may support the suggestion that CD14 uses the closely associated or the same region to capture both ligands and may suggest that CD14 function as a tethering receptor for both ligands. Therefore it may be the signalling partners within the local region that recognise the patterns displayed by CD14 in order to promote macrophage responses. Alternatively specific residues within these common ligand-binding regions of CD14 may be responsible for recruiting the signalling partners following ligand (LPS or apoptotic cell) binding.

Previously Devitt *et al.*(2004) found that sCD14-Fc bound to apoptotic cells, therefore here sCD14 and point mutants was considered to map apoptotic cell binding to CD14. Supernatant taken from HEK293 transfected with sCD14-Fc plasmid was used as a source of sCD14-Fc to stain apoptotic cells. However the ability of sCD14 to bind to apoptotic cells was inconsistent and due to the level of variation no significant differences in the binding of apoptotic cells to CD14 point mutants was observed. Any variation in the binding of sCD14-Fc could be a result of varying batch and possibly concentration. However when considering CD14 binding to LPS *in vivo*, CD14 is considered to cluster

within lipid raft at the cell surface and form a dimer (Kim *et al.*, 2005, Pfeiffer *et al.*, 2001, Triantafilou *et al.*, 2002). Therefore variation in binding may be due to lack of CD14 clustering however due to the Fc regions present, it is possible that sCD14-Fc may form a dimer in solution. CD14 may need to aggregate and form clusters to bind and possibly tether apoptotic cells, like membrane-bound form. Therefore in order to map the binding of CD14 to apoptotic cells, making each of the sCD14 point-mutants membrane-bound was considered.

Results 4: Mapping apoptotic cell binding to mCD14

6.1 Introduction

LPS binding studies coupled with mutagenesis have identified four regions of CD14 (figure 43) required for LPS binding (Juan *et al.,* 1995a, Juan *et al.,* 1995b, Stelter *et al.,* 1997, and Cunningham *et al.,* 2000), while apoptotic cell binding to CD14 has not been mapped. Previous data has shown that 61D3 and MEM18 block both LPS responses and apoptotic cell binding (Juan *et al.,* 1995a, Devitt *et al.,* 1998). Furthermore data here has shown that 61D3 and MEM18 compete for binding to CD14 which suggests that both apoptotic cells and LPS bind to the same or closely associated regions of CD14. 61D3 and MEM18 were used as surrogate markers, to map apoptotic cell binding to sCD14 point-mutants. The data suggested that residues E11, and D59 of CD14 may be required for apoptotic cells and LPS binding. Furthermore apoptotic cells have been suggested to display cell surface markers, termed ACAMPs that share common structural homologies with PAMPs therefore allowing phagocyte to recognise and engulf dying cells. Previous data here suggests that apoptotic cells display LPS-like patterns at the surface that may be recognised by CD14. This implies that three-dimensional patterns displayed by LPS and apoptotic cells are, to some degree, similar and both ligands therefore may bind to the same regions of CD14. If LPS and apoptotic cells interact with the same regions of CD14, it suggests that CD14 functions as a tethering molecule. Consequently any variation in response would most likely be caused by interaction with different signalling partners. However if both ligands display LPS-like pattern then apoptotic cell ligation with CD14 may also result in the activation of an inflammatory signalling pathway. While interaction with another phagocyte receptor, such as Mer may produce a dominant anti-inflammatory signal that intercepts, the inflammatory signalling and reprograms phagocyte response.

Alternatively apoptotic cells may interact with different residues within the LPS binding regions of CD14. This idea is supported by findings by Darveau *et al.*(1998) and Cunningham *et al.*(1999,2000) that reports that the low biological activity of some species of LPS is dependent on slight variations in the structure of LPS. Cunningham *et al.*(1999) suggested that LPS from *P.gingivalis* is less potent than *E.coli* due to the absence of an ester-linked phosphate group at position 4 on the diglucosamine head group of lipid A. This was also supported by a previous study by Coats *et al.* (2011) that found that the

phosphate position of lipid A determined the TLR4 response to LPS. Therefore any variation in the residues within CD14 that LPS and apoptotic cells interact with may result in activating inflammatory or anti-inflammatory signalling pathways. However due to the limitations posed by sCD14 in section 5.8 the mutations were expressed as membrane-bound CD14. Therefore having generated membrane versions of all the CD14 point mutants **the aim of this chapter was to map apoptotic cells binding in order to identify key residues in CD14 required for the binding of apoptotic cells.**

6.2 Expressing the mCD14 mutants

Gene transfection efficiency is often dependent on cell type, with epithelial cells regarded as being easily transfectable, while myeloid cells in particular are more difficult to transfect (Dokka *et al.,* 2000, Schnoor *et al.,* 2009). Myeloid cells are resistant to transfection due to their viral-defence and high levels of DNAases (Dokka *et al.,* 2000). However with the aim of trying to map apoptotic cell binding and LPS responses to CD14, myeloid cells were first considered as CD14 would be expressed within a normal situation with the required signalling partners.

6.2.1 THP-1

THP-1 are a human monocyte cell line that has been previously characterised for their LPS responsiveness (section 3.3.1) and ability to bind and clear apoptotic cells (section 3.3.5). Furthermore cell surface expression of CD14 was undetectable on THP-1 by flow cytometry, therefore THP-1 transfection was considered. However THP-1 are difficult to transfect and transfection is often reported to result in a loss of viability and functionality (Schnoor et al., 2009). Where gene transfection in monocytes is successful, it is known to produce low transfection efficiencies and be highly toxic to the cell or induce maturation (Dokka *et al.,* 2000). Data here has shown that maturation of THP-1 produces macrophage-like cells that express surface CD14. Dokka *et al.*(2000) compared several methods of transfecting monocytes and macrophages and found that lipofectAMINE was the most effective method of gene transfection assessed.

LT1 was previously used within this study and found to be an effective and efficient method of transfecting cells, which is non-toxic. As a preliminary experiment LT1 was used to transfect THP-1 with a reporter plasmid, expressing GFP and cell-associated fluorescence was determined by flow cytometry (figure 53). The spontaneous

differentiation of THP-1 following transfection was monitored by assessing cell surface CD14 expression.

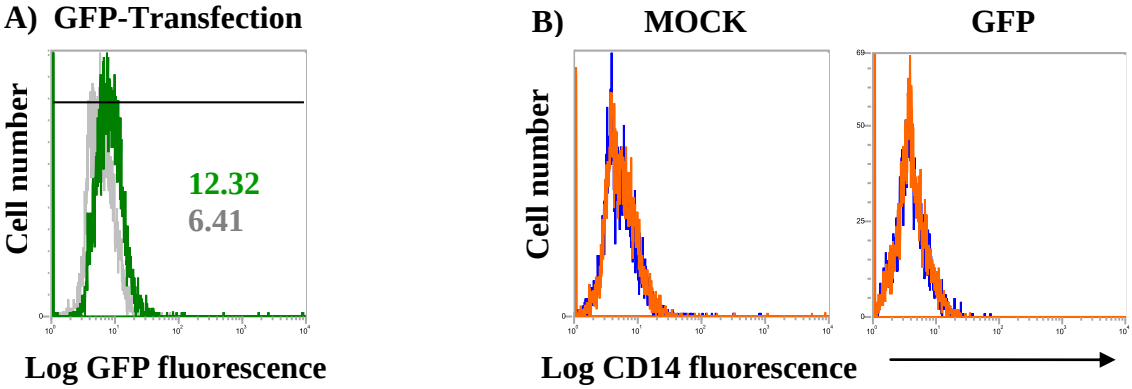

Figure 53: LT1-mediated transfection THP-1 with GFP
A. THP-1 was transfected with 2µg GFP reporter plasmid (green) or no DNA (grey) using LT1. 48 h following transfection cells were analysed by flow cytometry for any increase in FL1 fluorescence. *(Mean fluorescence intensity of total population shown).* **B.** Cell surface expression CD14 on GFP or mock (no DNA) transfected THP-1 was also assessed using indirect immunofluorescence staining with mAb 63D3 (orange) or MOPC 21 (blue) as isotype-matched control and secondary antibody, anti-mouse PE detected on FL2. This data shown is representative of two independent experiments.

GFP is frequently used as a reporter for protein expression as it is easily detectable using flow cytometry. The mean fluorescence intensity shown in figure 53 suggests a very slight increase in fluorescence of THP-1 transfected with GFP when overlaid with mock transfected cells (mock MFI=6.41 Vs GFP MFI=12.32). The effects of transfecting THP-1 with foreign DNA was considered, as differentiation of THP-1was previously shown to up-regulate CD14 expression. Therefore the spontaneous differentiation of THP-1, following transfection was assessed by assessing cell surface expression of CD14. The results in figure 53b suggests that transfection of THP-1 with GFP had no effect on cell surface expression of CD14. Overall the data in figure 53 suggest that THP-1 cells were successfully transfected and did not induce differentiation. Unfortunately the transfection efficiency was very low and whilst optimisation was considered, a more transfectable cell system was used. Also THP-1 are semi-adherent therefore the use of these cells in binding assays would have been difficult to undertake.

6.2.2 COS-1

Previously Devitt *et al.*(1998) transiently transfected COS-1 cells with mCD14 and observed an increase in interaction and phagocytosis of apoptotic cells, first implicating CD14 in apoptotic cell clearance. COS-1 cells are readily transfectable and have

previously been used to study CD14 function therefore here optimisation of transfection was sought.

6.2.2.1 DEAE-Dextran transfection

In order to optimise transfection efficiency, COS-1 was transiently transfected with a GFP reporter plasmid using DEAE-dextran-method of transfection at a range of co-incubation times. 24 hours following transfection cells were analysed by flow cytometry for any increase in fluorescence (figure 54).

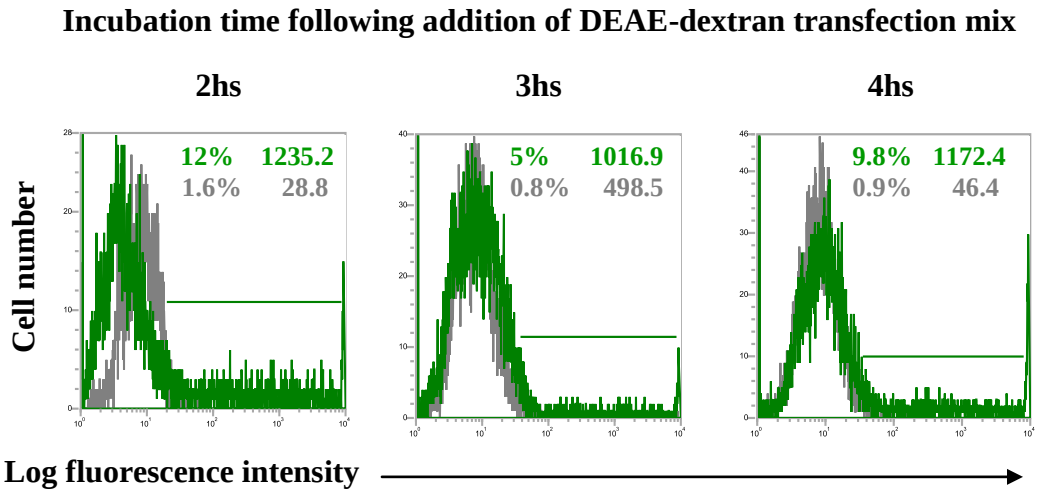

Figure 54: Optimising DEAE-dextran-mediated transfection of COS-1
COS-1 was transfected with 2µg of GFP reporter plasmid (green) or no DNA (grey) using DEAE-dextran transfection method. Cells were incubated in DEAE-dextran transfection mixture for 2, 3 or 4 h as indicated at 37°C before recovery in cDMEM for 24 h prior to analysis of GFP expression by flow cytometry. The percentage of cells and the mean fluorescence intensity of the positive gated cells are shown.

The results in figure 54 suggest that COS-1 have been transfected following incubation for two, three and four hours. However the results suggest that incubating the transfection mix with COS-1 for 2 hours was the most efficient method. Generally the transfection efficiencies were low and therefore it was decided to seek alternative methods of transfecting COS-1.

6.2.2.2 LT1-mediated transfection

An alternative technology that has been widely used for transfecting cells producing high transfection efficiencies was transfection reagent LT1. Therefore COS-1 was transfected with 2µg of GFP using LT1 at various ratios in order to find the highest transfection efficiency (figure 55).

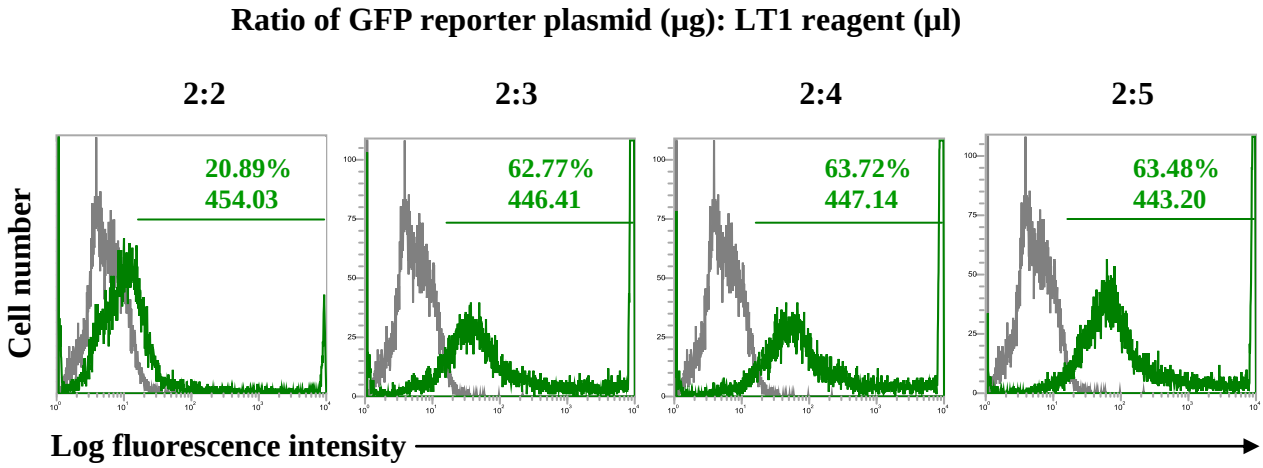

Figure 55: Optimising LT1-mediated transfection of COS-1
COS-1 were seeded at $3x10^5$cells/well in 6 well plates overnight prior to LT1-mediated transfection of 2µg of GFP reporter plasmid (green) using the indicated ratios of LT1 to DNA, to establish the optimum transfection efficiency. As a negative control COS-1 cells were also transfected with no DNA (grey) using only 3µL of LT1. 24 h following transfection, the cells were analysed, using flow cytometry for any increase in fluorescence. The percentage of cells and the mean fluorescence intensity of the positive gated cells are shown. *Mock 2.08% MFI: 20.88*

The flow cytometry results in figure 55 suggest that COS-1 are readily transfected with LT1 and produced high transfection efficiency particularly when used at the ratio of 2µg:4µL of LT1 to DNA. In light of this result, LT-1 at 2µg:4µL of DNA was used to transfect COS-1 with wildtype mCD14. The transfection efficiency and expression levels of mCD14 were assessed using indirect immunofluorescence with mAb 63D3 (figure 56).

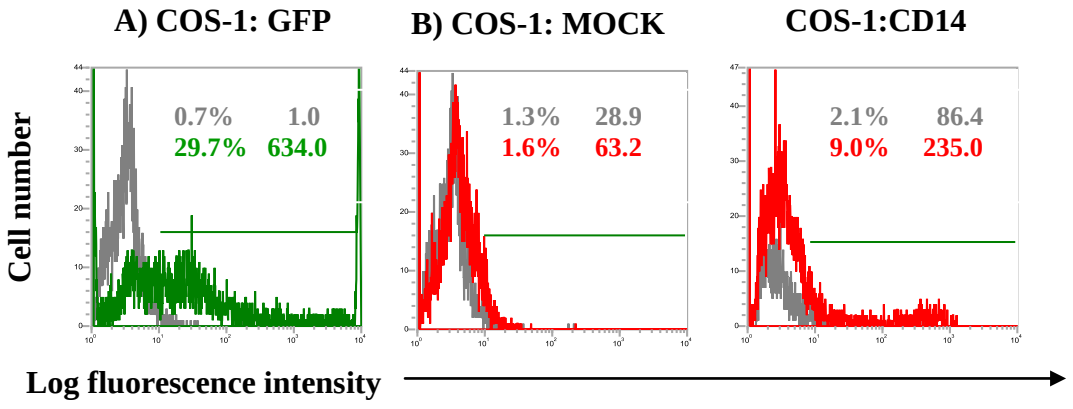

Figure 56: LT1-mediated transfection of COS-1 with mCD14
COS-1 was transfected with no DNA (Mock), mCD14 or reporter GFP (green) using LT1-mediated transfection method. 24 hs following transfection COS-1 was analysed for any increase in fluorescence (**A**) or an increase in cell surface expression of CD14 (**B**) with mAbs 63D3 (red) or as isotype-matched control MOPC 21 (grey). These results are representative of three independent experiments.

The results in figure 56B suggest the percentage of COS-1 that had been successfully transfected with wildtype mCD14 was reproducibly low. Furthermore Devitt *et al.*(1998) reported that COS-1 transfected with CD14 were not as efficient at apoptotic cell clearance compared to macrophages. Therefore due to low expression levels of CD14 and poor interaction with apoptotic cells, COS-1 was considered unsuitable.

6.2.3 HeLa

The human cervical epithelial cell line, HeLa had previous been used within our research group and are easily transfected with the transfection reagent LT1, producing very high transfection efficiencies. Subsequent data (figure 38) also suggested that HeLa interact with apoptotic cells in a CD14-independent manner. Therefore in order to map the binding of apoptotic cells to mCD14, HeLa were transfected with mCD14 mutants and expression assessed using indirect immunofluorescence staining with mAb 63D3 (figure 57).

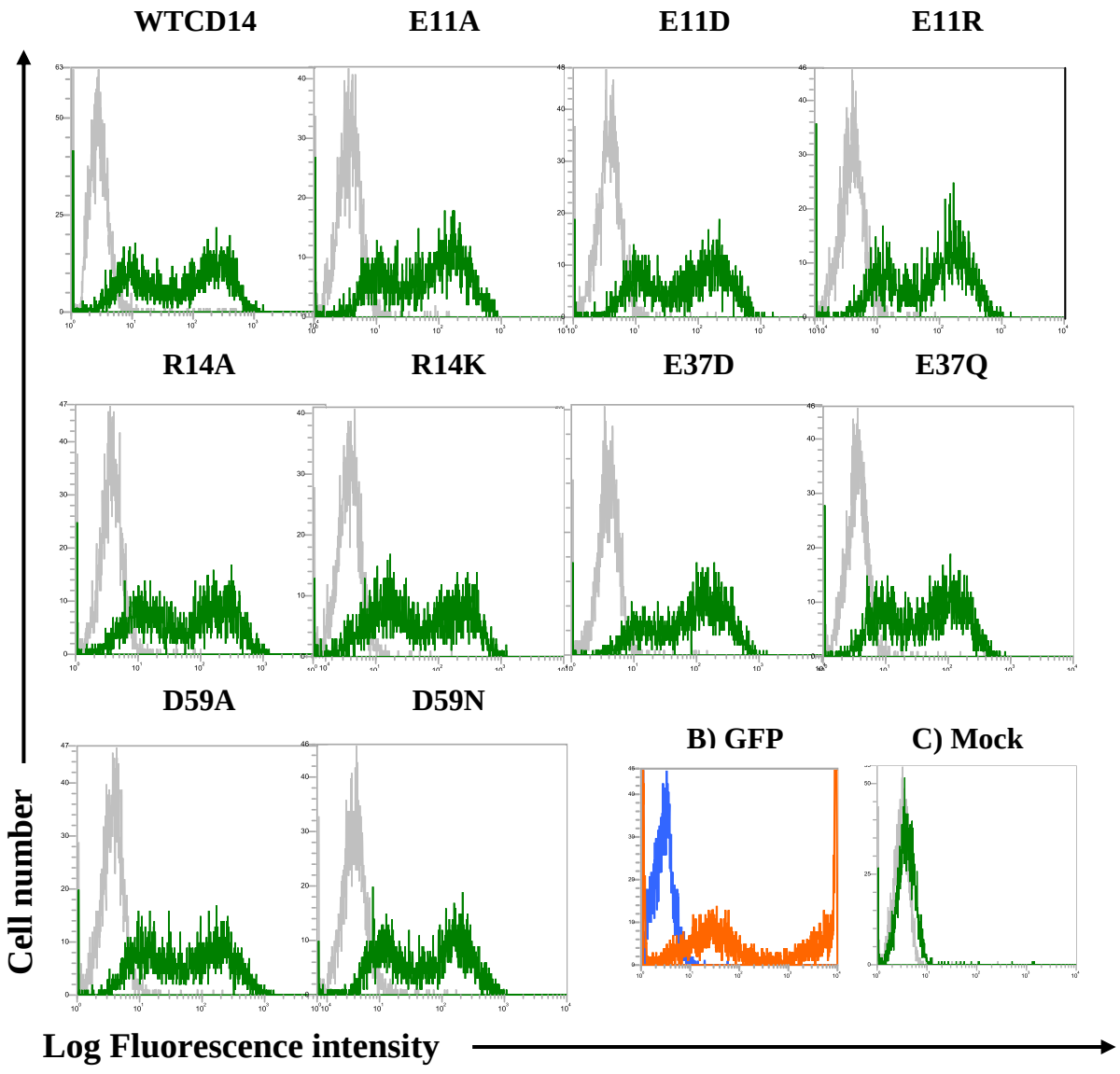

Figure 57: Flow cytometric analysis of HeLa transfected with mCD14 mutants
HeLa was transfected using LT1-mediated transfection with **A.** wildtype or mutant mCD14, **B**. reporter plasmid expressing GFP or **C**. no DNA (mock). 24 h following transfection, cell surface expression of CD14 was assessed on HeLa cells transfected with mCD14 and mutants using indirect immunofluorescence staining with the mAb 63D3 (green) or isotype-matched control MOPC 21 (grey). **B.** HeLa was transfected with GFP reporter to assess transfection efficiency and compared to mock (blue). The results are representative of three independent experiments.

Flow cytometric results (figure 57) demonstrate the successful transfection and subsequent expression of all mCD14 mutants. The mAb 63D3 binds to the C-terminus of CD14 (Viriyakosol *et al.*, 2000), away from the mutated N-terminal sites therefore this mAb was used to verify expression. Following transfection, a bimodal distribution of HeLa was evident, which varied in the level of CD14 expressed and the populations are described as CD14 low or CD14 high. However a bimodal distribution was also observed following transfection with the reporter GFP plasmid suggesting that the distribution is not dependent on gene expression. It is likely that subpopulations produced by HeLa following transfection are caused by two heterogeneous strains which vary in the phase of the cell cycle. This may suggests that during a particular phase of the cell cycle HeLa may be more transfectable. Another possible explanations for the difference in protein expression between subpopulations of HeLa may include variations in the uptake of DNA, activity of enzymes used to replicate plasmid DNA or protein trafficking.

6.3 MEM18 and 61D3: surrogate markers of apoptotic cell binding

The N-terminal pocket of CD14 is known to contain all four regions required for LPS binding (Viriyakosol and Kirkland, 1995, Cunningham *et al.*, 2000, Kim *et al.*, 2005). Juan *et al.*(1995) found that residues 57-64 of CD14 were required for MEM18 binding and LPS responses, currently considered as the fourth LPS binding region of CD14. Previously Devitt *et al*.(1998) showed that both mAbs 61D3 and MEM18 blocked LPS responses and apoptotic cell interaction with macrophages, suggesting that the same or closely associated regions of CD14 interact with both ligands. MEM18 and 61D3 have been mapped to sCD14 as surrogate markers of apoptotic cell binding and data here has suggested that residues E11 and D59, within the first and fourth LPS binding regions of CD14 may be required for apoptotic cell binding. Therefore here we attempt to map the binding of these two mAbs, as surrogate markers for LPS and apoptotic cell binding, to membrane-bound CD14 using a panel of CD14 point mutants.

6.3.1 Mapping 61D3 binding to mCD14

As previously stated, the epitope of 61D3 is unknown but a major part of this epitope is considered to involve residue 11 of CD14 (figure 48), located within the first LPS binding region. 63D3 is known to bind towards the C-terminus (Viriyakosol *et al.*, 2000) of CD14 and previous findings (figure 57) have shown that 63D3 binding is unaffected by mutations at the N-terminus. Previously HeLa, following transfection were shown to express

wildtype mCD14 or mutants, therefore transfectants were used to map the binding of 61D3, as a surrogate marker of apoptotic cell binding to membrane CD14 (figure 58).

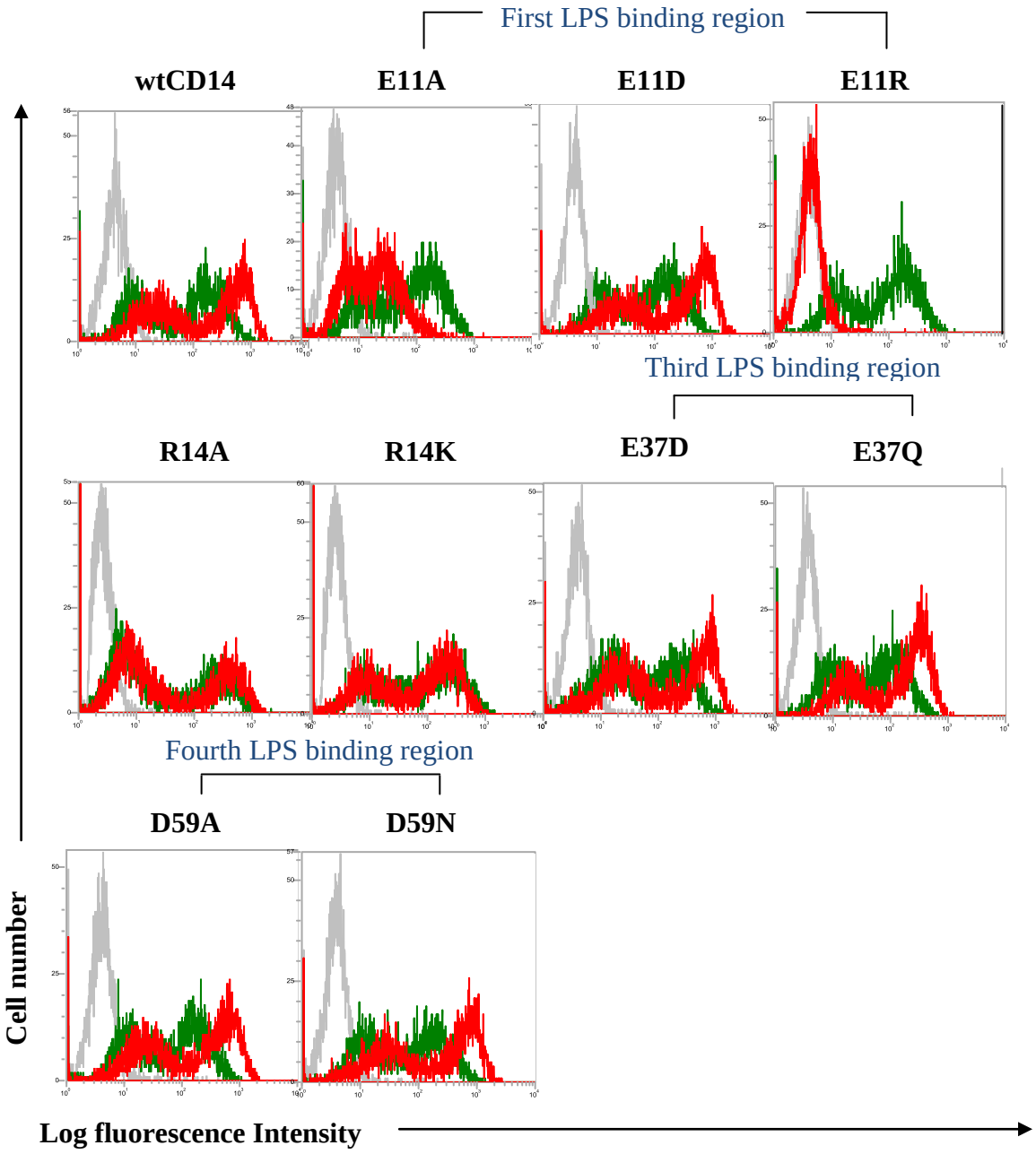

Figure 58: Mapping 61D3 binding to HeLa transfected with mCD14 mutants
HeLa were transfected using LT1-mediated transfection with wildtype or mutant mCD14. 24 h following transfection, cell surface expression of CD14 was assessed using indirect immunofluorescence staining with mAbs 61D3 (red), 63D3 (green) or isotype-matched control MOPC 21 (grey). Binding was detected using flow cytometry and the results are representative of four independent experiments. The locations of the amino acid residues with respect to the LPS binding regions of CD14 are shown. Residue R14 is not labeled as this residue is outside the first LPS binding region.

The flow cytometric results (figure 58) show that all transfectants express membrane wtCD14 or mutants at the cell surface as denoted by 63D3 binding. With respect to 61D3 binding, substitution of glutamic acid at position 11 for aspartic acid had no overall effect

on 61D3 binding. However alanine replacement (E11A) caused a reduction in 61D3 binding supporting the requirement of a negatively charged region for antibody binding. This is further demonstrated when glutamic acid is substituted for arginine, which has an overall positive charge, resulting in a complete loss of 61D3 binding. The flow cytometric data also suggests that mutations R14A and R14K also cause a slight reduction in 61D3 binding when compared to wildtype CD14. Furthermore E37 an D59 mutations analysed had no effect on 61D3 binding to CD14. This data supports previous findings, that the first LPS binding region of CD14 is required for 61D3 binding and possibly apoptotic cell binding.

6.3.2 Mapping MEM18 binding to mCD14

Previous studies have identified the requirement of residues 57-64; within the fourth LPS bind region, for MEM18 binding (Juan *et al.*, 1995a and Juan *et al.*, 1995b). However previous data (figure 47) suggested MEM18 and 61D3 compete for binding to CD14, suggesting that the epitopes were the same or closely associated. Having established that 61D3 binds to residue 11 within the first LPS binding region of CD14 it was decided to map the binding of MEM18 using mutations within the first and fourth LPS binding regions of CD14 (figure 59).

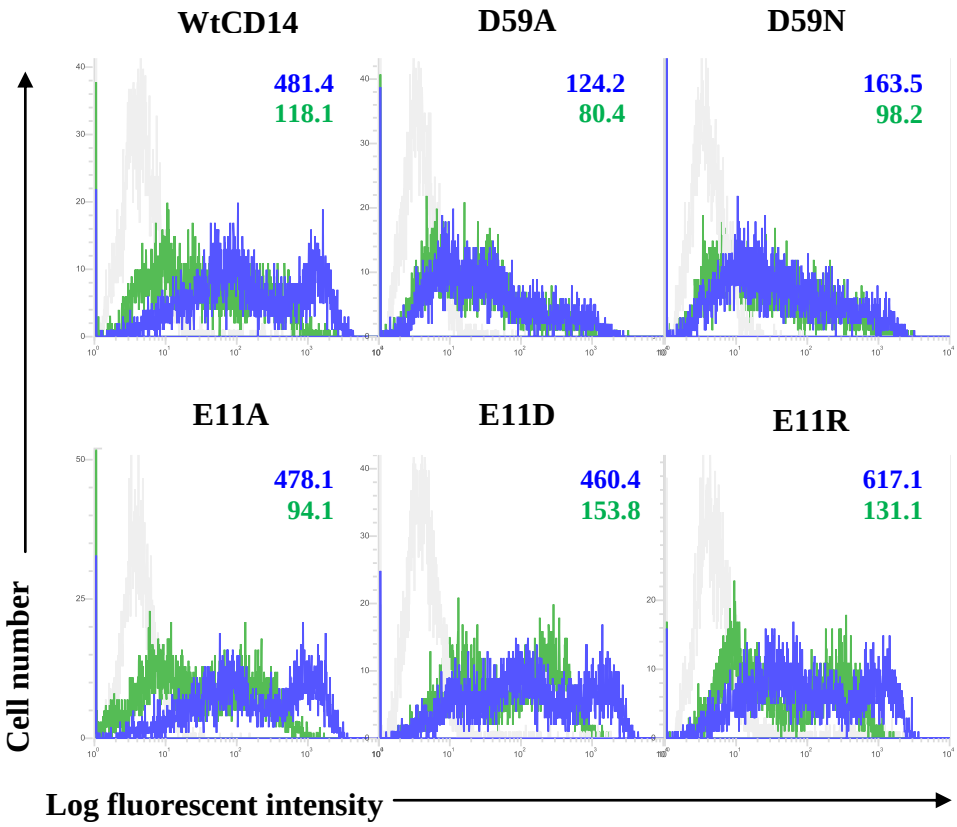

Figure 59: Mapping MEM18 binding to HeLa transfected with mCD14 mutants
HeLa were transfected using LT-mediated transfection with wildtype or mutant mCD14. 24 h following transfection cell surface expression of CD14 was assessed using indirect immunofluorescence staining with the mAb MEM18 (blue), 63D3 (green) or isotype-matched control MOPC 21 (grey). Binding was detected using flow cytometry and the results are representative of three independent experiments. The mean fluorescence intensity for MEM18 (blue) and 63D3 (green) is shown.

The flow cytometric results shown in figure 59 suggest that all cells express wildtype or mutant mCD14 as denoted by 63D3 staining. With respect to MEM18, a reduction in binding was observed with mutations D59A and D59N compared to wtCD14. This data illustrates that mutation of residue 11 has no effect on MEM18 binding compared to

wtCD14, unlike 61D3 binding (figure 58). While substitution of residue 59 (aspartic acid) with alanine or asparagine was shown to reduce MEM18 binding in agreement with sCD14 mapping data (figure 49). Alanine and asparagine are both neutrally charged amino acid, therefore it is possible that a loss of the negative charge associated with aspartic acid is responsible for reducing the level of MEM18 binding.

6.4 Mapping apoptotic cell binding to mCD14

The precise residues of CD14 responsible for apoptotic cell binding are unknown. However earlier data presented here have mapped MEM18 and 61D3 binding to msCD14 and results have indicated that some of the region required for LPS binding may also be involved in apoptotic cell binding. Therefore in order to assess the binding of apoptotic cells to mCD14, HeLa were transfected with wildtype or mutant CD14 and the ability of cells to interact with apoptotic cells was assessed (figure 60).

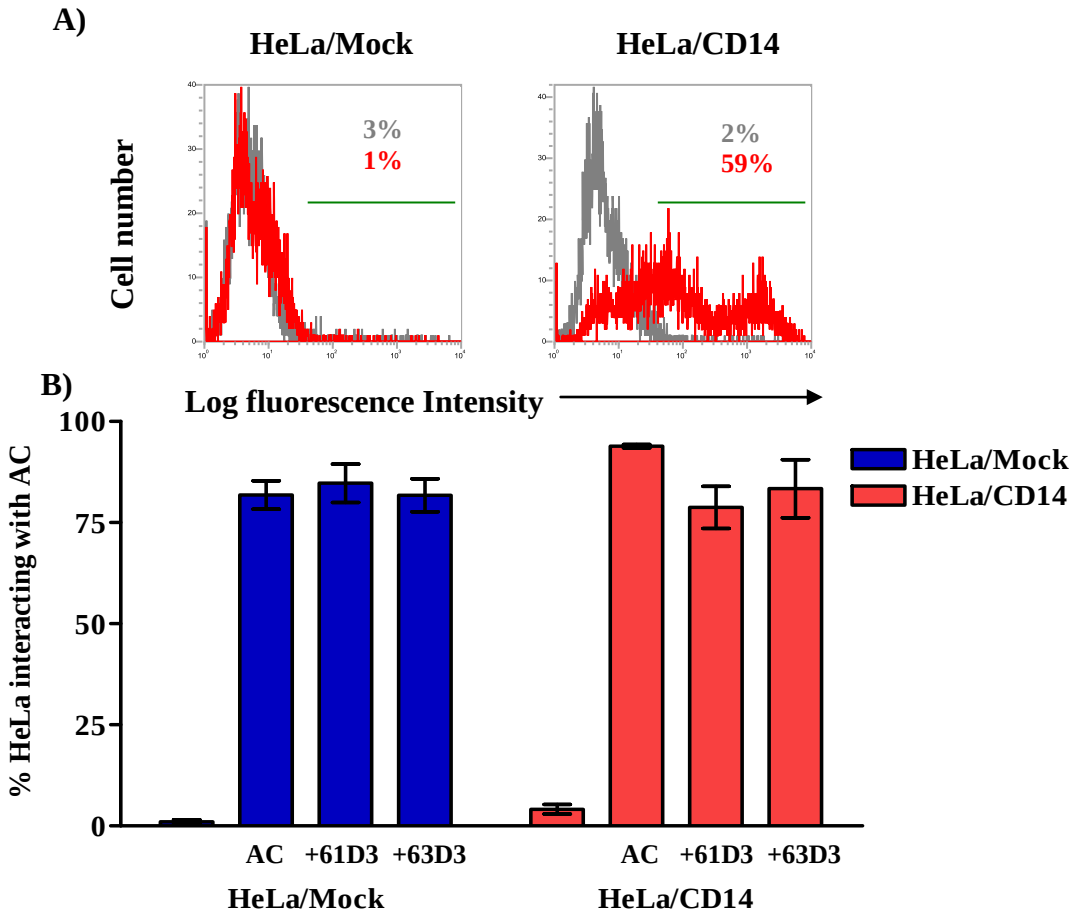

Figure 60: HeLa interact with apoptotic cells in CD14-independent manner
HeLa were transfected using LT-mediated transfection with wildtype CD14 or no DNA (Mock). 24h following transfection HeLa, with and without CD14 were seeded onto four-well glass slides and incubated overnight to allow for attachment. **A**. Cell surface expression of CD14 was assessed using indirect-immunofluorescence staining with mAb 63D3 (red) or isotype-matched control MOPC 21 (grey). The results are representative of three independent experiments **B.** HeLa cells were co-cultured with apoptotic Mutu for 1 h

at 37°C. Slides were then washed in ice cold PBS and fixed in 1% (w/v) formaldehyde prior to Jenner-Giemsa staining. Interaction was assessed using light microscopy and values scored as a percentage of HeLa interacting with apoptotic cells. The results shown are the mean ± SEM of three independent experiments. *Statistical analysis conducted was ANOVA followed by Bonferroni post-test.*

The flow cytometric data shown in figure 60a supports previous data (figure 35) that suggests HeLa express undetectable levels of mCD14. However following transfection cell surface expression of CD14 was assessed and the results suggest cells are expressing mCD14. The results shown in figure 60b support previous findings (figure 38) that suggest HeLa interact with apoptotic cells in a CD14-independent manner. Following transfection (HeLa/CD14) incubation with apoptotic cells in the presence of 61D3, caused a slight reduction in the level of interaction. Therefore in order reveal any contribution of CD14 in apoptotic cell clearance; the assay was conducted at 4°C, for various times to reduce the rate of interaction and to solely consider binding (figure 61).

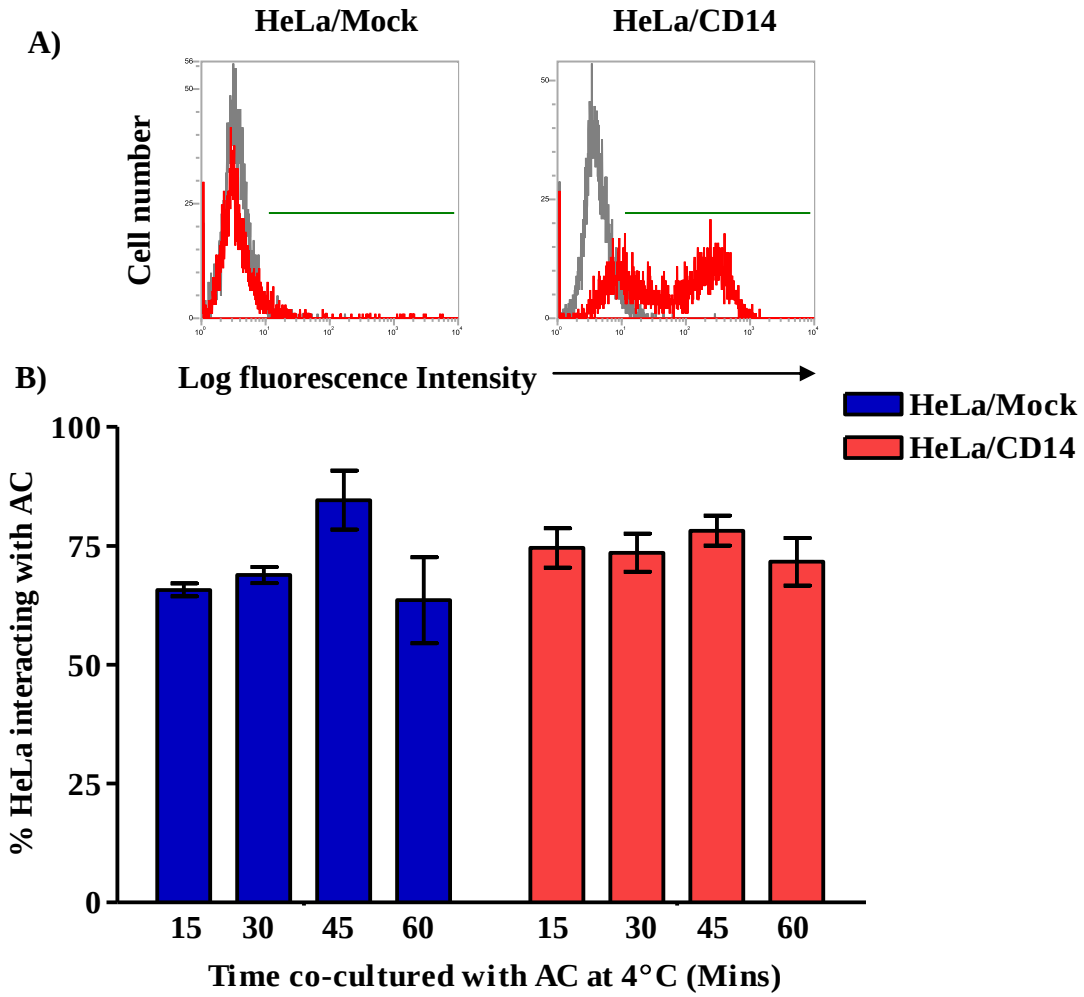

Figure 61: The CD14-independent binding of apoptotic cells to HeLa
HeLa was transfected using LT-mediated transfection with wildtype CD14 or no DNA (Mock). 24 h following transfection HeLa, with and without CD14 were seeded onto four-well glass slides and incubated overnight to allow for attachment. **A**. Cell surface

expression of CD14 was assessed using indirect-immunofluorescence staining with 63D3 (red) or isotype-matched control MOPC 21 (grey).**B**. HeLa was co-cultured with apoptotic Mutu for 15, 30, 45 or 60 min at 4°C. Prior to Jenner-Giemsa staining, slides were washed in ice cold PBS and fixed in 1% (w/v) formaldehyde. Interaction was assessed using light microscopy and values scored as a percentage of HeLa interacting with apoptotic cells. The results shown are the mean ± SD of four wells.

The results in figure 61 suggest that co-culturing HeLa with apoptotic cells at 4°C causes a slight reduction in the level of binding, when compared to figure 60. Whilst the level of CD14-dependent binding of apoptotic cells at all incubation times was not statistical significant, some observations were made which allowed experimental development. As the incubation time increased, the level of interaction between HeLa (HeLa/Mock) and apoptotic cells increased, peaking at 45 minutes. However the level of CD14-dependent binding (HeLa/CD14) did not change with time, therefore suggesting that lower incubation times had a higher level of CD14-dependent binding. This would suggest that mCD14 accelerates the initial binding of apoptotic cells to the phagocyte. Therefore to reveal the role of CD14 in the initial binding of apoptotic cells, the assay was conducted at 4°C and incubated for 5min and 15min to solely consider the initial binding events (figure 62).

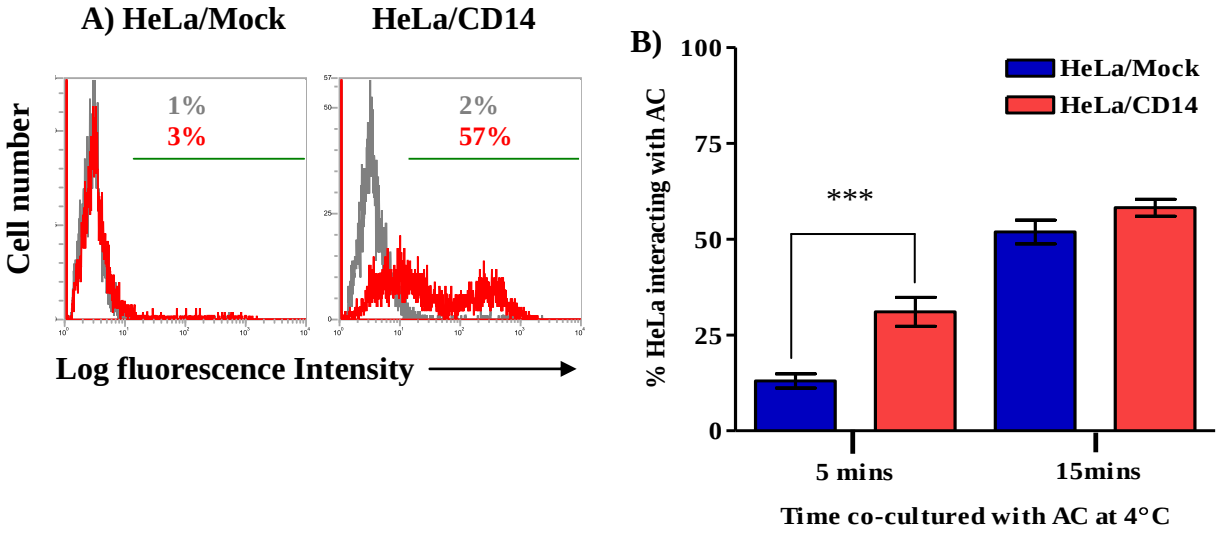

Figure 62: CD14 mediates initial binding of apoptotic cells to HeLa
HeLa was transfected using LT-mediated transfection with wildtype CD14 or no DNA (Mock). 24h following transfection HeLa, with and without CD14 were seeded onto four-well glass slides and incubated overnight to allow for attachment. **A**. Cell surface expression of CD14 was assessed using indirect-immunofluorescence staining with mAb 63D3 (red) or isotype-matched control MOPC 21 (grey). The results are representative of three independent experiments **B**. Prior to the assay cells were incubated at 4°C for 30 min then co-cultured with cold apoptotic Mutu for 5 or 15 min, washed and incubated at 4°C for a further 30 min. Prior to Jenner-Giemsa staining, slides were washed in ice cold PBS and fixed in 1% (w/v) formaldehyde. Interaction was assessed using light microscopy and values scored as a percentage of HeLa interacting with apoptotic cells. The results shown

are the mean ± SEM of three independent experiments. *Statistical analysis was conducted using ANOVA followed by Bonferroni post-test. (* ** P<0.001)*

The results in figure 62 show that following 5 min of incubation with apoptotic cells, the level of CD14-dependent binding doubled compared to CD14-independent binding. Following incubation for 15 minutes, the level of CD14-independent and CD14-dependent binding of apoptotic cells to phagocytes appears the same. This supports the previous suggestion that mCD14 accelerates the initial binding of apoptotic cells to phagocytes. Therefore having established the assay conditions required to analyse the role of mCD14 in apoptotic cell binding the assay was used to map the binding of apoptotic cells to mCD14 point mutants (figure 63).

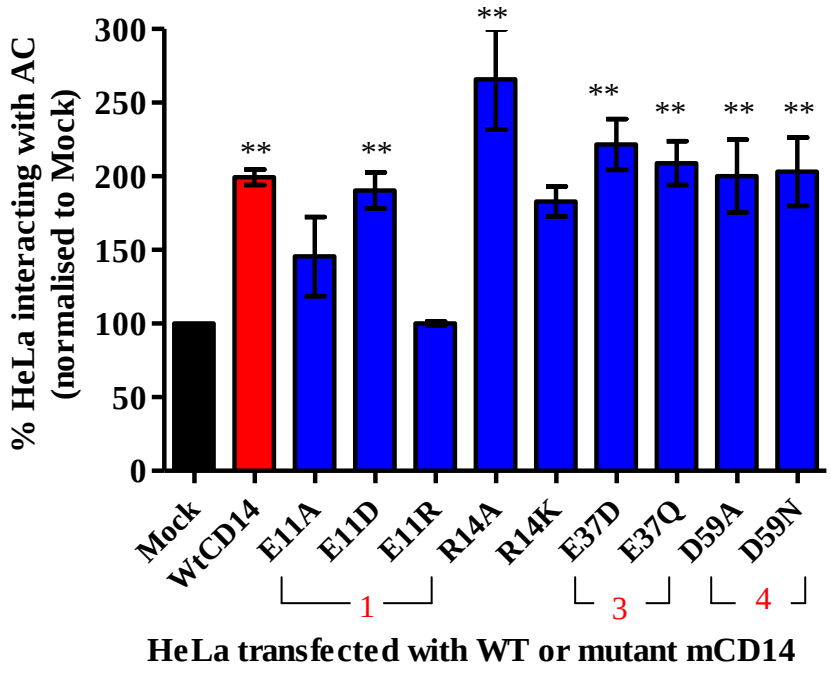

Figure 63: Mapping the binding of apoptotic cell to mCD14
HeLa was transfected using LT-mediated transfection with wildtype or mutant mCD14 or no DNA (Mock). 24 h following transfection HeLa were seeded onto four-well glass slides and incubated overnight to allow for attachment. Prior to assay, HeLa were incubated at 4°C for 30 min then co-cultured with cold apoptotic Mutu for 5min. Slides were then washed in ice cold PBS and fixed in 1% (w/v) formaldehyde prior to Jenner-Giemsa staining. Interaction was assed using light microscopy and values scored as a percentage of HeLa interacting with apoptotic cells. The location of the residues mutated, with respect to four LPS binding regions of CD14 are also shown. The results shown are the mean ± SEM of three independent experiments that were first normalised to mock alone. *Statistical analysis was conducted using ANOVA with Dunnets Post-test (wtCD14 and mutants compared to Mock) (*P<0.05, **P<0.01).*

Glutamic acid at residue 11 is within the first LPS binding region of CD14 (Cunningham *et al.*, 2000). Replacement with alanine (E11A) or arginine (E11R) significantly reduced apoptotic cell binding when compared to wtCD14. However replacement of residue 11

with aspartic acid, (E11D) had no significant effect on apoptotic cell binding to CD14. This suggests that residue 11 is required for the binding of apoptotic cells to mCD14. Furthermore alanine replacement of residue 14 (R14A) caused a significant increase in apoptotic cell binding to CD14 while replacement with lysine had no effect on apoptotic cell binding to CD14 (compared to wtCD14). Mutation of residue 14 may affect apoptotic cell binding to CD14, due to the close proximity to residue 11. The mutations of E37 and D59 assessed, had no significant effect on apoptotic cell binding to wtCD14.

6.5 Mechanistic differences underlying the response of mCD14 to PAMPs Vs ACAMPs

CD14 is found within microdomains in the Triton-X 100-insoluble fraction of the plasma membrane (Pugin *et al.*, 1998, Triantafilou *et al.*, 2001b). Isolation of the microdomains containing CD14 led to the discovery other LPS-binding molecules, including Hsp60 and Hsp70 (Byrd *et al.*, 1999, Triantafilou *et al.*, 2002, Wallin *et al.*, 2002). Following LPS stimulation MD2/TLR4 and other LPS-binding molecules (including CXCR4 and GDF5) required for both CD14-dependent and CD14-independent cellular activation, are recruited to the plasma membrane (da Silva Correia *et al.*, 2001, Triantafilou *et al.*, 2002). For CD14-dependent responses, LPS binds CD14 and recruits MD2/TLR4 resulting in the expression of TNF-α and other proinflammatory cytokines (Akira and Takeda, 2004 and Saitoh and Miyake, 2006).

Having previously established LPS and apoptotic cells share conformational epitopes and bind a region of CD14 involved in TLR4 signalling (the first LPS binding region)(Juan *et al.*, 1995b & Stelter *et al.*, 1999) it seems logical to assess the possibility that like LPS, apoptotic cells may also activate LPS-signalling pathways.

6.5.1 CD14-dependent IL-8 response

To elucidate the role of CD14 in apoptotic cell clearance, the mechanistic differences underlying the responses of CD14 to LPS and apoptotic cells must be determined. Therefore here the ability of CD14, following ligation with apoptotic cells or LPS, to activate NF-κB-inducible genes was assessed. Having established the function of membrane wtCD14 in a HeLa-based assay sytem, this system was then used to investigate cellular response to LPS by measuring cytokine expression following LPS stimulation. Sharma *et al.*(2011) also used HeLa to examine the LPS-induced expression levels of cytokine (IL-6 and IL-1α) and chemokines (IL-8 and MCP-1) in culture medium and cell

lysates. Here the same method was used to seed and stimulate HeLa transfected with no DNA and wildtype mCD14, following LPS stimulation cellular response was measured by IL-8 ELISA on culture supernatants and cytoslic fractions (figure 64).

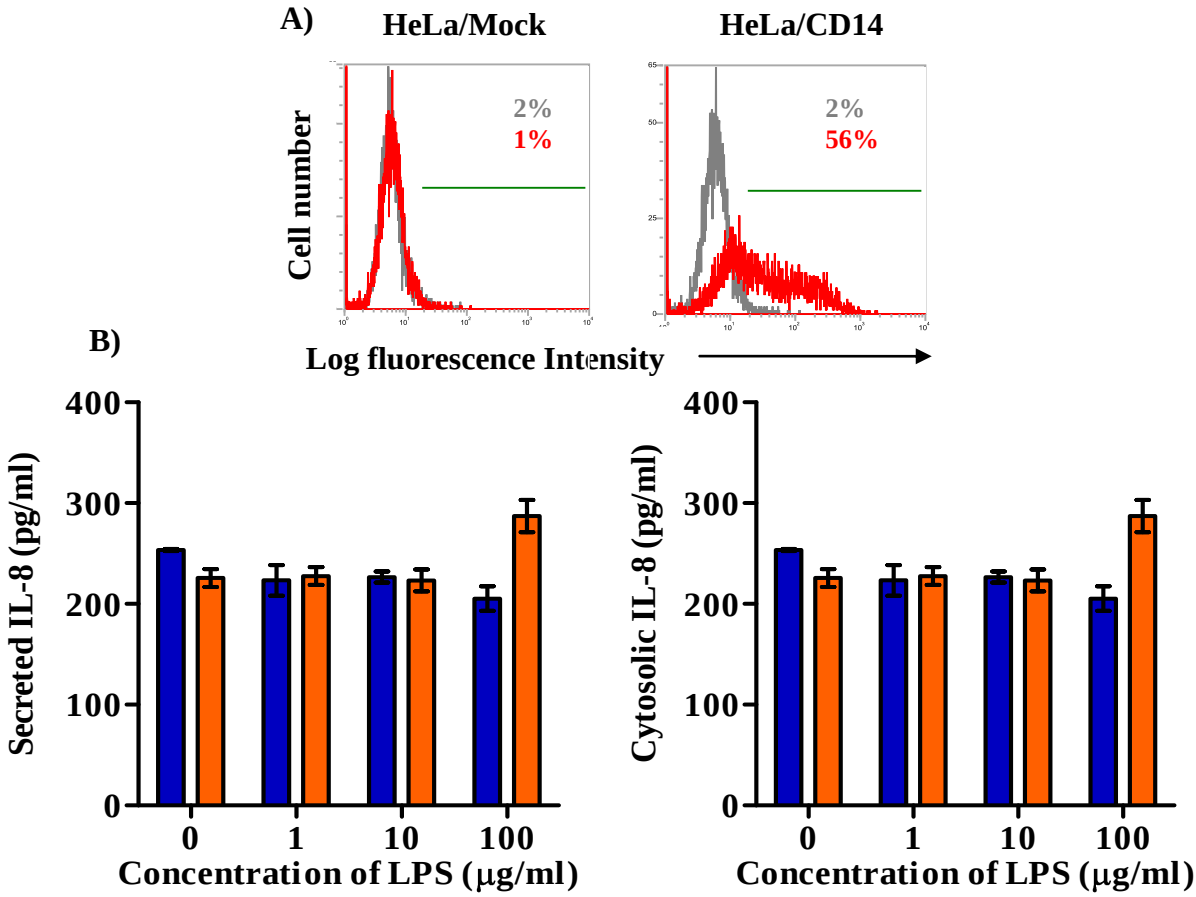

Figure 64: LPS-responsiveness of HeLa in the absence or presence of mCD14
Following transfection, HeLa with (orange) or without mCD14 (blue) were seeded in triplicate at a density of $3.5x10^5$/well in 96-well plates and incubated overnight to allow for attachment. **A**. Cell surface expression of CD14 was analysed using indirect-immunofluorescence staining with 63D3 (red) or isotype-matched control MOPC 21 (grey).**B**. Cells were stimulated with 200µl of medium alone, 1µg/ml, 10µg/ml or 100µg/ml of LPS in the presence of 10% NHS for 24 h. Following incubation, secreted and cytosolic IL-8 was measured by ELISA. The data shown is the mean ± SD of triplicate well assayed in duplicate by ELISA and is representative of two independent experiments.

The results in figure 64 suggest that in the absence or presence of mCD14 expression, HeLa are unresponsive to LPS stimulation. However following stimulation with 100µg/ml of LPS there is a insignificant trend upwards in IL-8 response produced from HeLa expressing mCD14. This would suggest that at high concentrations of LPS, HeLa produce a CD14-dependent IL-8 response. However this trend coincides with a decrease in the basal level of IL-8 produced by mock transfected HeLa. This may suggest that high concentrations of LPS are toxic and induce apoptosis which reduced basal IL-8 production, yet cells displayed no morphological signs of apoptosis. Given the lack of IL-8 response

following LPS stimulation, another method of measuring LPS-induced activation of HeLa, in the absence or presence of mCD14 was explored.

6.5.2 CD14-dependent NF-κB activation

To characterise the role of CD14 in NF-κB-mediated inflammation to LPS, HeLa were transfected with mCD14 and a plasmid containing several NF-κB response element upstream of a luciferase reporter gene. Following LPS stimulation, CD14-dependent responses was assessed by measuring luciferase activity and the light emitted (figure 65).

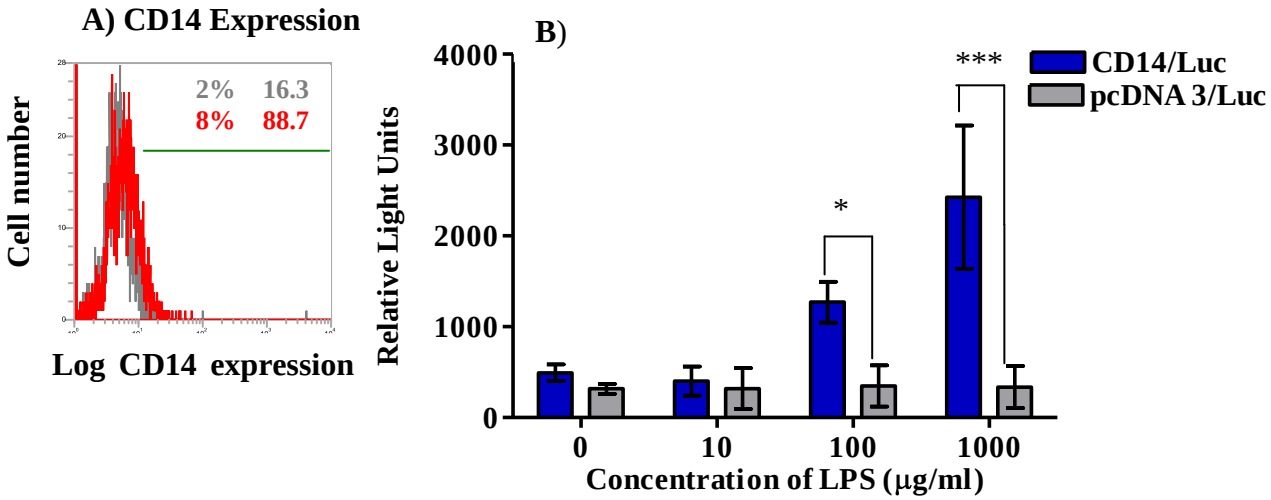

Figure 65: Titrating an LPS-induced response in HeLa expressing mCD14
A. HeLa was transfected with an NF-κB reporter using LT-mediated transfection along with wildtype mCD14 (red) or pcDNA3 (empty plasmid) (grey). Following transfection cells were seeded at a density of $1.5x10^4$/well in 96-well plates and incubated overnight to allow for attachment. Cell surface expression of CD14 was analysed using indirect-immunofluorescence staining with 63D3 **B.** Cells were washed and then stimulated with 100µl of medium alone, 10µg/ml, 100µg/ml or 1000µg/ml of LPS in the presence of 10% (v/v) NHS for 5 h. Following incubation, 100µl of One-Glo was added to each well and the plate incubated for 3min prior to measuring luminescence. The data shown is the mean ± SD of quadruplicate wells. *Statistical analysis was conducted using ANOVA followed by Bonferroni post-test. (* $P<0.05$, *** $P<0.001$)*

The flow cytometric results shown in figure 65a suggest mCD14 expression is relatively low compared to previous experiments and is most likely due to the nature of the double transfection. However results in figure 65b suggest LPS stimulation of HeLa expressing mCD14 resulted in a dose-dependent increase in NF-κB activation. As a control, HeLa were transfected with an empty pcDNA3 plasmid but to ensure that NF-κB activation was specific to mCD14, another membrane protein, ICAM-3 was expressed (figure 66).

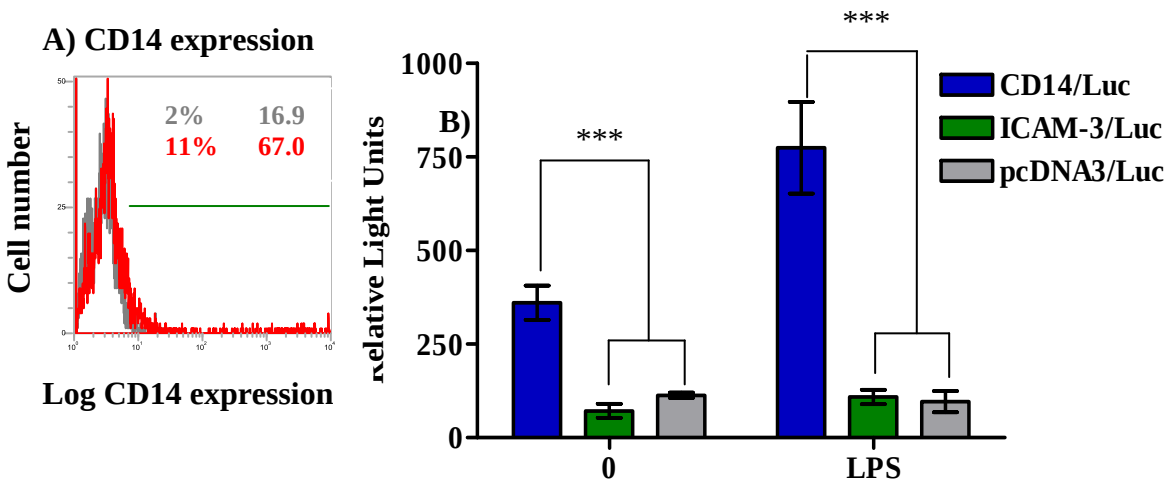

Figure 66: CD14-dependent LPS-induced response in HeLa expressing mCD14
A. HeLa was transfected with an NF-κB reporter using LT-mediated transfection along with mCD14 (red) or pcDNA3(empty plasmid) (grey). Following transfection cells were seeded at a density of $1.5x10^4$/well in 96-well plates and incubated overnight to allow for attachment. Cell surface expression of CD14 was analysed using indirect-immunofluorescence staining with mAb 63D3 **B.** Cells were washed and then stimulated with 100µl of medium alone or 100µg/ml of LPS in the presence of 10% (v/v) NHS for 5 h. Following incubation, 100µl of One-Glo was added to each well and the plate incubated for 3 min prior to measuring luminescence. The data shown is the mean ± SD of quadruplicate wells. *Statistical analysis was conducted using ANOVA followed by Bonferroni post-test. (* P<0.05, *** P<0.001)*

As shown in figure 66 HeLa expressing both the NF-κB reporter and mCD14 are LPS-responsive therefore suggesting that NF-κB activation is CD14-dependent. CD14 does not contain a cytosolic signalling domain and often TLR4 is required to confer an LPS-dependent response (Hoshino *et al.*, 1999,Miyake, 2004). The expression of TLR4 on HeLa was assessed with direct immunofluorescence staining (figure 67).

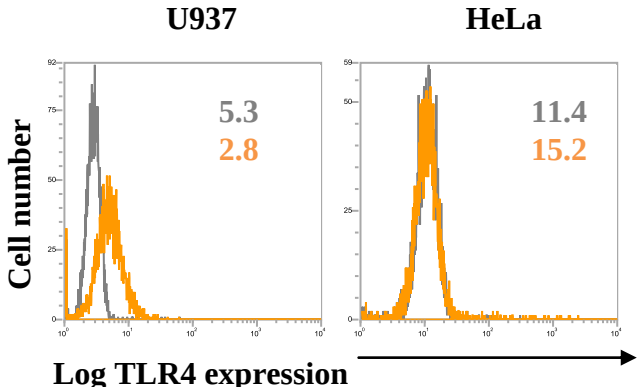

Figure 67: Surface expression of TLR4 on HeLa
Surface expression of TLR4 on HeLa was detected with flow cytometric staining using direct immunofluorescence with the monoclonal antibody HTA125-PE (Orange) or IgG2a-

PE (Isotype-matched control, (grey)). U937 were used as a positive control. The results are representative of three separate experiments.

Cell surface expression of TLR4 was undetectable on HeLa following flow cytometric analysis (figure 67). This data is supported by Guillot *et al.*(2002) that suggested lack of TLR4 expression on HeLa prevented constant inflammation when exposed to resident flora. Werner *et al.,* (2011) also found that HeLa was unresponsive to LPS stimulation possibly due to lack of TLR4 expression. Both studies assessed expression of TLR4 by flow cytometry, however levels may be below the level of detection. Alternatively TLR4 may be recruited to the surface following LPS binding to mCD14. CD14 is also considered to interact with heat shock proteins (Kol *et al.,* 2000b), also found with microdomains (Byrd *et al.,* 1999, Triantafilou *et al.,* 2002, Wallin *et al.,* 2002). Therefore LPS ligation of CD14 may signal in a TLR4-independent manner though the inflammatory signalling pathway used by heat shock proteins. However Ohashi *et al.*(2000) found that heat shock protein 60 used a TLR4-dependent complex to induce inflammation. Taken together it seems likely that CD14-dependent activation of NF-κB is dependent on TLR4-signalling. This would suggest that TLR4 is recruited to the cell surface following LPS binding to mCD14 or LPS ligation resulted in uptake which activated intracellular TLR4.

6.5.2.2 CD14-dependent response to LPS or apoptotic cells

Given the previous data, that demonstrated CD14-dependent activation of NF-κB following LPS stimulation, here we assessed if the point at which response pathways to LPS and apoptotic cells diverge , is prior to, or following NF-κB activation (figure 68).

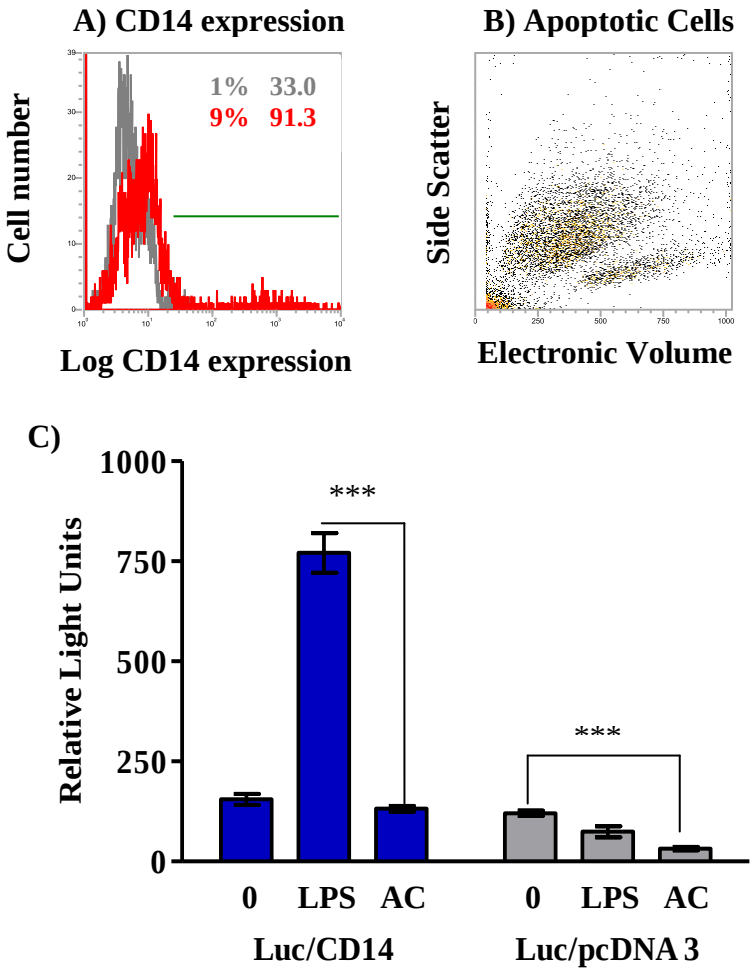

Figure 68: Apoptotic cells binding to mCD14 does not activate NF-κB response
A. HeLa was transfected with an NF-κB reporter using LT-mediated transfection along with mCD14 (red) or pcDNA3 (empty plasmid) (grey). Following transfection cells were seeded at a density of $1.5x10^4$/well in 96-well plates and incubated overnight to allow for attachment.Cell surface expression of CD14 was analysed using indirect-immunofluorescence staining with mAb 63D3 and the data is representative of three independent experiments **B.** Mutu were induced to undergo apoptosis by UV-treatment then incubated overnight and apoptosis was assessed by flow cytometry **C.** Cells were washed and then stimulated with 100µl of medium alone, 100µg/ml of LPS in the presence of 10% (v/v) NHS or $1.5x10^6$/well apoptotic cells alone for 5 h. Following incubation, 100µl of One-Glo was added to each well and the plate incubated for 3min prior to measuring luminescence. The data shown is the mean ± SEM of three independent experiments. *Statistical analysis was conducted using AVONA with Bonferroni post test. (*** P<0.001)*

The results in figure 68 suggest that incubation of apoptotic cells in the presence or absence of mCD14 expression did not result in NF-κB activation. This suggests that that unlike the binding of LPS to mCD14, apoptotic cells do not induce NF-κB activation. The data also illustrates that incubation of HeLa with apoptotic cells in the absence of mCD14 expression, reduced basal levels of NF-kB activation. This data suggest that apoptotic cell interaction produces an immunosuppressive and anti-inflammatory effect and is supported by Voll *et al.* (1997) and Fadok *et al.* (1998). Overall this data suggest that the

immunosuppressive and anti-inflammatory effect of apoptotic cell interaction can be uncoupled from CD14 expression. This may suggest that CD14 is not required for the anti-inflammatory response of apoptotic cells therefore suggesting mCD14 function simply for tethering apoptotic cells to phagocytes. Alternatively phagocytes may express a variety of receptors capable of mediating the anti-inflammatory response of apoptotic cells in the absence of CD14.

6.5.2.3 Is TLR4 recruited following apoptotic cell ligation with CD14?

Given previous findings that residue 11 of CD14 is required for both apoptotic cell and LPS binding the differences in signalling following ligation of CD14 may be a result of interaction with signalling partners. Therefore the requirement for TLR4 in the phagocytic synapse following LPS stimulation or incubation with apoptotic cells was assessed. Double-stimulated THP-1 were previously found to express mCD14 (section 3.2.2) and TLR4 (section 3.3.3) therefore following stimulation with LPS or apoptotic cells for 1 hour indirect immunofluorescence staining for CD14 (FITC) and TLR4 (PE) was undertaken and slides were analysed by confocal microscopy. Following titration of cell number and concentrations of antibodies, problems with the effects of the laser on fixative methods used arose. Failure to overcome these issues resulted in the use of fluorescent microscopy. However due to time limitations the problems with non-specific binding of the secondary antibodies could not be overcome and while overlays of all images were conducted the results were inconclusive. As part of the future work of this project, the use of FRET would be considered to investigate the recruitment of TLR4 to lipid rafts following CD14 ligation. If TLR4 is recruited to the cell surface following CD14 ligation with apoptotic cells, this would suggest that LPS-like patterns on the surface of apoptotic cells are also recognised by TLR4. Therefore this would suggest that inflammatory signalling becomes reprogrammed as a result of apoptotic cell ligation with CD14, resulting in a dominant anti-inflammatory response.

6.6 Discussion

CD14, expressed by monocytes and macrophages is well characterised for binding LPS and promoting inflammation (Ulevitch and Tobias, 1999 and Wright *et al.,* 1990). CD14 is also known to participate in the clearance of apoptotic cells, a process known to have immunosuppressive and anti-inflammatory effects (Voll *et al.,* 1997 and Fadok *et al.,* 1998b). This led to the suggestion that CD14 was involved in the assembly of two distinct ligand-dependent macrophage responses, possibly due to variations in ligand-binding. LPS

binding to CD14 has been mapped to four regions within the N-terminal pocket of CD14 (Stelter *et al.,* 1997, Juan *et al.,* 1995a, Juan *et al.,* 1995b, Cunningham *et al.,* 2000 and Kim *et al.,* 2005). Apoptotic cell binding to CD14 has not been finely mapped but previous work by Devitt *et al.*(1998) with CD14-blocking mAbs, MEM18 and 61D3, suggested that CD14 uses the same or closely-associated regions to bind both LPS and apoptotic cells. This was supported by Gregory *et al.* (2000) and finding here that apoptotic cells expose LPS-like structures at their surface (section 3.3.7). Previous work within this thesis using soluble CD14 point mutants has suggested that apoptotic cells bind to residues located within the LPS-binding regions of CD14. However limitations with the use of soluble CD14 point mutants led to a need for the mutations to be membrane-bound and these were expressed within HeLa.

6.6.1 Mapping mAb binding to mCD14

Having generated the panel of membrane-bound CD14 point mutants, the binding of MEM18 and 61D3 were mapped, with flow cytometry, as surrogate markers of apoptotic cell binding. The flow cytometric data suggests that residue 11 within the first LPS binding region of CD14 is required for 61D3 binding. The first LPS binding region is primarily composed of aspartic and glutamic acid residues producing an overall negative charge likely required for LPS binding. Consequently, substitution of glutamic acid at position 11 for aspartic acid had no overall effect on 61D3 binding. However a loss of the negative charge caused a reduction in 61D3 binding, suggesting that charge may dictated antibody binding. This is further demonstrated when glutamic acid is substituted for arginine, which has an overall positive charge, resulting in a complete loss of 61D3 binding. However Cunningham *et al.*(2000) and Shapiro *et al.* (1997) also found that charge reversal mutations had the greatest effect on LPS binding. Taken together the results suggest that the first LPS binding region of CD14 contains part or the whole epitope for 61D3. Furthermore the results shown here suggest that the glutamic acid (residue 11) of CD14 may be a contact residue required for 61D3 binding. Residue 14 is two amino acids outside the first LPS binding region and therefore located between a negatively charged region of the protein, and a neutral, non-polar region which is involved in disulphide bridge formation. These data suggested that due to the close proximity of residue 14, a loss of the highly basic amino acid; arginine, affects the three-dimensional shape of the surrounding area therefore reducing 61D3 binding.

Juan *et al.* (1995b) and Stelter *et al.* (1997) suggest that the first LPS-binding region is also required for TLR4 signalling, as the ability of sCD14 to mediate LPS response was impaired following mutation of residues 7-14. This work was conducted with sCD14 and using cells that do not express CD14. Therefore this may suggest that residues 7-14 are required for sCD14 to interact with cell-bound receptors, such as TLR4, to induce inflammation. However it may also suggest that residues 7-14 of mCD14 are required to interact with TLR4 and mediate inflammatory signalling. Given this suggestion, 61D3 may directly block interaction of TLR4 with CD14, therefore suggesting that LPS binds to CD14 but signalling is blocked. However mutation of residue 11 prevents LPS and 61D3 binding therefore this would suggest that 61D3 binds CD14 which then block LPS binding. Given the involvement of residues 7-14 in TLR4 signalling and findings here, that residue 11 of CD14 is required for apoptotic cell binding, TLR4 may be involved in the binding of apoptotic cells to CD14. Therefore a future experiment to consider would be assess the role of TLR4 in the CD14-dependent binding of apoptotic cells and could be conducted using FRET analysis. This may establish if TLR4 is associated with CD14 when apoptotic cells are bound.

The third LPS binding region of CD14 spans amino acids 35-39 and is predominately composed of neutral and non-polar residues. However due to the presence of two glutamic acid residues, this region is likely to have an overall negative charge. The data suggests that a loss of charge or a slight change in the dimension of residue 37 had no effect on 61D3 binding. The fourth region of CD14 spans amino acids 57-63 and is located on the rim of the NH-terminal pocket (Kim *et al.,* 2005). Overall this region has neutral charge but most likely exhibits polarity. The data here suggests that a loss of charge or a reduction in the length of the side chain of residue 59 had no effect on 61D3 binding. Due to competition for binding to CD14, the epitopes of 61D3 and MEM18 are considered the same or closely related, however MEM18 binding had been mapped to the fourth LPS-binding region (Juan *et al.,* 1995a). The data here supports these findings as a loss of charge and or change in dimension of residue 59 reduced MEM18 binding. With respect to location, the first LPS-binding region (shown to contain 61D3 epitope), is located close to the wall of the N-terminal pocket of CD14, while the fourth LPS binding region (to which MEM18 binds), is at the rim of the pocket (Kim *et al.,* 2005). The pocket is described as relatively large with dimensions of 8Å wide, 13Å long and 10Å deep (Kim *et al.,* 2005). However compared to an antibody, the epitopes of MEM18 and 61D3 would probably be within close proximity that the binding of MEM18 would sterically hinder the binding of

the other 61D3. In comparison, an apoptotic cell is bigger than an antibody therefore the mapping studies with MEM18 and 61D3 may suggest that the first and fourth LPS binding regions are involved in apoptotic cell binding. Therefore a future experiment to be considered would be to map the binding of the Fab-fragments of 61D3 and MEM18 to CD14.

6.6.2 Mapping apoptotic cell binding to mCD14

Membrane CD14 point-mutants were then used to map apoptotic cell binding to HeLa as they were previously shown to function as amateur phagocytes (figure 38). HeLa were very efficient at clearing apoptotic cells, however the results suggest that CD14 accelerates the initial binding of apoptotic cells to these amateur phagocytes. In order to focus on the initial binding events, the temperature and incubation time of the assay was reduced and apoptotic cell binding to CD14 was mapped. The data supported previous findings, suggesting that residue 11 was important for apoptotic cells binding. This data present here supports the suggestion that both charge and the three dimensional conformation in relation to residue 11 are important for apoptotic cell binding to CD14. Comparison of the binding of apoptotic cells with 61D3 binding, found that alanine replacement or a charge reversal reduced 61D3 binding. Residue 14 is two amino acids outside the first LPS binding but within the first suggested TLR4 binding domain (Juan *et al.,* 1995b, Stelter *et al.,* 1999), therefore mutation of residue 14 may affect TLR4-mediated responses but not binding. However the data suggested that the removal of the side chain on arganine at residue 14 enhanced apoptotic cell binding to CD14, possibly by reducing steric hindrance. Alternatively a loss of charge conferred by arginine, may result in changes to the three dimensional conformation of the surrounding area which includes the first LPS binding region, thereby favouring apoptotic cell binding. Comparison of apoptotic cell binding to residue 14 with 61D3 binding, found that alanine replacement also reduced 61D3 binding suggests that a loss of charge alters the three-dimensional shape of the surrounding regions. Although, considering that 61D3 binding to residue 11 was also reduced upon loss of charge, this may simply suggest that 61D3 binding is dictated by charge rather than three-dimensional changes.

Mutations at residue 37 had no effect on apoptotic cell binding or 61D3 binding compared to wtCD14, suggesting apoptotic cells did not bind to the third LPS binding region of CD14. Further work is required to confirm this for example by investigation of the effect of charge reversal as previously these mutations were found to have the greatest effect on

LPS binding (Shapiro et al., 1997). Residue 59, within the fourth LPS binding region of CD14 is located on the same side the N-terminal pocket at the first and third LPS binding regions. Replacement of aspartic acid (residue 59) with asparagine and alanine, both containing neutrally charged side chains, had no effect on apoptotic cell binding to CD14. However to rule out the possibility that apoptotic cells interact with the fourth LPS binding region, a charge reversal mutation should be considered. It seems feasible, considering the positioning at the edge of the pocket and close proximity to the first LPS binding site that apoptotic cells may interact with this region of CD14. This region is important for MEM18 binding, previously shown to block LPS response (Juan *et al.*, 1995a) and apoptotic cell binding to CD14 (Devitt *et al.,* 1998). Comparison of apoptotic cell and MEM18 mapping data found that while MEM18 binding to CD14 was reduced following loss of the negative charge apoptotic cell binding was unaffected. This again suggests that mAb binding is predominantly dictated by charge of the epitope rather than small changes in the three-dimensional shape of the epitopes. Taken together, mAbs and apoptotic cell mapping data, suggest that apoptotic cells interact with the first LPS binding region of CD14. This may suggest that CD14 presents negatively charged hydrophilic residues on one surface of the protein to interact with the lipid back bone of LPS and apoptotic cells. However a question raised is how the LPS-like patterns at the surface of apoptotic cells are recognised by CD14 to result in different downstream signalling events? One possible suggestion is that apoptotic cells may interact with the first LPS binding domain, as this region may also be responsible for TLR4 signalling (Juan *et al.,* 1995b, Stelter *et al.,* 1999) and block interaction with CD14 when apoptotic cells are bound. Alternatively TLR4 also recognises the LPS-like pattern presented by apoptotic cells, (like CD14) however another phagocytic receptor within the phagocytic synapse induces an inhibitory signal that interferes with the inflammatory response.

6.6.3 Mechanistic differences underlying the ligand-dependent responses of CD14

To determine the mechanistic differences underlying the ligand-dependent responses of CD14, the ability of apoptotic cells and LPS to activate inflammatory signalling was assessed. As an indicator of inflammatory signalling, the production of IL-8 following LPS stimulation was examined in HeLa. However HeLa in the absence and presence of mCD14 expression LPS treatment did not produce a significant IL-8 response. This contrasts with previous finding by Sharma *et al.*(2011) that found upon LPS-induced stimulation, HeLa increased NF-κB levels and produced proinflammatory cytokines (IL-6 and IL-1α) and

chemokines (IL-8 and MCP-1). Sharma *et al.*(2011) also found that LPS-induced cell activation was mediated though TLR4 but did not comment on CD14 expression or involvement in cellular activation. One possible explanation is the type of LPS used to stimulate cells, Sharma *et al.*(2011) used LPS isolated from vaginal strains of *E. coli* therefore HeLa may be more sensitive to these stains. However this seems unlikely as the lipid A region is responsible for the biological activity of LPS and its structure is highly conserved with only slight variations between bacterial strains. CD14 is known to recognise the lipid A portion of LPS and induce inflammation in a TLR4-dependent manner (Rietschel *et al.,* 1994 and Wright *et al.,* 1990). Previously Werner *et al.*(2011) found that a range of cervical epithelial cells including HeLa were unresponsive to LPS and findings suggests it was due to low RNA production and lack of cell surface TLR4 expression. Fichorova *et al.*(2002) found that cervical epithelial cells failed to express TLR4 or MD2 mRNA but were able to respond to gram negative infections in the absence of MD2/TLR4.

Prior to an IL-8 response the transcription factor, NF-κB is activated leading to transcription of proinflammatory mediators. Therefore using an NF-κB reporter assay to assess the ability of CD14 to induce inflammatory signalling following ligation with LPS or apoptotic cells was established. The data suggests that the point of divergence between these two signaling pathways may occur upstream of NF-κB activation, as incubation of apoptotic cells for 5 hours with HeLa expressing CD14 did not lead to NF-κB activation. These findings are supported by Tassiulas *et al.*(2007) that reported macrophages incubated with apoptotic cells alone did not induce degradation of IκBα and therefore prevented NF-κB activation. Following TLR4 activation, MyD88-independent pathway is activated resulting in STAT 1 activation; however Tassiulas *et al.*(2007) also found that following incubation with apoptotic cells alone, macrophages did not phosphorylate STAT1. Interpretation of these findings, along with data presented here, may suggest that apoptotic cells binding residue 11 of CD14 and block TLR4 interaction therefore resulting in the exclusion of TLR4 from the phagocytic synapse. This is supported by Shiratsuchi *et al.*(2004), that investigated the ability of LPS and apoptotic cells, following uptake by mouse peritoneal macrophages, to induce translocation of NF-κB to the nucleus. The findings suggested that incubation of macrophages with apoptotic thymocytes for an hour did not induce NF-κB translocation. Taken together with results shown here, these data suggest that apoptotic cell binding to CD14 does not induce inflammatory signalling.

However the inhibitory signal induced by apoptotic cell uptake may occur upstream of IκB kinase complex responsive for NF-κB activation and subsequent translocation. One such inhibitor of the innate immune system are the TAM receptor tyrosine kinases (Rothlin *et al.,* 2007), also involved the phagocytic clearance of apoptotic cells though association with PS-binding protein GAS6 or protein S (Lemke and Rothlin, 2008 & Lemke and Burstyn-Cohen, 2010). TAMs are considered to inhibit TLR-dependent inflammation by inducing the expression of silencer of cytokine signalling proteins (SOCS) (Rothlin *et al.*, 2007). Following expression, SOCS1 and SOCS3 are considered to inhibit the JAK/STAT pathway, intercepting and therefore inhibiting TLR-mediated cytokine response (Rothlin *et al.*, 2007). Therefore ligation of CD14 with LPS or apoptotic cells may induce TLR4 signalling, however subsequent association of the Mer-tyrosine receptor with a bound apoptotic cell may inhibit proinflammatory signalling. This is supported by Scott *et al.*(2001) that reported a direct link between failed clearance due to truncated Mer tyrosine kinase receptor and increased auto-antibodies. TAMs are also considered to recruit crk-DOCK180-ELMO complex to the membrane resulting in Rac1 activation and consequently actin-dependent engulfment (Wu *et al.*, 2006).

Discussion

The innate immune system discriminates 'self' from 'infectious non-self' using a series of PRRs that recognise evolutionary conserved PAMPs (Janeway, 1992, Janeway, 2001). Research within the field had highlighted the involvement of many PRRs, including, CD14 in the clearance of apoptotic cells. This has led to the suggestion that apoptotic cells present surface patterns that share structural similarities with PAMPs, termed ACAMPs (Franc *et al.,* 1999 & Gregory *et al.,* 2000). However while LPS-binding to CD14 promotes inflammation (Wright *et al.,* 1990, Ziegler-Heitbrock and Ulevitch, 1993, Martin *et al.,* 1994), the clearance of apoptotic cells is considered an immunomodulatory and anti-inflammatory process (Stern *et al.,* 1996, Fadok *et al.,* 1998b, Voll *et al.,* 1997). Devitt *et al.*(1998) first implicated CD14 in the clearance of apoptotic cells by HMDM and further work suggests that CD14 functions as a tethering receptor (Devitt *et al.,* 2004). The CD14-dependent clearance of apoptotic cells has been supported here, by the development of several THP-1-derived macrophages models. While this data supports a role for CD14 in interaction between apoptotic cells and phagocytes, the potential involvement of CD14 in the anti-inflammatory effects which follows these interactions is currently unknown. Overall data here suggests that the immunosuppressive effects of apoptotic cells are independent of CD14 and expression of its signalling partner, TLR4. These findings are supported by Devitt *et al.*(2004) that reported that CD14-deficient mice had persistent apoptotic cells at specific sites without inflammation, suggesting that the anti-inflammatory consequences of apoptotic cell clearance can be uncoupled from CD14-dependent binding events. This points to a scenario, in which CD14 functions simply, as a tethering receptor which may enhance apoptotic cell interaction with other phagocyte-receptors that mediate uptake and the anti-inflammatory response. Alternatively, it may suggest receptor redundancy with several receptors capable of mediating the anti-inflammatory response to apoptotic cells in the absence of CD14.

7.1 The role of non-myeloid CD14

The macrophage is the professional scavenger of apoptotic cells that efficiently and rapidly phagocytoses apoptotic cells when amateur phagocytes are overwhelmed (Gregory and Devitt, 2004). The clearance of apoptotic cells is mediated by both professional and amateur phagocytes, with clearance in both cases resulting in this characteristic anti-inflammatory response (Patel *et al.,* 2010). Data presented here using a series of non-

professional oral, pulmonary, mammary, cervical epithelial cells and pulmonary fibroblasts supports the role of amateur phagocytes in the clearance of apoptotic cells. The receptors used by amateur phagocytes to clear apoptotic cells are largely unknown, however Monks *et al.* (2005) found that mammary epithelial cells utilised a series of PRR also used by macrophages to clear apoptotic cells. Therefore the role of CD14 in the clearance of apoptotic cells by a series of amateur phagocytes was examined. Originally, CD14 expression was considered to be limited to myeloid cells and B cells (Ziegler-Heitbrock and Ulevitch, 1993). Fearns *et al.*(1995), using a mouse model found that epithelial cells in certain tissues, including the lung expressed CD14. Furthermore, Devitt *et al.*(2004) observed that CD14-deficient mice had persistent apoptotic cells in the thymus, spleen, lung, liver and gut, which implies CD14-mediated clearance is crucial at these sites. However due to the repertoire of receptors expressed by macrophages that interact with apoptotic cells it is likely that molecular redundancy would facilitate clearance. Therefore the persistence of apoptotic cells at these sites, within CD14-deficient mice, may suggest that it is the absence of CD14 from amateur phagocytes that results in persistent apoptotic cells. This has been supported by the findings here that pulmonary epithelial cells, Beas-2B and Calu-3 expressed cell surface CD14, were LPS responsive and participated in the CD14-dependent clearance of apoptotic cells. However, in direct comparison, cell surface expression of CD14 was undetectable in the cervical epithelial cell line, HeLa which were unresponsive to LPS stimulation and interaction with apoptotic cells was not inhibited by CD14-blockade. Taken together the data along with findings by Devitt *et al.*(2004) may suggest that the expression and consequently, involvement of CD14 in the innate immune responses is tissue specific.

With respect to the lungs, failed clearance of neutrophils would be massively detrimental with proinflammatory consequences. While innate immune components provide defence against invading pathogens, many have been implicated in the clearance of apoptotic cells, including, the mucocilliary escalator, immunoglobulins, complement factors, surfactant proteins A and D along and alveolar macrophages. However, pulmonary epithelial cells maintain mucosal integrity by modulating the immune responses and therefore express a variety of innate immune receptors to mediate responses to infectious agents (Diamond *et al.*, 2000). With several innate immune receptors suggested to be involved in the clearance of apoptotic cells, it is likely that pulmonary epithelial cells utilise multiple receptors to interact and mediate uptake of apoptotic cells. In terms of CD14, interpretation of the data here suggests that expression of CD14 on pulmonary epithelial cells facilitates efficient

and rapid removal of apoptotic cells compared to other amateur phagocytes analysed. The theoretical mechanism proposed, is that apoptotic cells are rapidly tethered to mCD14, which enhances the interaction between apoptotic cell-associated ligands and engulfment receptors, such as, Mer or TIM-4 (Hoffmann *et al.*, 2001). Therefore could the expression of CD14 on phagocytes dictate their professional status? Data here illustrates that the expression of mCD14 in HeLa enhances the initial binding of apoptotic cells. This would suggest that the delay in the uptake of apoptotic cells by amateur phagocytes observed by Parnaik *et al.*(2000) may be PS-dependent. However Hoffmann *et al.* (2001) found that PS-exposure alone was insufficient to induce binding or uptake of apoptotic cells, but combined with any tethering receptor, PS triggers engulfment. This may suggest tethering receptors expressed by amateur phagocytes are limited or low. In turn this would suggest that amateur phagocytes, depending on site and location, express certain PRRs, such as CD14 in an attempt to modulate local immune responses and improve their professional status.

7.2 Mechanistic differences underlying the responses of CD14 to PAMPs Vs ACAMPs

Data here suggests that specific tissues require CD14 to modulate local immune responses. The role of CD14 in the anti-inflammatory consequences of apoptotic cell clearance is unknown. While the divergence in ligand-dependent responses of CD14 is currently unknown, findings by Hoffmann *et al.*(2001) and Devitt *et al.*(2004) suggest that CD14 functions as a tethering receptor. However Gregory(2000) suggested that CD14 may induce an immunosuppressive signal; following apoptotic cell binding, in a manner similar to LPS stimulation. This suggests that the divergence in ligand-dependent responses of CD14 is a result of the mechanistic difference of PAMPs and ACAMPs recognition (figure 69).

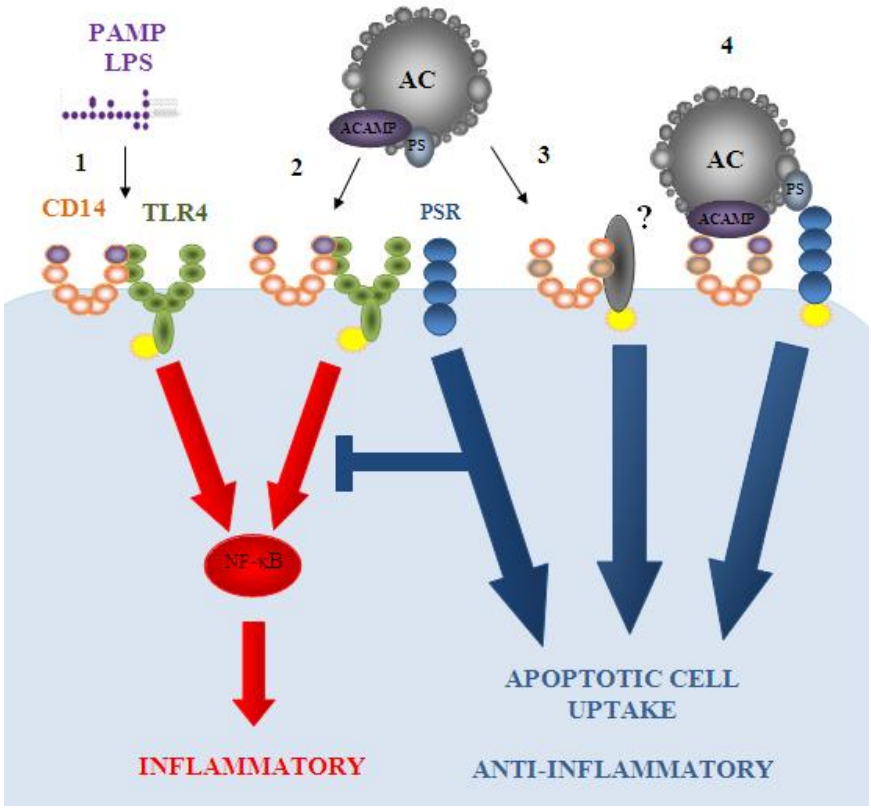

Figure 69 : Possible scenarios involved in CD14-dependent responses
CD14 dependent response to LPS: **1.** LPS binds CD14 (purple domains) and induces a pro-inflammatory signal (red arrows) though association with TLR4, resulting in NF-κB translocation and an inflammatory response. Possible CD14-dependent responses to apoptotic cells: **2** ACAMP on apoptotic cells bind to CD14 in an identical manner to LPS (purple domains) and induce a pro-inflammatory signal as seen in scenario one. However pro-inflammatory signalling is suppressed following further phagocyte receptor ligation (e.g. PS-PS receptor) which produces a dominant anti-inflammatory signal (blue arrows). **3** ACAMP binding to CD14 differs from LPS binding (grey domains), thereby dictating the recruitment of an alternative signalling partner to CD14 resulting in anti-inflmmatory signalling **4.** CD14 here functions very simply just to tether the apoptotic cell to the phagocyte. The tethering effects of CD14 may be mediated thought ACAMP interaction with LPS-binding regions of CD14 (purple domains) or another region of CD14 (grey domains), but interaction does not result in signalling. Therefore interaction of the apoptotic cell with another phagocyte receptor independently mediates corpse uptake and the anti-inflmmatory response. *Adapted from Gregory and Devitt, 2004*

7.2.1 Scenario 1

Data present here along with mutagenesis studies identified the importance of the amino terminal region of CD14 for LPS binding and responses (Viriyakosol and Kirkland, 1995 & Juan *et al.*, 1995c). Further, separate mAb mapping and point mutations have been used to identify four hydrophilic regions, within the amino-terminal that were independently required for LPS binding (Juan *et al.*, 1995a, Stelter *et al.*, 1997, Shapiro *et al.*, 1997, Cunningham *et al.*, 2000). Cunningham *et al.*(2000) found that residues located within these regions of CD14 contribute to LPS binding. This is supported by the LPS data

shown here, that found that mutations within one significantly reduced LPS binding. The protein sequence of mouse CD14 shares 67% homology with human CD14, with all LPS binding regions conserved. Furthermore mouse models have previously been used to assess the function of CD14 and findings have suggest mouse CD14 functions the same manner as human CD14, in terms of LPS responses (Haziot *et al.,* 1996) and apoptotic cell clearance (Devitt *et al.,* 2004). Therefore while there is currently no crystal structure for human CD14, the crystal structure of mouse CD14 suggests that the LPS-binding regions identified, are located within a pocket. LPS-binding regions one and two are located at the wall, region three located at the base and region four located on the rim of the pocket (Kim *et al.,* 2005). Previous findings by Cunningham *et al.,* (2000) and Kim *et al.*(2005) along with data presented here, suggests that the LPS-binding site of CD14 is dependent on the tertiary structure of several residues which are within close proximity. As demonstrated by 61D3 binding to region one which hinders MEM18 binding to region four. Prior to the mouse crystal structure, Cunningham *et al.*(2000) suggested that the first, third and fourth LPS binding regions of CD14 are located on one side of the protein and proposed that CD14 presents several charged residues to interact with LPS and possibly other microbial ligands. This theory was supported by the findings that serine or alanine replacement of residues throughout the amino terminal of CD14 had little effect on LPS binding (Cunningham *et al.,* 2000). As supported by LPS-binding data here, charge reversal had the greatest effect on LPS-binding probably by reversing local surrounding charges preventing LPS from docking. Another PRR, the scavenger receptor, has broad ligand binding specificity and is also considered to interact with ligands by presenting several charged residues which act as a "molecular fly paper" (Krieger *et al.*1992, 1993).

7.2.2 Scenario 2 Vs 3

Whilst LPS-binding to CD14 is well characterised, the nature of apoptotic cell interaction with this receptor is relatively unclear. Consequently characterisation of this interaction has formed a key element of this work. Previously Devitt *et al.*(1998) found that CD14-blocking mAbs, MEM18 and 61D3 inhibited both the binding and phagocytosis of apoptotic cells, along with LPS-induced production of TNF-α by HMDM. This led to the suggestion that MEM18 and 61D3 binding defined both LPS and apoptotic cell binding to CD14. Therefore MEM18 and 61D3 binding was mapped to CD14 as a surrogate marker of apoptotic cell binding. The data here suggested that both apoptotic cells and LPS interact amino acids E11 (within region one) and D59 (within region four). However unlike LPS binding, results suggested mAb binding were more susceptible to changes in

charge. Alanine replacement of E11 reduced 61D3 binding to background levels but had no effect on LPS binding. Furthermore, aspartic acid replacement of E11 reduced LPS binding but had no effect on 61D3 binding. This suggested that mAb binding was dictated by charge and could therefore be considered the limiting factor in this case. Taken together these findings suggest that the ligand binding site of CD14 is a conformational epitope composed of several critical hydrophilic residues throughout the N-terminal pocket that capture both LPS and possibly apoptotic cells. This was further supported by data here that suggests apoptotic cells present ACAMPs that share structural similarities with LPS. Interpretation of this data would suggest that due to a resemblance in their binding patterns, both LPS and CD14 interact with several charged residues presented by CD14.

Using a panel of membrane-bound CD14 point mutants, apoptotic cell binding was mapped. The data suggested that residue E11 (i.e. LPS binding region one) was important for apoptotic cell binding while alanine replacement or asparagine replacement of residue D59 (LPS binding region four) has no effect on apoptotic cell binding. This suggests that both charge and the three dimensional conformation in relation to residue E11 was important for apoptotic cell and LPS binding to CD14. However this data also suggests that 61D3 binding defines the apoptotic cell binding site of CD14. Therefore the ability of MEM18 to inhibit apoptotic cell binding is likely to be a result of the size of the antibody and close proximity to residue 11.

Findings by Juan *et al.*, (1995b) and Stelter *et al.*(1999) using a series of sCD14 point mutants identified that amino acids 9-14 were required for cellular responses to LPS in cells that do not express mCD14. Mutations within amino acids 9-14 had the most detrimental effects on LPS responses. However this region is located within the first LPS binding region of CD14, therefore any mutation that affected LPS-binding would be expected to impact on cellular responses. Stelter *et al.*(1999) reported that the inability of mutations to induce a response is not due to an impaired capacity to bind to CD14, suggesting that LPS binding and cellular response are separate functions of CD14. This would suggested that an apoptotic cell may bind to the first LPS binding region of CD14 and results in no inflammation as proinflammatory signalling partner, TLR4 is excluded. One possible way of assessing the association of TLR4 with CD14 when LPS or apoptotic cells are bound may be to use FRET analysis.

7.2.3 Scenario 2

Previous data within this study has suggest that apoptotic cells bind to the first LPS-binding site of CD14 also required for pro-inflammatory signalling (Juan *et al.*, 1995b & Stelter *et al.*, 1999). TLR4 is also a PRR therefore if apoptotic cells present LPS-like patterns recognised by CD14 it seems likely that binding would induce TLR4 signalling resulting in activation of an inflammatory response. Again, this would suggest that LPS and apoptotic cells have similar binding patterns, but that the downstream responses are reprogrammed to be immunosuppressive or anti-inflammatory. To determine the mechanistic differences underlying the ligand-dependent responses of CD14, the ability of apoptotic cells and LPS to activate inflammatory signalling was assessed with an NF-kB reporter assay. However, the data here suggested that if apoptotic cells do induce an inflammatory signal then the point of divergence between these two signaling pathways may occur upstream of NF-κB activation. This data is supported by Tassiulas *et al.*(2007) that reported macrophages incubated with apoptotic cells alone did not induce NF-κB activation. Tassiulas *et al.*(2007) also found that following incubation with apoptotic cells alone macrophages did not phosphorylate STAT1. This is supported by Shiratsuchi *et al.*(2004), that investigated the ability of LPS and apoptotic cells, following uptake by mouse peritoneal macrophages to induce translocation of NF-κB to the nucleus. The findings illustrated that co-incubation of macrophages with apoptotic thymocytes for an hour, did not induce NF-κB translocation therefore upon ligation with CD14, regardless of the LPS-like structures displayed, apoptotic cells do not induce inflammatory signalling.

Alternatively the inhibitory signal induced by apoptotic cell uptake may occur upstream of the IκB kinase complex responsive for NF-κB activation and subsequent translocation. A family of inhibitors of the innate immune system are the TAM receptor tyrosine kinases (Rothlin *et al.*, 2007) which are also implicated in the phagocytic clearance of apoptotic cells though their association with PS-binding protein GAS6 or protein S (Lemke and Rothlin, 2008 & Lemke and Burstyn-Cohen, 2010). TAMs are considered to inhibit TLR-dependent inflammation in dendritic cells by inducing the expression of SOCS (Rothlin *et al.*, 2007). Following expression, SOCS1 and SOCS3 are considered to inhibit the JAK/STAT pathway, intercepting and therefore inhibiting TLR-mediated cytokine response (Rothlin *et al.*, 2007). Therefore given the role of TAMs in apoptotic cell clearance in macrophages SOCS may also have a role in inhibiting TLR-dependent signalling following apoptotic cell ligation with CD14. The role of SOCS in the

immunosuppressive and anti-inflammatory effects of apoptotic cell clearance could be assessed using siRNA-mediated SOCS knockdown or SOCS-deficient macrophages. If apoptotic cell interaction with CD14 then results in inflammation it would suggest scenario 2 (figure 69). ACAMPs on surface of apoptotic cells bind to CD14 in an identical manner to and induce a pro-inflammatory signal. However pro-inflammatory signalling is suppressed following further phagocyte receptor ligation which produces a dominant anti-inflammatory signal.

7.2.4 Scenario 4

Alternatively apoptotic cell bound by CD14 may interact with TLR4 in a manner that does not induce pro-inflammatory signalling or responses. This was suggested following findings by Coats *et al.* (2011) that the phosphate position of lipid A determined TLR4 response to LPS. This may suggest that a specific part of the LPS molecule, such as a phosphate group of lipid A, is required for TLR4 activation. This would therefore suggest that CD14 may function simply to tether apoptotic cells to the phagocyte. Therefore apoptotic cell ligation of CD14 does not result in a signalling event. This would suggest that engulfment receptors, for example, Mer tyrosine kinase or TIM-4 are required to mediate the uptake of apoptotic cells and anti-inflammatory signalling. This is supported by Hoffmann *et al.*(2001) following findings that PS exposure alone was insufficient to mediate the phagocytic uptake of apoptotic cells. Following these findings Hoffmann *et al.*(2001)suggested that apoptotic cells engulfment by phagocytes occurs in two distinct phases, tethering and uptake therefore addition phagocyte receptors were required to tether the apoptotic cell to the phagocyte prior to PS-dependent uptake. Fadok *et al.* (1992) illustrated the important of PS exposure following activation of apoptotic programme and how failure to expose PS resulted in defective clearance. Mer tyrosine kinase is a member of TAMs family which are reported to recruit crk-DOCK180-ELMO complex to the membrane resulting in Rac1 activation and consequently actin-dependent engulfment (Wu *et al.,* 2006). The association of apoptotic cells with TAMs is considered to involve the bridging molecule GAS 6 and protein S which binds to PS. Therefore suggesting that the inhibitory effects of apoptotic cells is PS-dependent as considered by Fadok *et al.*(1998), Hoffmann *et al.*(2001) and Huynh *et al.*(2002).

7.3 Conclusions

The data within this study collectively suggest that the expression of CD14 on professional and amateur phagocytes facilitates the clearance of apoptotic cells. Findings shown here

suggest that CD14 functions as a true PRR that presents several charged regions at one surface of the protein which are responsible for ligand-binding may be unable to discriminate between LPS and LPS-like patterns presented by apoptotic cells. However ligand-binding to CD14 may enhance interaction with other phagocyte receptors (PS receptor, Mer tyrosine kinase or TLR4) that recognise critical features in the patterns bound by CD14 and mediate cellular responses. In an inflammatory capacity, CD14 binds LPS and the signalling partner, TLR4 is recruited with respect to apoptotic cells, CD14 tethers apoptotic cells to the phagocyte and enhances interaction with engulfment receptors which mediate clearance and an anti-inflammatory response. The findings in this study support previous data that suggest apoptotic cell interaction with phagocytes expressing CD14, does not induce inflammatory signalling. Therefore, this would suggest that although CD14 recognises and may bind the LPS-like patterns on the surface of apoptotic cells, a critical component of this interaction may be unrecognisable to TLR4. Therefore suggesting that CD14 enhances apoptotic cell interaction with other phagocyte (both professional and amateur) receptors that are responsible for engulfment and the anti-inflammatory responses.

7.4 Future work

This study supports previous findings that apoptotic cells and LPS interact with similar regions of CD14. Here a panel of CD14 point mutations were used to map the crucial regions of CD14 required for LPS and apoptotic cell binding. The data found that a charge reversal mutation of residue E11 reduced apoptotic cell binding to background level, demonstrating the requirement of this residue for apoptotic cell binding. Charge reversal mutations were previously found to have the greatest effect on LPS binding. Therefore further characterisation of the binding patterns of apoptotic cells in comparison with LPS may be facilitated by using a series of charge reversal mutations. The discovery that LPS and apoptotic cells have the same binding patterns would suggest that the downstream responses are reprogrammed to be immunosuppressive or anti-inflammatory. However CD14 is a GPI anchored glycoprotein that requires a signalling partner to induce cellular responses. Ligation of PAMPs, including LPS with CD14 is known to recruit TLRs to the signalling complex resulting in an inflammatory response. Previous work and data shown here has suggested that TLR4 expression can be uncoupled from the anti-inflammatory response of apoptotic cells. Furthermore work with NF-κB reporter construct has suggested that apoptotic cells do not activate proinflammatory signalling pathways. Therefore in order to characterise the signalling molecules recruited to CD14 following ligation,

immuno-precipitation of the lipid raft following ligation with LPS and apoptotic cells would be carried out. Identification of co-precipitated molecules would be analysed or identified by mass spectrometry and western blot analysis. Successful identification of partners considered to be involved in the anti-inflammatory responses of apoptotic cells, could be expressed with mCD14 and association assessed using FRET analysis following apoptotic cell binding.

Should such findings suggest that both apoptotic cells and LPS recruit the same signalling partners to CD14, a divergence from the inflammatory signalling pathway activated must occur to produce the contrasting ligand-dependent macrophage response. However work within this study, may suggest that the inhibitory signal induced by apoptotic cell uptake occurs upstream of NF-κB activation. Therefore activation of signalling events prior to IκB phosphorylation, including, the initial receptor-mediated events that induce signalling could be investigated. Alternatively as mention previously, a known inhibitor of the innate immune, inflammatory signalling pathway, are the TAM receptor tyrosine kinases (Rothlin *et al.,* 2007). TAMs are considered to inhibit TLR-dependent inflammation by inducing the expression of SOCS proteins that are considered to inhibit the JAK/STAT pathway, intercepting and therefore inhibiting TLR-mediated cytokine response (Rothlin *et al.,* 2007). The role of SOCS in the anti-inflammatory signalling could be assessed using RT-PCR to measure an upregulation of SOC following apoptotic cell uptake. Additionally macrophages could be transfected with shNA for SOCS and the effects of down-regulation on the anti-inflammatory effect of apoptotic cell clearance observed.

Apoptotic cells are considered to present cell surface molecules, termed ACAMPs that are considered to share structural similarities with PAMPs. Results within this study using anti-LPS antibodies found that apoptotic cells and LPS have common conformational epitopes, suggesting that the ACAMPs recognised by CD14 are LPS-like. Immuno-precipitation of apoptotic–associated ligands with anti-LPS antibodies and sCD14-Fc followed my mass spectrometry may lead to the identification of the ACAMPS recognised by CD14.

CD14 also exists in a soluble form present in serum and is elevated following LPS stimulation (Fearns *et al.,* 1995). Previous studies have shown that sCD14 binds LPS and confers LPS responsiveness to cells that do not express mCD14 (Frey *et al.,* 1992, Pugin *et al.,* 1993). While the role of sCD14 in the clearance of apoptotic cells is unknown, a

previous study by Devitt *et al.*(2004) along with data supported here has shown that sCD14-Fc binds to apoptotic cells but not viable cells. This may suggest that sCD14 binds to apoptotic cells and acts as a soluble bridging molecule that facilitates apoptotic cells clearance. Having established a HeLa-based system to assess the role of mCD14 in the clearance of apoptotic cells, the same system could be utilised to assess the role of sCD14 in the clearance of apoptotic cells by amateur phagocytes.

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