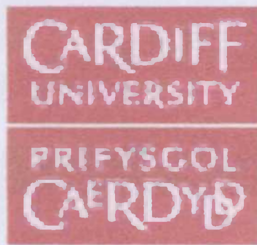

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Complement Plays a Key Role in Tailoring Innate Immune Recognition of Apoptotic and Necrotic Cells

A dissertation for the degree of Doctor of Philosophy

at

Cardiff University

by

Kristina Elward

September 2005

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This project was funded by the Medical Research Council (MRC)

Acknowledgements

I wish to express my sincere thanks to my supervisor Dr Philippe Gasque for all his help and guidance throughout my PhD. Also, to Johan, many thanks for the mentoring during my first year of study. To members of BIIG, past and present (Cecile, Mathieu, Jennifer, Mark, Lloyd, Stephanie, Jean Jacques and Claudine) and to members of medical biochemistry, I am very grateful for your support and assistance over the past few years.

To my loving family, massive hugs, as through difficult times, they always endeavoured to provide me with the constant support and encouragement I needed, and I will always be eternally grateful to have them at my side.

To my bestest friend Jayne, it was made so much easier having you around with words of encouragement. Thanks so much for all our stress relieve chats, you always kept my spirits high.

A big thank you to friends in medical biochemistry (especially Miz, Masashi, Claire and Mike) who were always ready to listen and give advice.

To friends from home (Andrea, Heather, Mari, and Rhiannon) and to university friends (Rachel, Beth and Donna) many thanks for all your support and encouragement over the past few years.

I am grateful to the following people for their kind donation of antibodies and cells:

Dr. V. Horejsi	Institute of Molecular Genetics, Prague, Czech Republic
Prof. J. Atkinson	Washington University, Saint Louis, USA
M. Glennie	Tenovus Research Laboratory, Southampton
Dr. E. Rubenstein	INSERM U268, Paris, France
Dr Leif Andersson	Department of Pathology, University of Helsinki, Finland
Dr. G. Dawson	Department of Biochemistry, University of Chicago, USA

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LIST OF ABBREVIATIONS

Abbreviation	Full name
Ab	Antibody
ABC1	ATP Binding Cassette Transporter 1
ACAMPs	Apoptotic Cell-Associated Molecular Patterns
AD	Alzheimers Disease
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
ANXAI	Annexin I
Apaf 1	Apoptotic protease-activating factor1
APP	Amyloid β-Precursor Protein
APS	Ammonium Persulphate
ASGP-R	Asialoglycoprotein Receptor
BAE	Bovine Aortic Endothelial
BLOX-1-CHO	Chinese Hamster Ovary cells expressing LOX-1
BMDM	Bone Marrow Derived Macrophages
BSA	Bovine Serum Albumin
CAD	Caspase Activated DNAse
CAG	Cysteine, Adenine, Guanine
C4bp	C4 binding Protein

CD200R	CD200 Receptor
cDNA	Complementary DNA
CHO	Chinese Hamster Ovary cells
CNS	Central Nervous System
CR1	Complement Receptor 1
CR3	Complement Receptor 3
CR4	Complement Receptor 4
CRP	C-Reactive Protein
CRT	Calreticulin
DAF	Decay Accelerating Factor
DAPi	4'-6-Diamidino-2-phenylindole
DISC	Death Inducing Signalling Complex
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide trisphosphates
dTT	
ECL	Enhanced chemiluminescent
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
EtBr	Ethidium Bromide
FACS	Fluorescence Activated Cell Sorter
FADD	Fas-Associated Death Domain

Fas L	Fas Ligand
FBS	Foetal Bovine Serum
fH	Factor H
FITC	Fluorescein Isothiocyanate
Gas 6	Growth arrest specific gene 6
β_2GPI	β_2 Glycoprotein I
HAT	Hypoxanthine, Aminopterin, Thymidine
HCl	Hydrochloric acid
HD	Huntington's Disease
HMDM	Human Monocyte Derived Macrophages
HRPO	Horse radish peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
IAP	Integrin-Associated Protein
ICAM-3	InterCellular Adhesion Molecule-3
ICAT	Isotope Coded Affinity Tags
ICC	Immunocytochemistry
i.p	intra peritoneal
ITIM	Immunoreceptor Tyrosine Inhibitory Motif
Kb	Kilo base
kDa	Kilo Dalton
LOX1	Lectin-like Oxidised Low density

	Lipoprotein 1
LPS	Lipopolysaccharide
mA	milliamps
mAb	monoclonal antibody
MAC	Membrane attack complex
MASP	Mannose-binding lectin-Associated Serine Proteases
MCP	Membrane Cofactor Protein
MBL	Mannose Binding Lectin
MER	Myeloid Epithelial Reproductive Receptor Tyrosine Kinase
MFG-E8	Milk Fat Globule-Epidermal growth factor 8
MMLV-rT	Moloney murine leukaemia virus reverse transcriptase
mRNA	messenger ribonucleic acid
MER	Myeloid Epithelial Reproductive
NaCl	Sodium Chloride
NaH_2PO_42H2O	Sodium Dihydrogen Orthophosphate Dihydrate
Na_2HPO_4	Di-sodium hydrogen Orthophosphate Anhydrous
NHS	Normal Human Serum
OxLDL	Oxidised Low Density Lipoprotein

OxRBCs	Oxidatively damaged red blood cells
pAb	Polyclonal antibody
PAMPs	Pathogen Associated Molecular Patterns
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PM	Plasma Membrane
PMNs	Polymorphonuclear cells
PRRs	Pattern Recognition Receptors
PS	Phosphatidylserine
PS-OX	Oxidised Phosphatidylserine
PSR	Phosphatidylserine Receptor
rPE	Red Phycoerythrin
PECAM-1	Platelet-Endothelial Cell Adhesion Molecule-1
PEG	Poly-ethylene Glycol
PI	Propidium Iodide
PMA	Phorbol 12-Myristate 13-Acetate
PMSF	Phenyl methylsulphonyl fluoride

PP2	4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo
PS	Phosphatidylserine
PSR	Phosphatidylserine Receptor
pmol	Pico-molar
RBC	Red blood cell
RNA	Ribonucleic acid
RNAsin	RNAs inhibitor
ROS	Reactive Oxygen Species
RPM	Revs per minute
RT	Reverse transcription
rPE	Red Phycoerythrin
SAMPs	Self Associated Molecular Patterns
SAP	Serum Amyloid Protein
s.c.	Subcutaneously
SDS	Sodium dodecyl Sulphate
SDS-PAGE	Sodium dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SIRPα	Signal Regulatory Protein α
SHIP	SH2-containing Inositol Phosphatase
SLE	Systemic Lupus Erythematosus
SPA	Surfactant Protein A
SR-A	Scavenger Receptor-A
SR-B	Scavenger Receptor-B

TAE	Tris Acetate EDTA
TEMED	NNN'N Tetramethylethylenediamine
TMRM	Tetramethylrhodamine Methyl Ester Perchlorate
TNFR	Tumour Necrosis Factor Receptor
TPM	Thioglycollate-elicited peritoneal macrophages
TRADD	TNFR1 Associated Death Domain Protein
TRITC	Tetramethyl Rhodamine Isothiocyanate
TSP	Thrombospondin
UV	Ultraviolet
V	Volts
VnR	Vitronectin Receptor
WB	Western Blot
w/v	weight per volume

Publications

Elward, K., Griffiths, M., Mizuno, M., Harris, C.L., Neal, J.W., Morgan, B.P., Gasque, P. 2005. CD46 plays a key role in tailoring innate immune recognition of apoptotic and necrotic cells. Journal of Biological Chemistry. 280(43): 36342-36354

Elward, K., Gasque, P. 2003. "Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. *Molecular Immunology*. 40: 85-94

van Beek, J., **Elward, K.**, Gasque, P. 2003. Activation of Complement in the Central Nervous System. *Ann.N.Y.Acad.Sci.* 992: 48-63

Summary

Complement is the canonical innate immune system involved in host defence and tissue repair with the clearance of apoptotic cells and other toxic cell debris.

The aim of the study was to shed new light on the role of complement activators – C1q, C3b, and complement regulators – CD46, CD55 and CD59 in selective recognition and finely tuned removal of "altered self" (i.e. apoptotic versus necrotic cells) in the CNS and to ascertain the capacity of macrophage and brain cells to express complement and other phagocyte receptors involved in the clearance of apoptotic cells. Key differences were observed for complement regulators with a rapid release of soluble forms of CD46, CD55 and CD59 from necrotic cells along with detection of the lytic membrane attack complex (MAC), while only CD46 was dramatically reduced on apoptotic cells to promote complement C3 opsonisation without the MAC. Although CD46 is evenly distributed on normal cells, a remarkable clustering of CD46 to apoptotic blebs at associated site of phosphatidylserine exposure was observed. CD46 was removed from nuclear and cytoplasmic/membrane stores to apoptotic blebs without involving Src kinases, and released from the cell in microparticles together with phosphatidylserine, C1q, C3b and iC3b. Interestingly, nucleic acid exposure at the apoptotic cell surface was observed. DNAse/RNAse treated camptothecin-induced apoptotic Jurkat cells displayed markedly reduced levels of PI staining (NA exposure) at the cell membrane, and the removal of NA was also shown to affect the activation of the classical pathway on apoptotic cells with reduced C1q binding and C3 levels at the cell surface. Therefore a possible central role for C1q in the early detection of membrane bound NA on apoptotic cells could be proposed.

Professional and amateur phagocytes were shown to express complement components but counter intuitively a significant decrease in complement biosynthesis was observed following phagocytosis of apoptotic cells. The analysis for receptor expression on professional (THP1) and amateur (T98G) phagocytes revealed THP1, stimulated with PMA had a good phagocytic activity and correlated with an increased expression of several phagocyte receptors. In contrast, T98G was shown to have a low level of phagocytic activity and IFNγ did not improve this activity. Furthermore, in agreement with their lower phagocytic activity we found T98G were expressing low levels of receptors. However, interestingly, the neuronal cell lines were shown to express an array of receptors and therefore could be considered as the most favourable amateur CNS phagocyte to aid the professionals in clearance of apoptotic cells.

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Chapter 1

General Introduction

1.1 Defining Cell Death - Apoptosis or Necrosis?

The term apoptosis (an ancient Greek word used to describe the "falling off" of petals from flowers or leaves from trees) was introduced by Kerr, Wyllie and Currie in 1972, when they published a paper entitled 'Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics' to describe a common type of programmed cell death associated morphological changes observed in various tissues and cell types and, distinct from the features observed in cells undergoing pathological, necrotic cell death (Kerr, *et al.* 1972, Kuan and Passaro 1998) (Table 1.1).

1.1.1 The Biochemistry of apoptosis

The biochemistry of apoptotic cell death can be characterised into two major stages: first a commitment to cell death, followed by execution resulting in recognition and safe engulfment of apoptotic cells by phagocytes (Cohen 1997). There are two pathways by which cells can be committed to apoptosis (Figure 1.1) - the receptor mediated or the receptor independent route. The receptor-mediated route is activated by tumour necrosis factor receptors (TNFR), which possess a death domain (e.g. Fas (CD95) and TNFR1 (CD120a)). Binding of Fas ligand (FasL) to Fas results in receptor clustering and formation of a death inducing signalling complex (DISC). Multiple pro-caspase 8 molecules are recruited to the complex via the adaptor protein FADD (Fas-associated death

domain), proteolytically activated to caspase 8 and then released into the cytoplasm. Once activated, caspase 8 can cause cleavage and activation of caspase 3. Activated caspase 3 is then able to act on its substrates to execute the apoptotic pathway (Hengartner 2000, Strasser, *et al.* 2000).

The receptor independent route (i.e. the mitochondria pathway) can be activated by a variety of external stimuli including growth factor withdrawal, irradiation, reactive oxygen species, and also by internal insults such as deoxyribonucleic acid (DNA) damage. The stress signal is targeted to mitochondria and the commitment to cell death at this stage is determined by pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) members of the Bcl-2 family, which decide whether the stress signal is strong enough to condemn the cell to death. If the pro-apoptotic signal is adopted, mitochondria membrane integrity is damaged leading to the release of cytochrome c that in turn binds to apoptotic protease-activating factor 1 (Apaf 1) and caspase 9 to form the apoptosome. Caspase 9 becomes activated by the apoptosome and can then cleave procaspase-3 to form activated caspase 3 to initiate the execution phase. The death receptor and mitochondria pathways converge at the level of caspase-3 activation. However, cross talk and integration between the two pathways may result via Bid, a pro-apoptotic Bcl-2 family member. Caspase-8 mediated cleavage of Bid increases its pro-death activity resulting in its translocation to mitochondria, where it promotes cytochrome c release (Amarante-Mendes and Green 1999, Hengartner 2000).

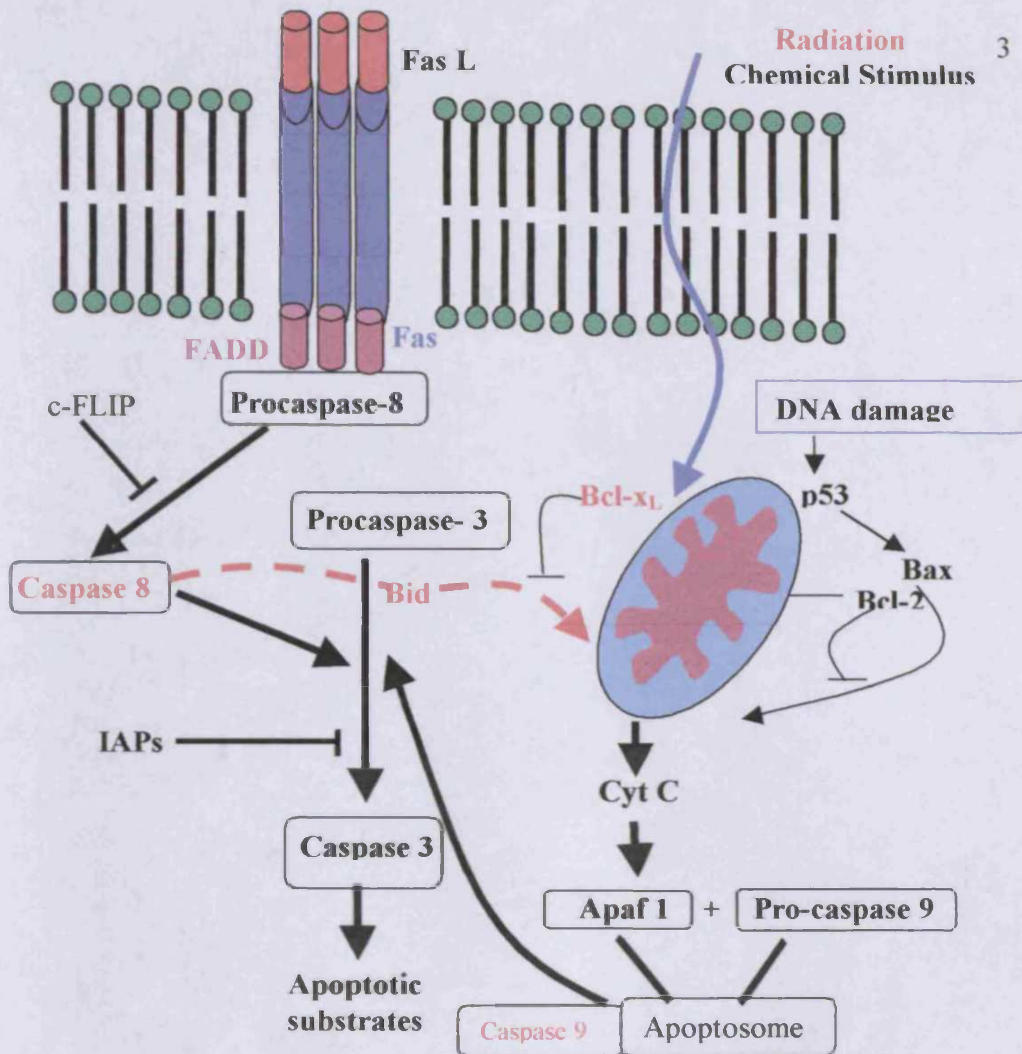


Figure 1.1 A Schematic of the Two Pathways by which Cells can undergo Apoptosis. Members of the death-receptor super family (i.e. Fas or TNFR) trigger the receptor-mediated route. Binding of Fas ligand to Fas induces receptor clustering and formation of a death inducing signalling complex (DISC). This complex recruits, via the adaptor molecule FADD (Fas-associated death domain protein), multiple procaspase-8 molecules, resulting in caspase-8 activation. Caspase 8 activation can be blocked by recruitment of the degenerate caspase homologue c-FLIP. The Receptor independent route (mitochondria pathway) is triggered by internal insults such as DNA damage or by external stimuli such as radiation or a chemical stimulus. The stress signal is targeted to the mitochondria, often through the activation of a pro-apoptotic member of the Bcl-2 family (Bax). Pro- (Bax) and anti- (Bcl-2 and Bcl-x_L) apoptotic Bcl-2 family members meet at the surface of mitochondria, where they compete to regulate cytochrome c exit. When cytochrome c is released from the mitochondria, it associates with Apaf-1 (Apoptotic protease-activating factor 1) and then pro-caspase 9 to form the apoptosome. Caspase 9 becomes activated in the apoptosome and can then go on to cleave pro-caspase 3. Both routes converge at the level of caspase-3 activation. IAP (inhibitors of apoptosis proteins) proteins antagonise caspase 3 activation and activity. Caspase 3 can then initiate the execution phase of apoptosis by acting on different apoptotic substrates. Cross talk and integration between the two routes is provided by Bid. Caspase-8 mediated cleavage of Bid greatly increases its pro-death activity, and results in its translocation to mitochondria, where it promotes cytochrome c exit.

The execution phase, which is totally dependent on the activation of caspases, is responsible for the dramatic stereotypic morphological and biochemical changes related to apoptosis including signalling to phagocytes (Cohen 1997). The morphological changes of cells under-going apoptosis, depicted in Figure 1.2, are characterised firstly by condensation of chromatin into typical dense crescents at the periphery of the nucleus adjacent to the nuclear membrane, followed by loss of cell surface features such as cell/cell junctions (Alison and Sarraf 1995) and modifications on the surface membrane (i.e. alteration of carbohydrate groups, and loss of phospholipid asymmetry resulting in phosphatidylserine (PS) exposure) to facilitate their recognition and removal by phagocytes (Savill and Fadok 2000). The cell begins to shrink with the compaction of cytoplasmic organelles, fragmentation of nuclear chromatin continues, plasma membrane blebbing ensues and disintegration of the cell into multiple vesicles results (Alison and Sarraf 1995, Barr and Tomei 1994, Martin, *et al.* 1994). These are then subsequently phagocytosed without damaging surrounding tissue.

1.1.3 The Biochemistry of Necrosis

Despite the devastating impact of necrosis on human health, and contrary to the evidence accumulated for the biochemistry of apoptosis, characterisation of the molecular mechanisms leading to necrosis had proceeded at a relatively slow pace (for review refer to (Syntichaki and Tavernarakis 2003). Unlike apoptosis, necrosis seemed devoid of a well-defined core set of hallmark features, and believed to result from chaotic breakdown of cells under intolerable conditions

(Kerr, *et al.* 1972, Walker, *et al.* 1988). A wide range of external and internal factors is known to trigger necrotic cell death such as hostile environmental conditions or mutated genes. Different triggers intrude on different aspects of cellular physiology (Walker, *et al.* 1988) . Intracellular and extracellular ion homeostasis has been implicated in many cases of necrotic cell death in a range of organisms. Acute-energy depletion such as during ischaemia and hyperglycaemia is an example of a necrosis triggering condition in neurones (Martin 1998). Without the energy needed to sustain ionic gradients, the resting potential of neurones collapses and depolarisation ensues. Unrestrained release of the excitatory neurotransmitter-glutamate at synaptic clefts occurs and energy shortage also impairs re-uptake of glutamate by the high affinity transporters of surrounding glial cells and neurones. Excessive build-up of glutamate at synapses induces hyper excitation, which will eventually lead to necrotic death of down-stream synaptic target neurones, a phenomenon known as excitotoxicity (Choi 1992). Additional disruptions to ionic homeostasis include alterations to the calcium and sodium influx (Koike, *et al.* 2000, Sattler and Tymianski 2000). During pathological conditions the regulatory mechanisms are overwhelmed and intracellular calcium concentration increases through calcium influx from extracellular stores and various channels, and under extreme conditions through the sodium/calcium exchanger, which under normal conditions is the route for calcium efflux (Ferri and Kroemer 2001, Kristian and Siesjo 1998). Calcium concentration can also increase by release from the endoplasmic reticulum (ER), the main compartment for calcium storage, through the ryanodine and inositol-1,

4,5-trisphosphate receptors. During extreme stress, ER calcium stores are rapidly mobilised, enhancing the massive increase of intracellular calcium concentration and signalling cell demise (Ferri and Kroemer 2001). Sodium influx is known to amplify acute neuronal swelling and facilitates calcium entry through voltage-gated channels and the Na^{+}/Ca^{+} exchanger (Sattler and Tymianski 2000).

Another example of induction of necrotic cell death includes the excessive accumulation of reactive oxygen species (ROS), which are produced as by-products of normal and atypical metabolic processes that use molecular oxygen (Bonfoco, *et al.* 1995). They usually inflict death by overwhelming regulatory and homeostatic mechanisms, or by compromising the structural integrity of the cell. For example, they are capable of inducing rapid increases in intracellular Ca^{2+} levels by stimulating Ca^{2+} influx from the extracellular environment and efflux from intracellular stores (Putney 1993).

Two additional ions that are involved in necrosis are magnesium and zinc. Magnesium entry through the NMDA (N methyl-D-aspartate) receptor channel and the consequent decrease in intracellular pH that follows NMDA-receptor-mediated calcium influx exacerbate necrotic neuronal death (Kim 1999, Stout, *et al.* 1996). Zinc, a known neurotransmitter and neuromodulator, has an important role in determining the mode of death during excitotoxic attacks. An overwhelming release of zinc into the extracellular space in certain disease states might be responsible for neuronal death (Kim, *et al.* 1999). Continued influx of zinc through voltage-gated calcium channels, NMDA-receptor channels and

calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors can induce necrosis on high-level exposure and apoptosis on less intense exposure (Jiang, *et al.* 2001, Kim, *et al.* 1999).

In addition to ion homeostasis, deviations from intracellular and extracellular pH homeostasis can result in necrosis. The cells have an array of specialised homeostatic mechanisms to keep their internal pH at a constant level (6.8 – 7.6). However, during pathological conditions such as ischaemia and trauma, the intracellular pH of the brain acidifies to 6.2-6.8. Ischaemia is associated with both hypoxia and acidosis owing to increased glycolysis, production of lactic acid and decreased intracellular pH, but the role of hypoxia remains unknown (Ding 2000).

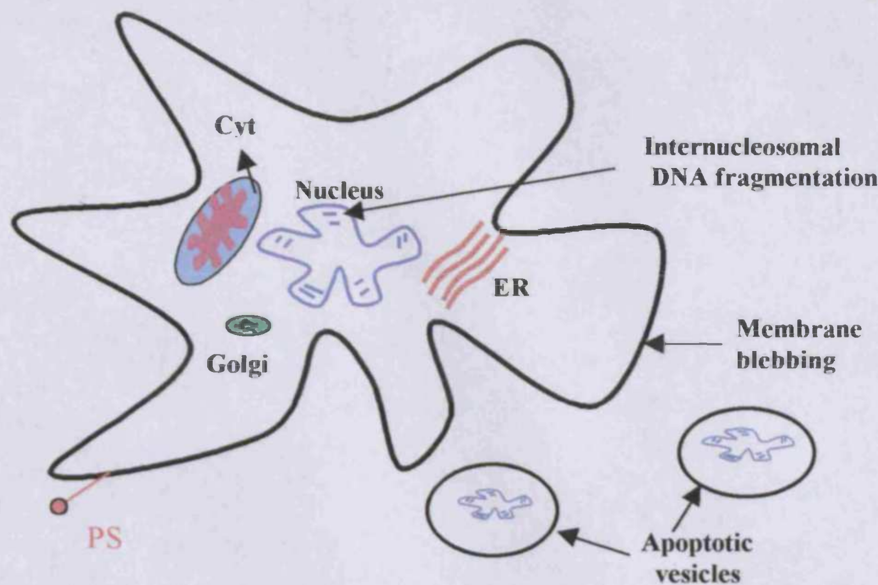
Proteolytic systems, including the lysosomal pathway have been implicated in the execution of necrosis in a number of pathological conditions such as in ischaemic injury of both the heart and brain. Lysosomes consist of an array of hydrolytic enzymes, and release of these proteins into the cytoplasm or delivery of cellular contents to the lysosome induces necrosis. Two mechanisms have been proposed to be involved in the spillage of these destructive enzymes from lysosomes. Firstly, the lysosomal membrane can become damaged by free radicals generated under conditions of extreme oxidative stress, and secondly, injury to the lysosomal membrane is inflicted enzymatically by the action of specific hydrolases (Xue, *et al.* 1999). Two classes of lysosomal proteolytic enzymes have been implicated in this process – aspartyl (cathepsin D) and

cysteine (cathepsin B, H, and L) proteases. Evidence to date has indicated cathepsin D in mediation of the execution of neuronal death induced by ageing, transient forebrain ischaemia and excessive stimulation of glutamate receptors during excitotoxicity (Adamec, *et al.* 2000) . Cathepsins B and L are believed involved in delayed neuronal cell death after global and focal cerebral ischaemia (Seyfried 2001).

The morphological features of necrosis are markedly different from those of apoptosis (Table 1.1) (Kerr, *et al.* 1972). They are characterised by the swelling of mitochondria, dilatation of rough endoplasmic reticulum, disruption of the Golgi complex, vacuolation of the cytoplasm and disappearance of plasma and nuclear membranes and heterochromatin. The cell swells and eventually lyses without the formation of vesicles, leaking the contents out and leading to inflammation and ultimately cell disintegration, often damaging neighbouring cells (Kuan and Passaro 1998). As the cell is dying, the cytoplasm becomes ill defined, the chromatin pattern becomes coarse and clumpy leading to karyolysis (disintegration of the nucleus) (Syntichaki and Tavernarakis 2003).

Table 1.1 Differences between Apoptosis and Necrosis

	NECROSIS	APOPTOSIS
Process	Unordered	Programmed
Energy	Passive	Active
Stimulus	External + internal extreme stress	External + Internal mild stress
Host response	Inflammatory	Anti-inflammatory
Cell membrane	Ruptured	Intact blebs
Mitochondria	Loss	Intact (release cytochrome c)
Nucleus	Disintegration	Nuclear compaction, chromatin condensation and internucleosomal cleavage of DNA
Result	Scar	Apoptotic bodies phagocytosed by macrophage



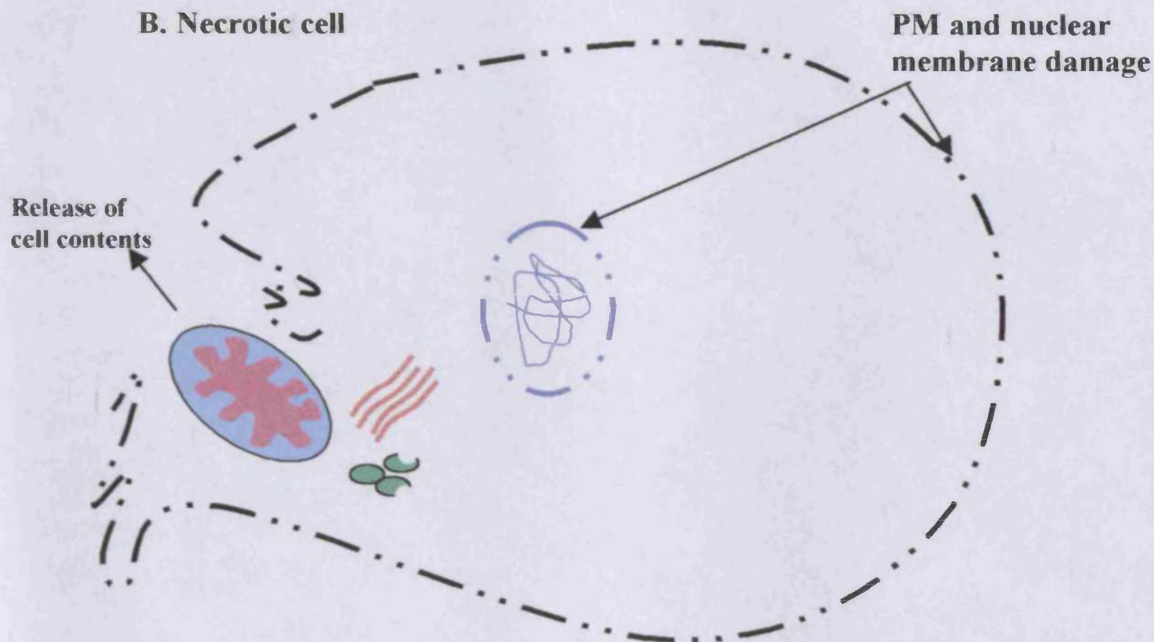


Figure 1.2 Morphological Features of Apoptosis and Necrosis
The morphological changes of cells under-going apoptosis are characterised by cell shrinkage, condensation of chromatin with internucleosomal DNA fragmentation, compaction of cytoplasmic organelles, release of cytochrome c from mitochondria, plasma membrane (PM) blebbing and disintegration of the cell into apoptotic vesicles (A). In contrast features of necrosis are characterised by the swelling of mitochondria, dilatation of rough endoplasmic reticulum, disruption of the Golgi complex, vacuolation of the cytoplasm and disappearance of plasma and nuclear membranes and heterochromatin. The cell swells and eventually lyses without the formation of vesicles, leaking the contents out and leading to inflammation and ultimately cell disintegration, often damaging neighbouring cells (B).

Removal of apoptotic cells is essential in a wide variety of different biological systems, from worm to mammal. Our very existence depends upon a willing exchange of old life for new. Optimum body maintenance means that approximately 10 billion cells within the human body will undergo apoptosis on a normal day to counteract the numbers of new cells produced through mitosis (Hengartner 2000). Cell death by apoptosis is a major mechanism of maintenance of tissue homeostasis, organogenesis, development, remodelling, protection against neoplasia, and maintenance of the immune system (Jacobson, *et al.* 1997, Kuan and Passaro 1998, Meier, *et al.* 2000).

1.2 Mechanisms Involved in the Safe Clearance of Apoptotic Cells

1.2.1 Apoptotic Cell Associated Molecular Patterns (ACAMPs) / "Eat me" Signals

An important stage in the apoptotic process involves molecular changes at the surface of apoptotic cells, which leads to their recognition and safe engulfment by phagocytes (Fadok, *et al.* 2001, Fadok and Chimini 2001, Gregory and Devitt 2004, Grimsley and Ravichandran 2003, Lauber, *et al.* 2004, Mevorach, *et al.* 1998, Savill, *et al.* 2002, Savill and Fadok 2000). However, the exact mechanism as to how this occurs is complex, and to date, not clearly understood. A number of surface alterations on apoptotic cells (apoptotic cell-associated molecular patterns (ACAMPs) in analogy to pathogen-associated molecular patterns (PAMPs) are known to provide 'eat me' signals recognised by phagocyte receptors (pattern recognition receptors, PRRs) (Figure 1.3). These

signals can include target structures that are newly expressed on the apoptotic cell surface as a result of the execution phase, such as externalisation of phosphatidylserine (PS) as opposed to its usual position on the inner membrane (Fadok, *et al.* 2000), or existing molecules, which during the process of apoptosis are altered by oxidation processes and modifications in sugar chains or surface charges (e.g. oxidised low density lipoprotein (OxLDL) sites) (for review refer to (Lauber, *et al.* 2004).

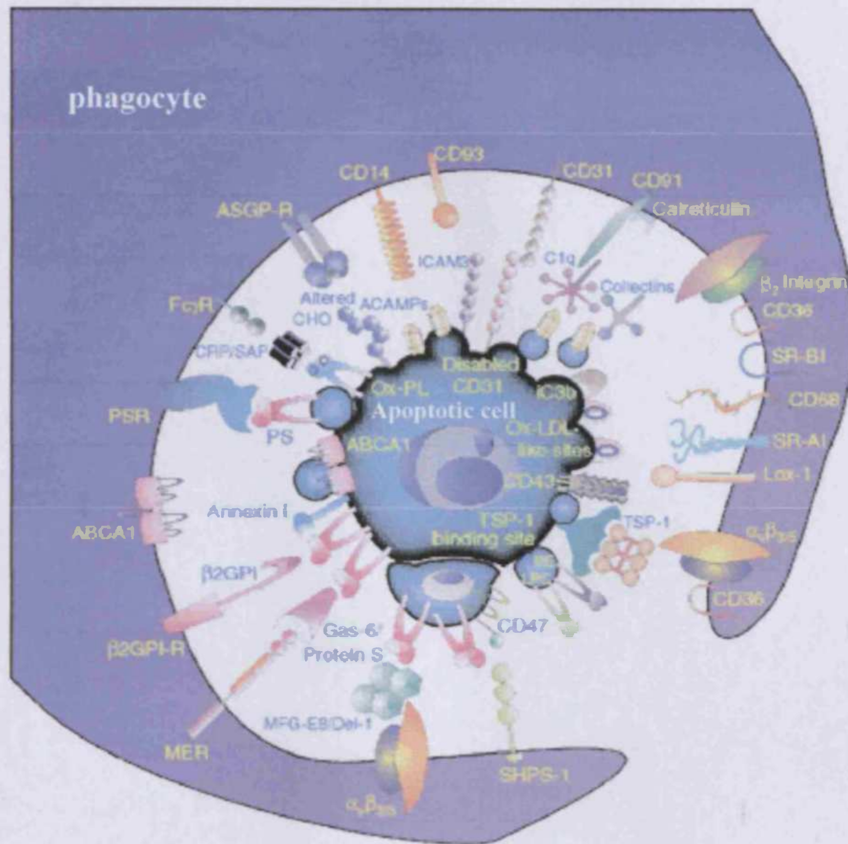


Figure 1.3 A Schematic View of Molecules Implicated in Interactions Mediating Recognition, Binding and Engulfment of a Theoretical Apoptotic Cell with a Theoretical Phagocyte. $\alpha_v\beta_3$, $\alpha_v\beta_v$ vitronectin receptor integrins; ABCA1, ATP-binding cassette transporter A1; ACAMPs, apoptotic cell-associated molecular patterns; ASGP-R, asialoglycoprotein receptor; β2GPI, β2 glycoprotein I; β2GPI-R, β2 glycoprotein I receptor; β2 integrins include CR3 and CR4; C1q, first component of complement; CHO, carbohydrate; CRP, C-reactive protein; Del-1, developmental endothelial locus-1; Gas-6, growth arrest specific gene-6; iC3b, inactivated complement fragment C3b; ICAM-3 (CD50), intercellular adhesion molecule-3; Lox-1, oxidised low density lipoprotein receptor 1; LPC, lysophosphatidylcholine; MER, myeloid epithelial reproductive tyrosine kinase; MFG-8, milk fat globule epidermal growth factor-8; Ox-PL oxidised phospholipids; PE, phosphatidylethanolamine; PS, phosphatidylserine; PSR, PS-receptor; SAP, serum amyloid protein; SHPS-1, Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1; SR-AI, scavenger receptor AI; SR-BI, scavenger receptor BI; TSP-1 thrombospondin-1.

Taken from (Gregory and Devitt 2004)

1.2.2 Self Associated Molecular Patterns (SAMPs) / "Don't eat me" signals

More recently, apoptosis has been found accompanied by down-regulation of a number of proposed 'self associated molecular patterns' (SAMPs), which may convey 'don't eat me' signals to specific inhibitory PRRs expressed by phagocytes to modulate the immune response (Elward and Gasque 2003, Grimsley and Ravichandran 2003, Medzhitov and Janeway 2002, Savill, *et al.* 2002). The concept of a 'missing self paradigm' was first described by Medzhitov and Janeway whereby they proposed that specialised markers of self expressed on normal healthy cells engage inhibitory receptors on phagocytes to prevent phagocytosis. However, the absence of these markers on microbial cells and down-regulation on infected, transformed, and senescent host cells render the cells susceptible to clearance by phagocytes (Medzhitov and Janeway 2002). To date, a small number of possible SAMPs have been characterised and include inhibitors of the complement system such as factor H (fH) (Meri and Pangburn 1990), OX2/CD200 (Barclay, *et al.* 2002, Hoek, *et al.* 2000), CD31 (Brown, *et al.* 2002), CD47 (Brown and Frazier 2001, Oldenborg, *et al.* 2000), and sialic acids (Crocker and Varki 2001).

Experimental down-regulation of OX-2 (CD200), a broadly expressed membrane glycoprotein, was shown to enable accelerated reactivity of resident tissue macrophages, including those in the central nervous system (CNS) (Hoek, *et al.* 2000). CD200 is known to be a ligand for a receptor restricted to myeloid cells - the CD200 receptor (CD200R). CD200 has a very short cytoplasmic tail and

hence does not signal, however, recent evidence has indicated that CD200R associates with SH2-containing inositol phosphatase (SHIP), consistent with a role in down-regulation of phagocyte activities (for review refer to (Barclay, *et al.* 2002).

CD31 (also known as platelet-endothelial cell adhesion molecule-1, PECAM-1) expressed by both leukocytes and macrophages, has been shown to prevent phagocyte engulfment of viable cells by transmitting 'detachment signals', whereas its function changes on apoptotic cells, relaying 'adhesive signals' to phagocytes and thereby promoting clearance (Brown, *et al.* 2002).

CD47 (integrin-associated protein, IAP) is a receptor for thrombospondin family members and a ligand for the transmembrane protein – signal-regulatory protein α (SIRPα), an immunoreceptor tyrosine inhibitory motif (ITIM)-containing inhibitory receptor expressed on macrophages (for review refer to (Brown and Frazier 2001). The CD47-SIRPα (CD172a) ligation bears many similarities to that of the CD200-CD200R interaction preventing phagocytosis of normal cells, and initiating phagocytosis of apoptotic cells with down-regulation of CD47 expression (Oldenborg, *et al.* 2000). CD47 is also known to interact with the Vitronectin receptor (VnR, αVβ3), a known phagocyte receptor, and thrombospondin (TSP), a known bridging molecule, indicating a possible role in clearance of apoptotic cells (Brown and Frazier 2001).

Sialic acids are also known to relay 'don't eat me' signals, which can be recognised by Siglecs, transmembrane inhibitory receptors containing ITIM motifs in their cytoplasmic regions (Crocker and Varki 2001). Sialic acids are also known to bind to fH of the complement cascade and thus inhibit formation of the C3 convertase on normal cells (Medzhitov and Janeway 2002).

In addition, neutrophil apoptosis has been found accompanied by down-regulation of the immunoglobulin superfamily members PECAM-1 (CD31), CD66acde, CD66b, and the integrin-associated proteins CD63 and urokinase plasminogen activator receptor (CD87) (Hart, *et al.* 2000).

Therefore it is paramount that a balance needs to exist between both "eat me" and "don't eat me" signals in binding to either activating or inhibitory PRRs to instruct a non-phlogistic phagocytic response to clear apoptotic cells when required.

1.2.3 Membrane Bound PRRs and Bridging Molecules Involved in the Clearance of Apoptotic Cells by Phagocytes

Mainly from *in-vitro* studies a wide range of phagocyte receptors (PRRs), which bind to their specific ligand on apoptotic cells have been identified (refer to Table 1.1). These include the phosphatidylserine receptor (PSR) binding to PS (Fadok, *et al.* 2000), scavenger receptors (SR) (e.g. CD68, SR-A, SR-B, lectin-like OxLDL-receptor 1 (LOX1), and CD36), which bind to oxidised low density lipoproteins (OxLDL's) (Erdosova, *et al.* 2002, Oka, *et al.* 1998, Platt, *et al.*

1996, Ren, *et al.* 1995, Sambrano and Steinberg 1995), members of the integrin family including the vitronectin receptor ($\alpha_5\beta_3$) (Savill, *et al.* 1990) and receptors for complement – complement Receptors 3 and 4 (CR3, CR4) binding to as yet unknown C3b/iC3b binding sites (Mevorach, *et al.* 1998, Takizawa, *et al.* 1996) (reviewed in more detail in the complement section of introduction (Section 1.5.4)). The phagocyte receptor CD14, has recently been reported to interact with intercellular adhesion molecule-3 (ICAM-3) exposed on apoptotic cells via a region that might be identical to the LPS (a known PAMPs) binding domain (Chowdhury, *et al.* 2004, Devitt, *et al.* 1998, Gregory 2000, Gregory and Devitt 1999) . However, unlike PAMPs, apoptotic cells expressing ACAMPs are cleared safely via a nonphlogistic response without release of pro-inflammatory cytokines from macrophages (Amarante-Mendes and Green 1999) (Figure 1.4).

In addition to the direct interaction of receptor to ligand, a number of bridging molecules have been identified to interact with the apoptotic cell surface and phagocytic receptors. Recently, the direct binding of PS to PSR, as a specific part of apoptotic cell clearance was put into question. Annexin I (ANXA I) was identified as an ACAMP on apoptotic cells, being recruited to the phosphatidylserine-rich domains of apoptotic cell surfaces in a caspase-dependent manner (Arur, *et al.* 2003, Parente and Solito 2004), and it has been suggested that it may also act as a bridging-molecule between PS and its receptor, PSR (Arur, *et al.* 2003). Further examples of bridging molecules binding to PS include milk-fat-globule-epidermal growth factor 8 (MFG-E8)

recognised by the vitronectin receptor ($\alpha5\beta3$) (Borisenko, et al. 2004, Hanayama, *et al.* 2002); growth arrest specific gene 6 (Gas 6) and protein S known to bind to receptors of the myeloid epithelial reproductive (MER) receptor tyrosine kinase family (Anderson, *et al.* 2003, Ishimoto, *et al.* 2000, Scott, *et al.* 2001), and β_2 glycoprotein I (β_2-GPI) and corresponding β_2-GPI receptor (Balasubramanian, *et al.* 1997). CD36 in cooperation with the vitronectin receptor has also been shown to bind to thrombospondin (TSP) binding-sites via the TSP bridging molecule (Navazo, *et al.* 1996, Savill, *et al.* 1992). Complement proteins - C1q, mannose binding lectin (MBL) and C3b/iC3b along with surfactant protein A (SP-A) have been characterised as bridging molecules between, as yet, unidentified collectin/C1q/C3b/iC3b binding sites on apoptotic cells and the phagocyte calreticulin (CRT)/CD91 complex (Vandivier, *et al.* 2002).

Table 1.2 *In-vitro* Studies for Identification of Membrane Bound and Soluble PRRs

In-vitro model	**Receptor identified**	**Results that confirmed identification of receptor**	**Reference**
Human monocyte-derived macrophages (HMDM) phagocytosing aged neutrophils	Vitronectin receptor (VnR, $\alpha5\beta3$)	Aged neutrophil phagocytosis inhibited by RGD-bearing proteins vitronectin. Monoclonal Ab's for $\alpha5$ and $\beta3$ subunits of VnR bound to macrophages and inhibited phagocytosis	**(Savill, *et al.* 1990)** Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. **Nature, 343: 170-173**
Healthy hepatocytes phagocytosing apoptotic newborn hepatocytes (induced apoptosis by hormonal treatments) in culture	Asialoglycoprotein receptor (ASGP-R)	Specific receptor Ab's and sugar moieties were capable of blocking binding and phagocytosis	**(Dini 2000)** The clearance of apoptotic cells in the liver is mediated by the asialoglycoprotein receptor **FEBS Lett. 296: 174-178**
Human monocyte-derived macrophages phagocytosing aged neutrophils	Vitronectin receptor and CD36 receptor in association with thrombospondin (TSP) bridging molecule	Both macrophage and aged neutrophil bound TSP, and phagocytosis was inhibited by excess soluble TSP and TSP Abs. mAbs to TSP, CD36, VnR exerted inhibitory synergistic effects on both Macrophage recognition of apoptotic neutrophils and macrophage adhesion to TSP	**(Savill, *et al.* 1992)** Thrombospondin cooperates with CD36 and the Vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. **J. Clin. Invest, 90: 1513-1522**
Aged human blood derived neutrophils, an apoptotic B lymphocyte cell line (serum free media, 48 h) and apoptotic fibroblasts, (serum free media, 24 h) were phagocytosed by Bowes melanoma or COS-7 cells, both containing vector with CD36 cDNA.	CD36	Transfection of CD36 into human Bowes melanoma cells and COS-7 cells greatly increased capacity to phagocytose apoptotic neutrophils, lymphocytes, and fibroblasts. Soluble CD36, and CD36 mAb inhibited phagocytosis by these transfected cells. Uptake was also inhibited by $\alpha_5\beta_3$ and TSP mAbs confirming previous observations of cooperation of CD36 with these structures. Therefore, findings revealed CD36 gene transfer could confer "professional" capacity of ingestion of apoptotic cells upon "amateur" phagocytes.	**(Ren, *et al.* 1995)** CD36 Gene Transfer Confers Capacity for Phagocytosis of Cells Undergoing Apoptosis. **J. Exp. Med. 181: 1857-1862**

Apoptotic thymocytes (dexamethasone), and oxidatively damaged red blood cells (OxRBCs) were phagocytosed by mouse peritoneal macrophages	OxLDL Receptor	PS liposome's and OxLDL but not Phosphatidylcholine (PC) or cholesterol (C) were both capable of inhibiting binding of OxRBCs to mouse peritoneal macrophages. Inhibition of RBC membrane phospholipid translocase by incubation with sodium vanadate resulted in a progressive increase in PS exposure and subsequently increased binding to macrophages, and OxLDL inhibited binding. OxLDL inhibited binding of sickled RBCs and apoptotic thymocytes to macrophages. However binding of thymocytes was only inhibited by 50%, therefore other receptors could be involved.	**(Sambrano and Steinberg 1995)** Recognition of oxidatively damaged and apoptotic cells by an oxidised low-density liproprotein receptor on mouse peritoneal macrophages: Role of membrane phosphatidylserine. **Proc. Natl. Acad. Sci. USA, 92: 1396-1400**
PS containing liposomes and apoptotic cells derived from mouse bone marrow (MKM) (IL3-depleted media for 48 h) phagocytosed by Chinese Hamster Ovary (CHO) cells transfected with SR-B1	Scavenger Receptor B1 (SR-B1)	Isolated CHO cells expressing SRB-1 and CHO transfectants were capable of phagocytosing PS-liposome's but not PC liposomes. They were also shown to phagocytose apoptotic cells. Control CHO cells and CHO cells expressing the type 1 scavenger receptor did not take up apoptotic cells	**(Fukasawa, *et al.* 1996)** SRB1, a Class B Scavenger Receptor, Recognises both Negatively Charged Liposome's and Apoptotic Cells. **Experimental Cell Research, 222: 246-250**
^{51}Cr-labelled apoptotic mouse thymocytes (γ irradiation) were phagocytosed by isolated mouse thioglycollate-elicited peritoneal macrophages (TPM) expressing ABC1.	ATP binding cassette transporter (ABC1)	An antibody –mediated blocking experiment of the intracellular ATP binding domain of ABC1 was performed. Phagocytosis was inhibited by ~80%. Control immunoglobulins showed no inhibition of phagocytosis.	**(Luciani and Chimini 1996)** The ATP binding cassette transporter ABC1, is required for the engulfment of corpses generated by apoptotic cell death. **The EMBO Journal, 15 (2): 226-235**
Apoptotic mouse thymocytes (dexamethasone) were phagocytosed by mouse thymic macrophages or TPM	Scavenger Receptor A (SR-A)	Phagocytosis of apoptotic thymocytes by thymic and TPM SR-A positive macrophages was partially inhibited by an anti-SRA mAb, and more completely by a range of scavenger receptor ligands, indicating a role for other scavenger receptors as well as SRA. Thymic macrophages derived from mice lacking the SRA receptor showed a 50% reduction in phagocytosis of	**(Platt, *et al.* 1996)** Role for the class A scavenger receptor in the phagocytosis of apoptotic thymocytes *in vitro* **Proc. Natl. Acad. Sci. USA, 93: 12456-12460**

		apoptotic thymocytes, indicating a role for SRA in clearance of apoptotic cells.	
Jurkat T lymphocytes induced to undergo apoptosis with cycloheximide or anti-Fas mAb (clone CH-11) or anti-Fas plus actinomycin D, and phagocytosed by THP1 (differentiated to macrophage phenotype with retinoic acid)	CR3 and CR4 recognition of C3b/bi binding sites	Enhancement of phagocytosis by treatment with normal human serum (NHS) was 2 fold higher than that without NHS. When serum treated cells were incubated with anti-human C3 F (ab')$_2$ enhanced phagocytosis was decreased to levels without NHS. This suggested opsonic effect of iC3b deposited on apoptotic cells had a role to enhance clearance. Enhanced phagocytic response with NHS was inhibited in medium containing anti-CR3 mAb or anti-CR4 mAb. Therefore enhanced phagocytosis could suggest interaction between iC3b deposited on apoptotic cells and CR3/CR4 on macrophages.	**(Takizawa, *et al.* 1996)** Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. **FEBS Letters, 397: 269-272**
PS-containing liposomes, β_2GPI-treated liposomes, and apoptotic thymocytes (methylprednisolone) phagocytosed by mouse peritoneal macrophages.	β2GPI	To model PS-dependent phagocytosis, the influence of adding β_2GPI antibodies on the uptake of PS liposome's and β_2GPI liposomes was determined. The addition of β_2GPI antibodies increased uptake 2 fold. Addition of β_2GPI and β_2GPI antibodies to PC liposomes did not increase uptake. ^{51}Cr-labeled apoptotic thymocytes were treated with β_2GPI alone or β_2GPI and β_2GPI antibodies. Uptake was increased 2 fold with cells treated with β_2GPI and β_2GPI antibodies. Apoptotic thymocytes stained for FITC anti-β_2GPI.	**(Balasubramanian, *et al.* 1997)** Immune Clearance of Phosphatidylserine-expressing Cells by Phagocytes: The role of β_2-Glycoprotein I in macrophage recognition. **Journal of Biological Chemistry, 49: 31113-31117**
Human monocyte-derived macrophages phagocytosing apoptotic leukocytes	CD14	Transient expression cloning in COS cells to determine molecule-possessing epitope that binds to monoclonal CD14 Ab. Reactivity with anti-CD14 Ab was detected in one clone and sequencing revealed it to be human CD14. Anti-CD14 mAbs substantially inhibited binding and phagocytosis of	**(Devitt, *et al.* 1998)** Human CD14 mediates recognition and phagocytosis of apoptotic cells. **Nature, 392: 505-509**

		apoptotic lymphocytes by HMDM.
Apoptotic murine thymocytes (γ irradiation), human neutrophils (absence of serum) and Jurkat T lymphocytes (γ irradiation) phagocytosed with human monocyte derived macrophages (HMDM).	CR3 and CR4 macrophage receptors for iC3b	In the presence of serum, blocking experiments with mAbs specific to complement receptors wer performed. mAbs to CR3 and CR4 inhibited uptake, and therefore were confirmed to be important in recognition and uptake of apoptotic cells opsonised with iC3b.
Aged RBCs, and apoptotic HL60 (cycloheximide) and Jurkat (anti-Fas IgM) cells were phagocytosed by Bovine aortic endothelial cells (BAE), and chinese hamster ovary (CHO) cells either expressing LOX1 (BLOX-1-CHO) or wild-type (CHO-K1)	LOX1 Receptor (OxLDL receptor in endothelial cells)	BAE and BLOX-1-CHO cells but not CHO-K1 bound and phagocytosed aged RBCs and apoptot cells. OxLDL, acetyl LDL and other LOX-1 ligar inhibited binding of aged RBCs and phagocytosi of apoptotic cells. Recombinant soluble LOX-1 also inhibited binding and distinguished aged RI from native RBC and apoptotic cells from native cells. PS liposome's inhibited LOX-1-mediated interactions with aged/apoptotic cells, suggestin LOX-1 is involved in PS recognition.
A gene recognising PS is transfected into B and T lymphocytes and apoptotic Jurkat T cells added	Phosphatidylserine receptor	Transfected cells were able to recognise and eng apoptotic cells in a PS specific interaction. Flow cytometry with mAb identified receptor, which recognises PS, and showed protein expressed on surface of phagocytes. MAb inhibited phagocyto
Apoptotic thymocytes (methylprednisolone) and PS-liposomes phagocytosed by peritoneal mouse macrophages.	Gas 6 bridging molecule	In the presence of Gas 6, macrophages phagocytosed a massive amount of fluorescent labelled PS-liposome's, 3 fold higher than witho Gas 6. Gas 6 had no effect on uptake of PC, PE, PI liposome's. RT-PCR revealed mouse macrophages expressed the Gas 6 receptor, Axl. Binding of [^{125}I] Gas 6 to macrophages was inhibited by presence of excessive non-labelled G

		6, and presence of Axl extracellular domain (Ax ECD) inhibited binding of Gas 6 and subsequen phagocytosis of PS-liposome's. Gas 6 known to interact with Axl *via* its c-terminal domain, therefore suggests Gas 6 binds to macrophages v its c-terminal domain. Gla domain (N-terminal deficient Gas 6 was unable to bind PS, and resul in no enhancement of uptake of PS-liposome's. Gas 6 also enhanced the uptake of ^{51}Cr labelled apoptotic thymocytes, 2 fold higher than withou Gas 6.
Apoptotic Jurkat cells and erythrocytes coated with MBL and C1q tails phagocytosed by HMDMs	Calreticulin (CRT) binding to C1q and MBL on apoptotic cell via signalling through association with CD91 on phagocyte cell surface	Antibodies to CRT blocked uptake of erythrocyt coated with MBL or C1q tails. MBL was able t inhibit C1q binding and vice versa. CRT and CI were shown to co localise on macrophage surfac Abs to CD91, erythrocytes with C1q tails, ligan for CRT and CD91 all individually were able to block uptake.
Apoptotic mouse thymocytes (dexamethasone) phagocytosed by primary macrophages isolated from mice possessing a cytoplasmic truncation of MER (mer^{kd})	Myeloid Epithelial Reproductive (MER) receptor tyrosine kinase family	Macrophages isolated from wild type and mer^{kd} mice showed negligible phagocytosis of untreate thymocytes. Wild-type macrophages were capal of phagocytosing apoptotic thymocytes, but mer macrophages were markedly deficient (~90%). Wild type and mer^{kd} macrophages were capable binding to apoptotic cells but mer^{kd} macrophage were deficient in phagocytosis. Mer^{kd} macropha were capable of phagocytosing particles (e.g. lat beads) other than apoptotic cells. Therefore evid that removal of apoptotic cells could be mediate by cytoplasmic signalling domain of MER.

Flow chamber system to analyse human leukocyte (PMNs or Jurkat T cells) binding to macrophage monolayers (human monocyte derived macrophages or THP1) under conditions of flow.	CD31	At 20°C, viable and apoptotic cells bound to macrophages. However at 37°C apoptotic cells remained attached but viable cells detached. To determine which surface molecules were involve in tethering, macrophages were screened at 20°C identify specific proteins by differential extractio with detergents. A single major polypeptide was identified by electophoretic mobility as CD31. Further experiments using CD31 coated cover sli confirmed the above attachment/detachment.
Histologically normal kidneys were collected from human embryos and fetuses (8-28 weeks), and routinely processed for immunohistochemical analysis.	CD68	Immunohistochemical methods using CD68 mA detected macrophages (CD68+). Apoptotic cells were detected by TUNEL. Double staining with CD68 and TUNEL revealed CD68+ macrophage were capable of phagocytosing 37-75% of apopto cells in the neogenous zone and the number of apoptotic cells phagocytosed increased in the 12t week.
Mouse peritoneal macrophages phagocytosing mouse apoptotic thymocytes (dexamethasone treatment)	Milk fat globule-EGF-factor 8 (MFG-E8) as a bridging molecule between phospholipids on apoptotic cells and $\alpha5\beta3$ receptor on phagocytes.	Armenian hamsters were immunised with thioglycollate-elicited mouse peritoneal macrophages, and hybridomas produced. One m increased % of macrophages that engulfed apoptotic cells. Further analysis and mass spectrometry identified mouse MFG-E8. Possess no membrane spanning region therefore suggeste secreted protein. MFG-E8 bound to apoptotic bu not normal thymocytes. Bound to apoptotic cells by recognising aminophospholipids (PS, PE). Macrophages expressing high levels of $\alpha5\beta3$ showed increased phagocytosis in presence of MFG-E8.

Apoptotic Jurkat or PMNs (UV irradiation) phagocytosed by HMDMs	Calreticulin and CD91 as a common collectin receptor complex	SP-A and SP-D bound to apoptotic cells, and initiated phagocytosis through engagement of calreticulin and CD91 on macrophage. Abs agai CRT or CD91 inhibited SP-A and SP-D amplifie phagocytosis. Erythrocytes coated with SPA or SPD bound to and were phagocytosed by HMDM and Abs against CRT and CD91 inhibited this action. Competitive modulation of macrophage surface CRT (C1q tails) or CD91 (CRT or α_2 macroglobulin) decreased phagocytosis of SPA o SPD coated erythrocytes
Apoptotic human Burkitt's lymphoma cells (etoposide) phagocytosed by HMDMs	Protein S bridging molecule	Phagocytosis of apoptotic cells increased in the presence of serum. Identification of the factor in serum responsible for this was Protein S. Protein depleted serum did not result in phagocytosis-stimulated activity. Protein S bound to apoptotic cells and co localised with Annexin V (recognise PS) staining. PS-containing liposome's were use as competitive inhibitors and inhibited serum stimulated phagocytosis of apoptotic cells.
Jurkat T lymphocytes induced to undergo apoptosis with anti-Fas IgM and phagocytosed by endothelial cells	Annexin I as a bridging molecule between PS and PSR	3D chromatography-isotope-coded affinity tags (3D-ICAT) and mass spectrometry were used to identify and simultaneously quantify complex membrane proteins on apoptotic Jurkat compared normal Jurkat cells. Human annexin I was identified. ICC, cell fractionation and western bl analysis revealed annexin I was specifically recruited from the cytosol and exported to the cell surface as discrete patches at PS-rich sites, in a caspase and calcium dependent manner. Presenc of Annexin I on outer plasma membrane (PM) wa also shown to promote clustering of PSR and was

		required for efficient tethering and internalisatio apoptotic cells. Using single and double silencin of annexin and PSR, implicated PSR and annexi in the same engulfment pathway.
Mouse apoptotic thymocytes (dexamethasone) phagocytosed by peritoneal macrophages prepared from thioglycolate-treated mice, or mouse macrophage cell lines (BAM3 cell line established by transformation of peritoneal macrophages with SV40)	Src Homology 2 domain – bearing protein tyrosine phosphatase substrate-1 (SHPS-1)	BAM3 phagocytosed apoptotic thymocytes but n a lymphoma cell line (WR19L), suggesting WR1 cells lacked molecules that were necessary for phagocytosis by BAM3 cells. mAbs against BA cells were prepared and screened for inhibition o phagocytosis of apoptotic thymocytes by BAM3. Two mAbs were identified, and purification of t antigen (Ag) revealed it was SHPS-1. Anti-SHP inhibited phagocytosis of apoptotic thymocytes b BAM3. Apoptotic thymocytes expressed CD47 and incubation with anti-SHPS-1 inhibited bindi of anti-CD47, suggesting a binding of SHPS-1 to CD47. WR19L expressed little CD47 and did n bind anti-SHPS-1. However, when they were transformed with CD47 and induced to apoptose they were successfully phagocytosed by BAM3. Blocking exposed PS on apoptotic thymocytes inhibited phagocytosis. Anti-SHPS-1 blocked binding and engulfment of apoptotic cells. Therefore macrophages require CD47 and PS on apoptotic cells for phagocytosis.

ELISA plates coated with various phospholipids, and phospholipid containing liposomes were incubated with recombinant, FLAG-tagged MFG-E8.	MFG-8 bridging molecule binding to oxidised PS (PS-OX) on apoptotic cells	MFG-E8 bound preferentially to PS; by 2 fold m compared to PC with negligible binding to ot phospholipids (PI, PE, PG). MFG-E8 bou preferentially to PS-OX (ELISA and liposome's) compared to the non-oxidised form.

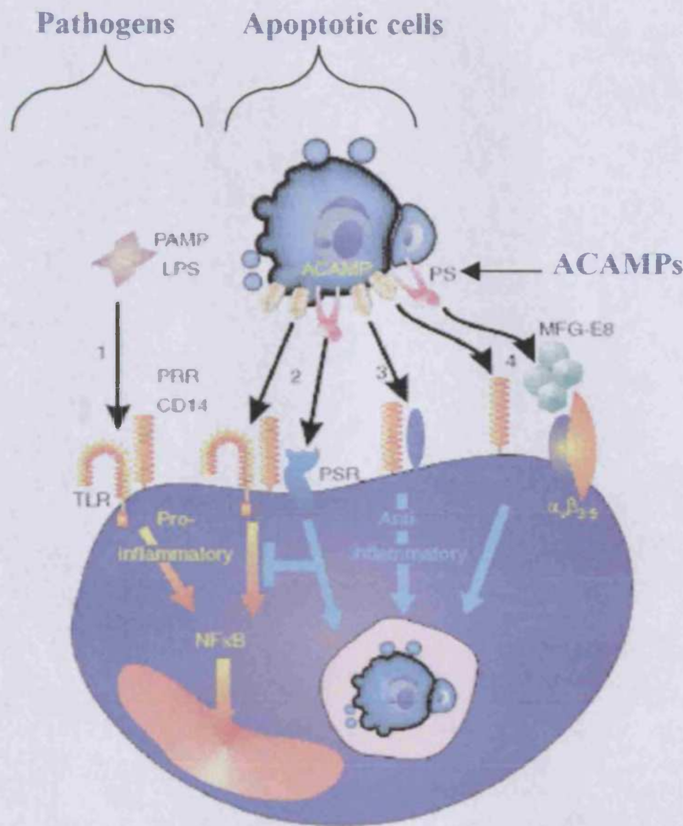


Figure 1.4 A Schematic Revealing the Mechanisms of Recognition by PRR's of PAMPs (pro-inflammatory) and ACAMPs (anti-inflammatory). 1: Proinflammatory response to a PAMP, LPS, in which GPI-anchored CD14 cooperates with toll like receptors (TLRs) to activate the NFκB and elicit proinflammatory responses. 2: engagement of CD14 by ACAMPs may engage a similar inflammatory signalling pathway, but here additional receptor ligand interactions, e.g. the PS/PSR interaction shown, dominantly suppress the proinflammatory response and may also engage anti-inflammatory signalling pathways. 3: Alternatively, in a ligand-dependent manner, CD14 may associate with different signal-transduction partners that activate anti-inflammatory rather than proinflammatory responses in the phagocyte. 4: CD14-ACAMP interactions may tether the apoptotic cell to the phagocyte and additional receptor/ligand interactions such as the illustrated vitronectin receptor ($\alpha_v\beta_{3/5}$)/MFG-E8/PS, activate anti-inflammatory responses in the macrophage.

Taken from (Gregory and Devitt 2004)

1.3 "Professional" and "Amateur" Phagocytes in the CNS

In tissues other than the CNS, immune responses involving both the innate (immediate ability) and adaptive (acquired ability) immune system (T cell, B cell, antibody) have pivotal roles in the efficient clearance of apoptotic cells (Fearon and Locksley 1996), (Gasque, *et al.* 2000). However, the brain is isolated from immunosurveillance with a limited ability of an adaptive immune response in the CNS to clear pathogens/apoptotic cells (Gasque, *et al.* 2000), (Schwartz, *et al.* 1999b). Therefore, it is vital that the brain has a mechanism to recognise and to initiate a local innate immune response against potential intruders expressing "eat me" signals to limit damage and initiate tissue repair (Barnum 2002), (Gasque, *et al.* 2000), (Elward and Gasque 2003). It has been proposed that a small number of cells within the brain act as phagocytes in clearing apoptotic cells from sites of injury. Microglia, the so called 'professional' phagocytes (approximately 10% of the total brain cell population), have been proposed to be the most important immune glial cells in the CNS (Magnus, *et al.* 2002, Perry, *et al.* 1993). However, astrocytes, the 'amateur' phagocytes (50-60% of the total brain cell population) could be the most strategic glial cells for the initiation and progression of CNS innate immune functions, due to their location at the blood brain barrier, a probable port of entry for most pathogens (Dong and Benveniste 2001). In addition, ependymal cells lining the ventricular system and choroid plexus in contact with the cerebrospinal fluid have been proposed to have a role in immune responses (Martino, *et al.* 2001). A subpopulation of neurones have also recently been characterised as important

innate immune cells in phagocyte activities during brain inflammation, restoring the immuno-privileged status of the brain while promoting tissue repair (Flugel, *et al.* 2000).

Discovering more about the receptor systems and downstream signalling processes involved in recognition of apoptotic cells in the CNS will enable further understanding of the role of professional and amateur brain phagocytes in maintaining homeostasis and preventing injury. Also, there is a significant clinical and medical need to understand the underlying repair mechanisms that could be implicated in the major CNS pathologies affecting an increasing number of people in an aging global population.

1.4 Apoptosis in Central Nervous System (CNS) Pathologies

Apoptosis features strongly in many diseases of modern man, and needs to be tightly regulated, as too little or too much cell death may lead to pathological symptoms and conditions, including developmental defects, stroke, neurodegeneration, acquired immunodeficiency syndrome (AIDS) (excessive apoptosis) or autoimmunity and cancer (insufficient apoptosis) (Barr and Tomei 1994, Carson and Ribeiro 1993).

In focusing on central nervous system pathologies for the purpose of this thesis, it is known that apoptosis of neurones occurs as a physiological process in the developing brain but is also a hallmark of neurodegenerative diseases -

Huntington's disease (HD), Parkinson's disease and Alzheimer's disease (AD) (Witting, *et al.* 2000, Yuan and Yankner 2000). Physiological apoptosis and pathological apoptosis share similar molecular mechanisms in the execution phase, but there are underlying differences between these conditions under which apoptosis is triggered. Whereas trophic-factor withdrawal is prominent in initiating apoptosis during development, there is little evidence to implicate trophic-factor withdrawal as a primary pathogenic cause in adult neurodegenerative disorders (Yuan and Yankner 2000). Rather, toxic insults resulting from biochemical or genetic accidents might trigger neurodegenerative diseases by co-opting apoptotic signalling pathways, for example through free-radical generation or caspase activation. The presence of neurotoxic proteins or aggregates symbolises the pathogenesis of neurodegenerative diseases, which results in neuronal death from accumulation of abnormal structures and activation of microglia (Yuan and Yankner 2000).

In Parkinson's disease, ultra structural studies of the dopaminergic neurones that constitute the pathological target of the disease revealed that they die by apoptosis due to toxicity of α-Synuclein protein (Cookson 2005). In HD, the Huntingtin protein has been revealed as the pathological protein itself, in which an underlying mutation is caused by a Cytosine, Adenine, and Guanine (CAG) triplet expansion in the first exon of the gene, resulting in a polyglutamine expansion of the protein. Normal individuals have between 10 and 35 repeats, whereas HD patients have from 37 to 121. The pathology of HD is characterised

by accumulation of neuronal nuclear inclusions throughout the brain (MacDonald 1993, Nijhawan, *et al.* 2000, Rangone, *et al.* 2004). AD is clinically associated with the development of amyloid plaques. It is believed that these plaques form from the improper folding and processing of amyloid β -precursor protein (APP) (Weiner and Selkoe 2002). Amyloid β is capable of inducing apoptosis by interaction with neuronal receptors, which initiate free radical production and result in the demise of the neurone (Yuan and Yankner 2000).

In addition to the need to remove apoptotic neurones produced during brain injury, apoptotic cell death of infiltrating auto reactive T cells, and their subsequent safe clearance, is paramount before secondary necrosis ensues. Insufficient clearance of auto reactive T cells can lead to pathology, an example of which is MS, where the proinflammatory response leads to damage of the myelin sheath and the underlying axon. Chan *et al* were the first to demonstrate *in vitro* the phagocytic clearance of apoptotic thymocytes and encephalitogenic T cells (isolated from a rat experimental autoimmune encephalomyelitis (EAE), a model of organ-specific autoimmunity in the CNS that serves as a paradigm for some aspects of the human disease multiple sclerosis) by the brain-specific phagocyte, the microglial cell (Chan, *et al.* 2001).

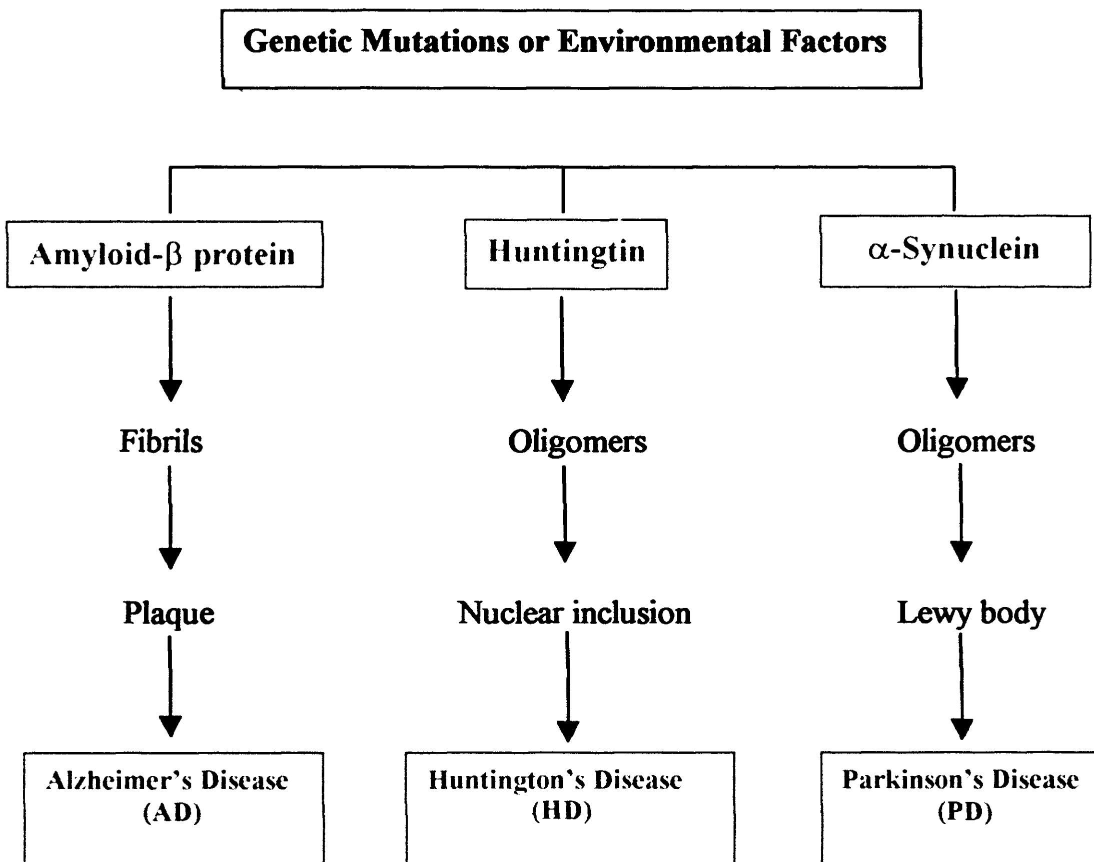


Figure 1.5 Neurotoxic Proteins and the Pathogenesis of Neurodegeneration. Normal proteins might become pathogenic when subjected to genetic mutations or environment factors that promote the formation of abnormal structures in specific neuronal subpopulations.

(Adapted from (Yuan and Yankner 2000))

1.5 Role of the Innate Immune Complement System in the Clearance of Apoptotic Cells

1.5.1 History

The innate immune system is an evolutionary ancient form of host defence found in most multicellular organisms. For over a century, the complement system has been known to play a central role in innate immune defence by the interaction of its 35 soluble and cell-surface proteins in the recognition, opsonisation and clearance or killing of invading pathogens and altered host cells (e.g. apoptotic or necrotic cells), while preserving normal 'self' cells (Sim and Tsiftsoglou 2004), (van Beek, *et al.* 2003). Having preceded the development of adaptive immunity, the complement system has maintained a high degree of phylogenic conservation among both invertebrates and mammals, underlying the critical role of complement in tissue homeostasis (Sunyer, *et al.* 1998). Early evidence portrayed only a role for complement in mediating cell lysis by necrosis, a pro-inflammatory process. However, over the past few years there has been growing evidence to confirm a now recognised role for complement in non-inflammatory clearance of apoptotic cells (Fishelson, *et al.* 2001).

1.5.2 Complement Activation Pathways

Three main pathways are involved in initiation of complement activation (Figure 1.6): -

(i) The Classical pathway (involving C1q, C1r, Cls, C4, C2 and C3 components),

(ii) The Lectin pathway (involving Mannose-Binding Lectin (MBL), and Mannose-binding lectin-Associated Serine Proteases (MASP)-1 and -2, C4, C2, C3).

(iii) The Alternative pathway (involving C3, factor B, factor D and properdin) (Sim and Tsiftsoglou 2004, van Beek, *et al.* 2003).

The binding of C1q to antibody-antigen complexes triggers the Classical pathway. MBL binds to microbial saccharides (mannose, N-acetylglucosamine), activates a MASP and initiates the Lectin pathway. The Alternative pathway is activated following the spontaneous hydrolysis of the thioester bond of C3 and undergoes amplification on activating surfaces. The three pathways merge at the level of C3, whereby C3b generated by the C3 convertases of all pathways converge in forming a C5 convertase, which splits C5 into C5a and C5b. Generation of C5b subsequently results in the assembly of the terminal complement complexes (C5b-6, C5b-7, C5b-8), leading to the formation of the Membrane-Attack Complex (C5b-9) (van Beek, *et al.* 2003). The terminal complement complexes C5b-7, C5b-8 and Membrane Attack Complex (MAC) are known to bind directly to hydrophobic regions of the target cell without the need of a receptor. The MAC forms a pore in the phospholipid bilayer, inducing rapid cell-death with a classic necrotic morphology (Sim and Tsiftsoglou 2004). During complement activation, activated complement components and fragments are produced which have been identified as having roles in inflammation (i.e. the anaphylatoxins C3a, C4a, C5a) and opsonisation (iC3b). However, whereas

C5a exerts pro-inflammatory effects, C3a is believed to be anti-inflammatory (Ember 1998).

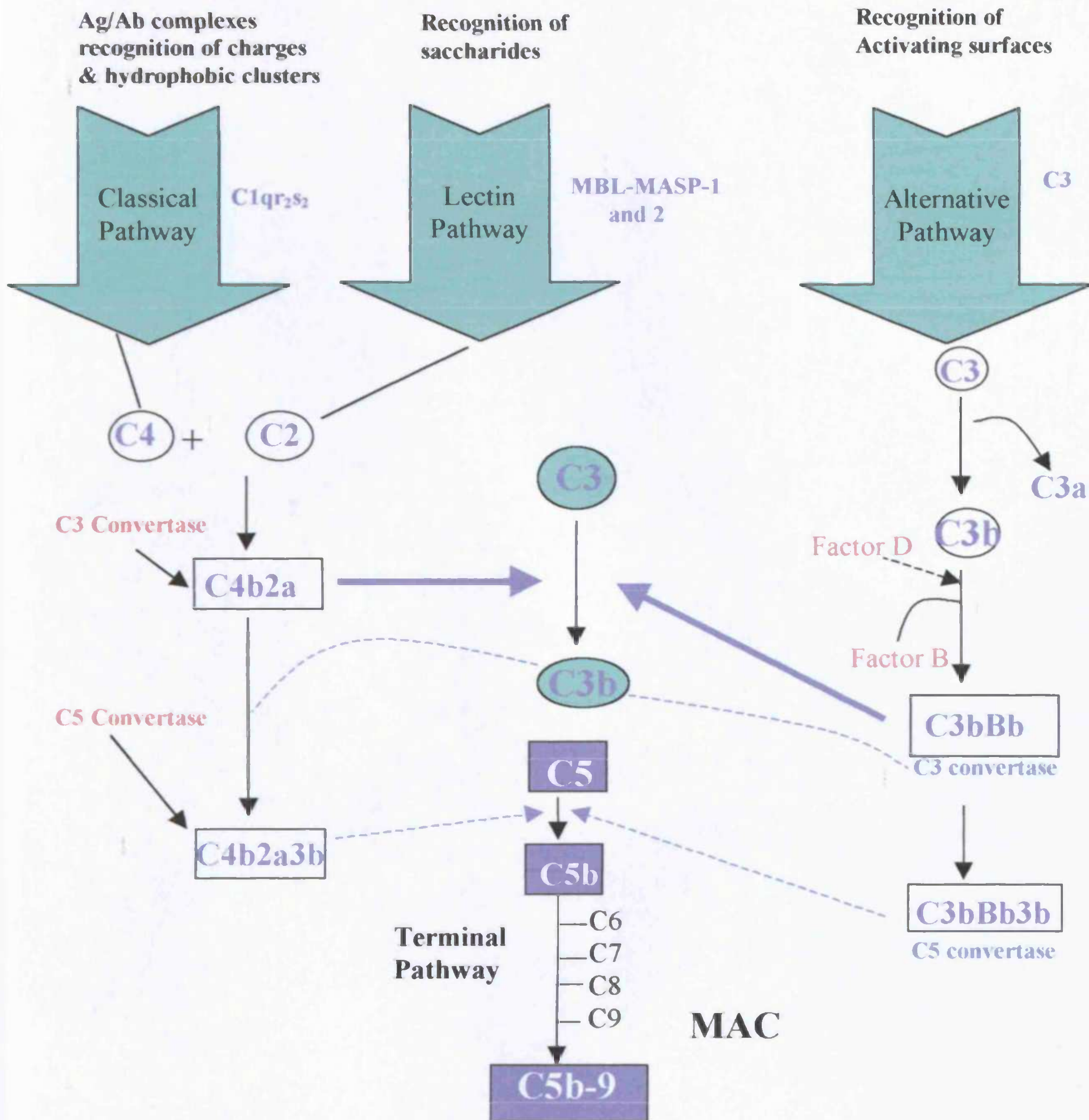


Figure 1.6 Schematic Representation of the Complement Cascade.

The Complement system can be activated *via* three pathways – the Classical, the Alternative and the Lectin pathway, leading to a common terminal pathway to form the MAC.

1.5.3 Complement Regulation

In order to protect 'self' cells against complement opsonisation and MAC mediated lysis, host cells express an array of regulatory proteins that inhibit either assembly of the C3 convertase or the formation of the MAC (Figure 1.7). C1 Inhibitor, C4 Binding Protein (C4bp), Factor H, Factor I, S-Protein and Clusterin are soluble inhibitors that are secreted and released in the fluid phase. Membrane Cofactor Protein (MCP, CD46), Decay Accelerating Factor (DAF, CD55), CD59 and Complement Receptor 1 (CR1, CD35) are membrane-associated complement inhibitors found on the surface of normal 'self' cells. These regulators target their inhibitory activity at three key steps in complement activation, namely C1 activation (C1 inhibitor), C3 activation (Factor H, C4b-Binding protein, Factor I, CR1, CD46 and CD55) or MAC formation (Clusterin, S Protein and CD59). CD55 binds to, and breaks up the classical and alternative C3/C5 convertase enzyme. CD46 acts as a cofactor for Factor I, allowing it to cleave C4b and C3b into biologically inactive fragments, C4d and iC3b, and in turn CD46 serves as an inhibitor of complement activation by inactivating the C3/C5 convertase enzyme. CD59 binds to C8 in the C5b-8 complex and blocks further incorporation of C9 to form the MAC (Morgan 1999), (Baldwin, *et al.* 2003), (van Beek, *et al.* 2003), (Spiller, *et al.* 2000) .

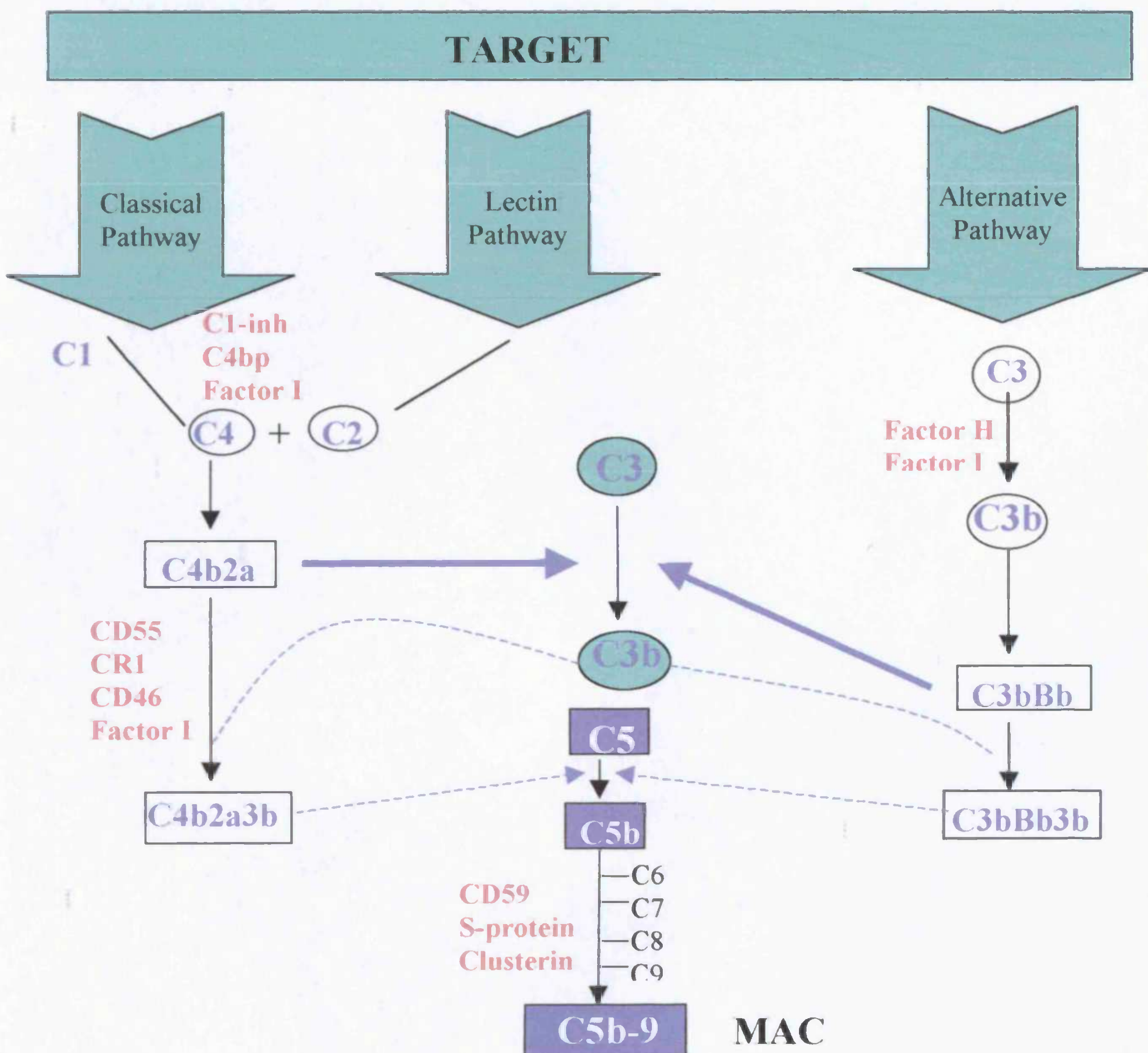


Figure 1.7 Regulation of the Complement Cascade.

Membrane associated (CD55, CD46, CD59 and CR1) or soluble (C1-inh, C4bp, Factor I, Factor H, S-protein and Clusterin) inhibitors regulate the different pathways.

1.5.4 Complement and Apoptosis

Several lines of evidence suggest that a number of proteins in the three pathways of the complement cascade are required for efficient removal of apoptotic cells (Table 1.2).

1.5.4.1 Role of the Alternative Pathway

First reported evidence concluded apoptotic cells activate the Alternative pathway of complement via initiating C3b deposition on their surface (Tsuji, *et al.* 1994), (Matsui, *et al.* 1994), (Takizawa, *et al.* 1996)).

1.5.4.2 Role of the Classical Pathway

Several studies have portrayed an association between the Classical pathway and the clearance of early apoptotic and secondary necrotic cells, with specific binding of complement proteins to apoptotic cells (Korb and Ahearn 1997), (Gershov, *et al.* 2000), (Navratil, *et al.* 2001), (Nauta, *et al.* 2002), (Ogden, *et al.* 2001), (Kim, *et al.* 2002), see Table 1.2). The Classical pathway was implicated for the first time in the clearance of apoptotic cells by Korb *et al*, who described C1q, the first component of the pathway, binding specifically and directly to surface blebs of apoptotic human keratinocytes *in vitro* (Korb and Ahearn 1997). These bleb structures are formed during apoptosis to reorganise and package intracellular contents, resulting in membrane, cytoplasmic and nuclear elements appearing at the surface. This observation suggested C1q may promote clearance of apoptotic cells and, hence, of exposed autoantigen, preventing further activation of the complement cascade. This hypothesis was confirmed *in vivo* using C1q-deficient mice (Botto, *et al.* 1998), (Mitchell, *et al.* 1999). These mice

developed high levels of antinuclear antibodies and proliferative glomerulonephritis, characterised by an increased number of uncleared apoptotic bodies, independent of C3 activation. However, studies by Nauta *et al* revealed that the binding of C1q to apoptotic cells can lead to the activation of the Classical pathway and deposition of C4 and C3 on the apoptotic cell surface (Nauta, *et al.* 2002). These contrasting findings indicate a dual role for C1q in the clearance of apoptotic cells: a direct role for C1q itself in signalling between apoptotic cell and phagocyte, and a second role as activator of the Classical pathway.

1.5.4.3 Role of the Classical and Alternative Pathway

Further reports by Mevorach *et al*, revealed that the use of a heterologous system of murine apoptotic thymocytes and human complement resulted in the activation of both the Alternative and Classical pathway (Mevorach, *et al.* 1998). This observation was confirmed by *in vitro* phagocytosis assays using human monocyte-derived macrophages to ingest murine thymocytes in the presence of complement-depleted sera for C1q, C2 or C4 (Classical pathway components), factor B (Alternative pathway component), or C3 (for both pathways) and indicated a role for both the Classical and Alternative pathways of complement in the phagocytosis of apoptotic cells (Mevorach 2000).

To address the relative contribution of Classical and Alternative pathway complement proteins to phagocytic clearance of apoptotic cells *in vivo*, a peritoneal model of apoptotic cell clearance was established in complement deficient mice (Taylor, *et al.* 2000). The mice were initially injected

intraperitoneally with thioglycollate, to induce recruitment of inflammatory macrophages and, four days later, the same animals were injected with syngeneic apoptotic thymocytes. C1q-deficient but not C4- and C3-deficient mice showed a defect in phagocytosis of apoptotic cells by resident peritoneal macrophages, indicating C1q has the predominant role in physiological conditions, in the absence of inflammation. The phagocytic uptake of apoptotic thymocytes by inflammatory macrophages was decreased in both C1q- and C4-deficient mice, compared to wild-type controls. However, the uptake was only partially inhibited in C4-deficient compared with C1q-deficient mice, suggesting a hierarchy of importance within the Classical pathway in the clearance of apoptotic cells (Taylor, *et al.* 2000).

1.5.4.4 Role of the Lectin Pathway

MBL, a major recognition molecule of the Lectin pathway is structurally related to C1q, both belonging to the collectin family, and both possessing a collagenous domain that interacts with receptors. MBL has been shown to bind to apoptotic cells *via* its lectin domain, but there is conflicting evidence whether this leads to complement activation. Two studies by Ogden *et al* and Nauta *et al* revealed the interaction does not lead to complement activation *via* the Lectin pathway (Ogden, *et al.* 2001), (Nauta, *et al.* 2003). However, recent studies showed binding of MBL, L-ficolin and H-ficolin to apoptotic cells activated complement via the Lectin pathway (Kuraya, *et al.* 2005). In contrast to C1q, which is known to bind to early apoptotic cells, MBL was revealed to bind only to late apoptotic cells to mediate phagocytic clearance (Nauta, *et al.* 2003).

Table 1.3 Evidence for a Role for Complement in Clearance of Apoptotic Cells

Apoptotic Model		**Complement Activation**	**References**
In vitro	***In vivo***	**on Exposure to Apoptotic Cells**	
HUVEC cells cultured in absence of FGF		Activation of the alternative pathway *via* C3b deposition on apoptotic HUVEC	**(Tsuji, *et al.* 1994)**, Activation of the Alternative pathway of human complement by apoptotic human umbilical vein endothelial cells. ***J Biochem (Tokyo)* 116: 794-800**
Anti-Fas (CH11) treated Jurkat T lymphocytes		Activation of the alternative pathway *via* C3b deposition on apoptotic Jurkat	**(Matsui, *et al.* 1994)**, Activation of the alternative pathway of complement by apoptotic Jurkat cells. ***FEBS Lett* 351: 419-22**
Cycloheximide treated Jurkat cells		Activation of the alternative pathway *via* C3b deposition. Cleaved C3b to iC3b induces CR3-dependent phagocytosis by macrophages (differentiated THP1)	**(Takizawa, *et al.* 1996)**, Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. ***FEBS Let t* 397: 269-72**
UVB induced apoptotic human keratinocytes		C1q, the first component of the classical complement pathway, binds directly and specifically to surface blebs of apoptotic keratinocytes	**(Korb and Ahearn 1997)**, C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. ***J Immunol* 158: 4525-8**
	C1q deficient (C1qa$^{-/-}$) Mice	Homozygous deficiency in C1q, the first component of the classical pathway resulted in impaired clearance of apoptotic cells	**(Botto 1998)**, C1q knock-out mice for the study of complement deficiency in autoimmune disease. ***Exp Clin Immunogenet* 15: 231-4**

γ irradiated murine thymocytes		Classical and alternative pathways of complement are activated by apoptotic cells leading to deposition of iC3b on the apoptotic cell surface. Exposure of PS resulted in opsonisation of apoptotic cells by iC3b, as preincubation with Annexin V partially inhibited complement binding.	**(Mevorach, *et al.* 1998)**, Complement-dependent clearance of apoptotic cells by human macrophages. ***J Exp Med*** **188: 2313-20**
	C1q/C2/factor B deficient mice (C1qa/H2-Bf/C2$^{-/-}$)	Elevated numbers of apoptotic bodies were present in undiseased kidneys of the C1qa/H2-fB/C2$^{-/-}$ mice but not in H2-fB/C2$^{-/-}$, therefore C3 activation not required for the clearance of apoptotic cells but strong evidence for a role for C1q	**(Mitchell, *et al.* 1999)**, Cutting edge: C1q protects against the development of glomerulonephritis independently of C3 activation. ***J Immunol*** **162: 5676-9**
UVB induced apoptotic Jurkat T lymphocytes	C1qa$^{-/-}$, C4$^{-/-}$ & C3$^{-/-}$ mice injected i.p with Jurkat T lymphocytes	A hierarchical role for classical pathway complement proteins *in vivo* in the clearance of apoptotic cells. i.e. C1>C4>C2	**(Taylor, *et al.* 2000)**, A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. ***J Exp Med*** **192: 359-66**
Anti-Fas (CH11) treated Jurkat T lymphocytes		C-Reactive Protein (CRP) binds to the surface of apoptotic cells resulting in amplification of classical pathway activation, reduced terminal complement component assembly and increased phagocytosis by macrophages	**(Gershov, *et al.* 2000)**, C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an anti-inflammatory innate immune response: implications for systemic autoimmunity. ***J Exp Med*** **192: 1353-64**
UVB induced apoptotic HUVEC		The globular heads of C1q bind directly to apoptotic blebs of HUVEC, indicating blebs may have the ability to directly activate the classical pathway	**(Navratil, *et al.* 2001)** The globular heads of C1q specifically recognize surface blebs of apoptotic vascular endothelial cells. ***J Immunol*** **166: 3231-9**
Apoptotic murine thymocytes induced by serum deprivation		Complement needs to be activated for uptake of apoptotic thymocytes by human macrophages (C1q, C3, Factor B)	**(Mevorach 2000)** Opsonization of apoptotic cells. Implications for uptake and autoimmunity. **Ann N Y Acad Sci 926:226-35**

Apoptotic HUVEC following hypoxia/ reoxygenation		Complement activation occurs through direct activation of the Classical Pathway (C1q, C3d) with observed PS exposure on apoptotic cells. This activation is prevented with use of a pan-caspase inhibitor.	**(Mold and Morris 2001)** Complement activation by apoptotic endothelial cells following hypoxia/reoxygenation. ***Immunology*** **102: 359-64**
	Nephrotoxic nephritis in $C1qa^{-/-}$, $H2\text{-}Bf/C2^{-/-}$, $C1qa/H2\text{-}Bf/C2^{-/-}$ mice	Severe disease developed in C1q-deficient but not in C2-deficient mice alone. i.e. an anti-inflammatory effect either with C1q or C4 proximal to C2 in the Classical Pathway of complement activation.	**(Robson, *et al.* 2001)** Accelerated nephrotoxic nephritis is exacerbated in C1q-deficient mice. ***J Immunol*** **166: 6820-8**
Apoptotic primary murine keratinocytes induced by UVB	$C1qa^{-/-}$ mice used to investigate the effects of C1q deficiency on UVR induced keratinocyte apoptosis *in vivo*	C1q staining was revealed on apoptotic keratinocytes *in vitro* and *in vivo* following UVR. However, no difference in the rate of clearance of sun burnt cells in C1q deficient mice compared with wild type control mice. i.e. C1q not critical in the physiologic clearance of apoptotic keratinocytes following UVR *in vivo*.	**(Pickering, *et al.* 2001)** Ultraviolet-radiation-induced keratinocyte apoptosis in C1q-deficient mice. ***J Invest Dermatol*** **117: 52-8**
UV irradiated Jurkat phagocytosed by human monocyte derived macrophages		C1q and MBL bind to and facilitate clearance of apoptotic cells by human macrophages *via* ligation to the calreticulin/CD91 complex on macrophages.	**(Ogden, *et al.* 2001)** C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. ***J Exp Med*** **194: 781-95**
Etoposide induced apoptotic Jurkat		C1q binds via its globular head domain to surface of apoptotic cells and blebs, leading to a C1q-dependent activation of the Classical Pathway via C4, C3 deposition	**(Nauta, *et al.* 2002)** Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. ***Eur J Immunol*** **32: 1726-36**
Staurosporine induced Apoptotic Jurkat		IgM antibodies are important for C1q binding and C3b/iC3b activation on the apoptotic cell surface, and these natural antibodies bind to lysophosphatidylcholine that is exposed on cells undergoing apoptosis	**(Kim, *et al.* 2002)** I-PLA (2) activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. ***J Exp Med*** **196: 655-65**

Serum-withdrawal and Anti-Fas (CH11) induced apoptotic Jurkat		Opsonisation of apoptotic cells by iC3b results in their clearance by immature dendritic cells in a non-inflammatory process	**(Verbovetski, *et al.* 2002)** Opsonisation of apoptotic cells by autologous iC3b facilitates clearance by immature dendritic cells, down-regulates DR and CD86, and up-regulates CC chemokine receptor 7. ***J Exp Med*** **196: 1553-61**
Gamma irradiated induced apoptotic Jurkat		MBL binds to late apoptotic cells, apoptotic blebs and necrotic cells by its lectin domain. Binding was partly inhibited by C1q suggesting that they may bind to the same or adjacent structures. Activation of the Lectin Pathway was not observed, but MBL facilitated an anti-inflammatory uptake of cells by macrophages	**(Nauta, *et al.* 2003)** Recognition and clearance of apoptotic cells: a role for complement and pentraxins. ***Trends Immunol*** **24: 148-54**
Anti-Fas induced Apoptotic Jurkat, and staurosporine/etoposide induced Raji cells		Early apoptotic Jurkat and Raji cells subjected to antibody-dependent complement activation are efficiently lysed by complement.	**(Attali, *et al.* 2004)** Increased sensitivity of early apoptotic cells to complement-mediated lysis. ***Eur J Immunol*** **34: 3236-45**
Dexamethasone induced apoptotic mouse thymocytes		The *in vitro* clearance of apoptotic cells by murine bone marrow derived macrophages (BMDM), using sera from complement deficient mice, revealed activation of the classical complement pathway by IgM antibodies and C3 deposition on the apoptotic cell surface.	**(Quartier, *et al.* 2005)** Predominant role of IgM-dependent activation of the Classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages in vitro. ***Eur J Immunol*** **35: 252-60**
Actinomycin D induced apoptotic HL60, U937 and Jurkat cells		MBL, L-ficolin, and H-ficolin bind to apoptotic cells and activate complement via the Lectin Pathway.	**(Kuraya, *et al.* 2005)** Specific binding of L-Ficolin and H-ficolin to apoptotic cells leads to complement activation. ***Immunobiology*** **209: 689-97**

HUVEC = Human umbilical vein endothelial cells UVR = Ultraviolet radiation FGF = Fibroblast growth factor
BMDM = bone marrow-derived macrophages

1.5.5 Apoptotic Cell Targets on to which Activated Complement Components are deposited

To date, little is known about the target structure on apoptotic cells onto which activated complement proteins are deposited. One of the early signals on apoptotic cells is the externalisation of phosphatidylserine (PS) from the inner to the outer surface of the plasma membrane (Savill 2000). It has been suggested that this exposure of PS is responsible for opsonisation with iC3b, since preincubation with Annexin V partially inhibited complement binding (Mevorach, *et al.* 1998). This hypothesis was also confirmed by Mold *et al*, who found a correlation between PS-positive and C3b positive cells in a population of apoptotic HUVEC cells incubated with complement (Mold and Morris 2001). Other reported evidence has suggested the binding of IgM or C-Reactive Protein (CRP). Activation of the calcium-independent phospholipase A2 ($iPLA_2$) by caspase-3 cleavage during apoptosis induces its hydrolysis of plasma membrane phospholipids and exposure of the phospholipid antigen, lysophosphatidylcholine, which is subsequently recognised by natural IgM antibodies to initiate C1q binding and engaging the Classical pathway (Kim, *et al.* 2003), (Quartier, *et al.* 2005). CRP opsonisation was shown to increase subsequent deposition of complement components C1q, factor B and iC3b, but reduced binding of the MAC, indicating that prior binding of CRP is a prerequisite for complement deposition (Gershov, *et al.* 2000). It has become evident that C1q can bind to a wide variety of agents, including CRP, serum

amyloid protein (SAP), and DNA, to mediate activation of the complement system and thereby leading to clearance of the target (Gewurz, *et al.* 1993).

1.5.6 Complement Receptors and Clearance of Apoptotic Cells

Several receptors have been suggested to have a role in the complement mediated clearance of apoptotic cells. A number of C1q receptors have been described, including CR1 and collagen C1q receptor (cC1qR, calreticulin, CRT), but their exact role as receptors for C1q binding and phagocytosis remains to be fully ascertained (Ghiran, *et al.* 2002). C1qRp was originally proposed to be a putative C1q-receptor mediating enhanced phagocytosis by monocytes (Nepomuceno, *et al.* 1997). However, it was recently shown to be identical to CD93 and strongly portrayed as being incapable of binding C1q directly (McGreal, *et al.* 2002, Norsworthy, *et al.* 2004). CR1 has been characterised as a multifunctional receptor both in its ligand specificity and in the complement regulation activities. It is capable of binding to C1q, other complement opsonins (C4b, C3b, iC3b) and MBL, resulting in phagocytosis of the target (Nicholson-Weller and Klickstein 1999). Several studies support a role for cell-surface calreticulin, in association with CD91, in the clearance of complement-opsonised apoptotic cells (Ogden, *et al.* 2001), (Vandivier, *et al.* 2002). The structurally similar collagen-like tails of the collectins, to which C1q, MBL and surfactant protein A and D (SP-A and SP-D) are members, can all bind to calreticulin (Ogden, *et al.* 2001), (Vandivier, *et al.* 2002). There is substantial evidence for a role for complement receptor type 3 (CR3, CD11b/CD18) and type 4 (CR4, CD11c/CD18) in phagocytosis of targets opsonised with C3b and iC3b fragments

(Takizawa, *et al.* 1996), (Mevorach, *et al.* 1998), (Ehlers 2000). Ligation of iC3b-opsonised cells to CR3 and CR4 has been confirmed to be an anti-inflammatory process (Aderem and Underhill 1999).

1.5.7 Complement and Necrosis

Professional phagocytes have to remove apoptotic cells rapidly and safely. However, if cells are missed during this scavenging they may collapse, decompose and undergo secondary necrosis forming sub cellular debris, which harm tissue homeostasis. The cellular debris, including chromatin, needs to be recognised and safely removed to prevent tissue damage and autoimmunity. Evidence to date has shown that C1q enhances the activity of DNAse I in degrading secondary necrotic cell-derived chromatin and only in the presence of C1q was efficient removal by phagocytes observed (Gaipl, *et al.* 2004). Further evidence has shown that necrotic cells expose phospholipids that may serve as ligands for receptors and opsonins (Roos, *et al.* 2004). Therefore, the mechanisms for phagocytic clearance of necrotic cells may be closely related to the mechanisms of apoptotic cell clearance. MBL has been shown to bind to necrotic cells as well as to late apoptotic cells (Nauta, *et al.* 2003). Reports have also revealed that, in contrast to non–inflammatory phagocytosis of apoptotic cells, necrotic cell phagocytosis undergoes a pro-inflammatory response, resulting in macrophage activation by release of heat shock proteins from necrotic cells (Gallucci, *et al.* 1999), (Li, *et al.* 2001). It has been suggested that heat-shock proteins can signal to phagocytes via CD91 and scavenger receptor A via a similar mechanism to Clq binding to calreticulin (CRT)/CD91 (Ogden, *et*

al. 2001), but it is still unknown how the phagocyte distinguishes between signals coming from heat-shock proteins (necrotic cells) and CRT bound to C1q (apoptotic cells) (Basu, *et al.* 2001),(Berwin, *et al.* 2003) in order to promote a pro- or anti-inflammatory response.

1.5.8 Complement Regulatory Proteins and Apoptosis

It is still unclear whether the complement membrane regulatory proteins have a role in controlling and signalling in the apoptotic process. Evidence to date has shown a reduction in cell-surface expression of these proteins during apoptosis (Jones and Morgan 1995), (Hara, *et al.* 1996), (Tsuji, *et al.* 1994). A reduction in CD59, an inhibitor of the Membrane-Attack Complex, and CD55, an inhibitor of the C3/C5 convertase, was shown on a subpopulation of polymorphonucleated cells aged in culture (Jones and Morgan 1995). CD46 and CD55 expression on human umbilical vein endothelial cells (HUVEC) was decreased upon apoptosis (Tsuji, *et al.* 1994) and apoptotic human lung adenocarcinoma cells revealed decreased expression of CD55, CD46 and CR1 (Hara, *et al.* 1996).

1.5.9 Complement Deficiency and Pathology

The role of the innate immune response, with complement being an essential component, is to detect the presence of infectious organisms and noxious substances, to induce clearance of invading pathogens, and in turn decide whether to initiate a strong pro-inflammatory response to engage acquired immunity or an anti-inflammatory response favouring tissue repair. The

significance of the role of the complement system in clearance of apoptotic cells *in vivo* may be portrayed in the pathogenesis of Systemic Lupus Erythematosus (SLE) (Sturfelt, *et al.* 2000, Walport, *et al.* 1998) . Early complement deficiencies, acting together with genetic and environmental susceptibility factors, increase susceptibility to a SLE-like disease as a result of diminished clearance of apoptotic cells (Walport, *et al.* 1998). Therefore, this portrays the vital role complement has to play in clearing apoptotic cells before pathology ensues.

1.6 Research Aims

As described in the introduction, complement is known to play a central role in CNS innate immune defence and has recently been implicated in the clearance of apoptotic cells. The complement system is tightly controlled by regulatory proteins – CD46 (MCP), CD59 and CD55 (DAF) at different sites along the pathway to protect human cells from complement mediated lysis. A reduction in cell surface expression of these membrane regulatory proteins has been revealed during apoptosis. However, it is still unclear whether they have a role in signalling in the apoptotic process, and whether the observed down regulation is key to complement activation and initiation of the apoptotic cascade.

The aim of this thesis was to shed further light on the role of the complement activators - C1q, C3b, and complement regulators - CD46, CD55, CD59, in selective recognition and safe removal of "altered self" (i.e. apoptotic versus necrotic cells) within the CNS. Further characterisation of the signal

transduction mechanisms involved in apoptotic clearance in the CNS was required. To achieve this aim, a number of *in vitro* apoptotic (γ irradiation, camptothecin, anti-Fas) and necrotic (H_2O_2) models were developed in a human Jurkat T lymphocyte cell line and complement-mediated clearance mechanisms were characterised. Moreover, a search was also conducted to identify phagocytic receptors on professional (THP1) and amateur brain phagocytes (astrocytes, oligodendrocytes and neurones).

Chapter 2

Materials and Methods

2.1 General Reagents

All buffers commonly used in this thesis are described below:

Phosphate Buffered Saline (PBS): 5.8M NaCl, 0.3M Na_2HPO_4, 0.1M $NaH_2PO4.2H_2O$ (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK)

Flow Cytometry Buffer

1) Phosphate buffered saline (PBS) with 1% w/v bovine serum albumin (BSA) (Sigma Aldrich, Poole, Dorset, UK)
2) RPMI 1640 medium without L-Glutamine (Invitrogen, Paisley, UK)

Buffers for Western Blot Analysis:

Lysis Buffer: 1% Triton X100 (Sigma), 10mM EDTA (Fisher), 1mM PMSF, 1μg/ml pepstatin A (Sigma), 1μg/ml Leupeptin in PBS (Sigma)

PBS Milk: PBS with 5% w/v dry skimmed milk powder and 0.05% w/v thimerosal (Sigma)

PBS Tween: PBS with 0.1% Tween 20 (Fisher), pH 7.4

Sodium Dodecyl Sulphate –Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving Gel buffer (Lower): 1.5M Tris-HCl (Fisher), 0.4% SDS (Sigma, pH 8.8 with HCl)

Stacking Gel buffer (Upper): 0.5M Tris-HCl, 0.4% SDS, pH6.8 with HCl

10% ammonium persulphate (APS) (Bio-Rad, Hemel Hempstead, UK)

Acrylamide/Bisacrylamide 37.5:1, 40% Solution (Sigma)

0.1 % N,N,N,'N'-tetramethylethylenediamine (TEMED) (Sigma)

In general, resolving gels containing 10% w/v acrylamide in combination with a stacking gel of 4% w/v acrylamide, were used. The appropriate percentage gels were prepared from the stock solutions according to the following recipe:

Quantities sufficient to prepare 2 SDS PAGE gels in Hoeffer system

Stacking Gel	*4%*
Upper buffer	1.2 ml
40% Acrylamide	0.506 ml
dH_2O	3.193 ml
10% APS	0.05 ml
TEMED	0.005 ml

Resolving Gel	*10%*
Lower buffer	3.75 ml
40% Acrylamide	3.75 ml
dH_2O	7.25 ml
10% APS	0.15 ml
TEMED	0.015 ml

Non-reducing loading buffer (Laemmli buffer): 0.1M Tris, 10% Glycerol (Fisher), 2% SDS, with addition of few crystals of Bromophenol Blue dye (Sigma), pH 6.8

Running buffer: 25mM Tris, 191mM Glycine, 1% SDS

Electroblotting transfer buffer: 25mM Tris, 191mM Glycine (Fisher), 20% (v/v) Methanol (Fisher)

2.2 Tissue Culture

2.2.1 **Cell Lines**

Obtained from ECACC, Porton Down, Salisbury, UK:

Human Jurkat E6 T lymphocyte cell line

Human THP1 monocyte cell line

Mouse EL4 T lymphocyte cell line

Human T98G astrocytoma cell line

Human Kelly CNS neuronal cell line

Human Paju peripheral nervous system (PNS) neuronal cell line (obtained from Leif C. Andersson, Department of Pathology, University of Helsinki, Finland)

Human HOG oligodendrocyte cell line (obtained from Dr G. Dawson, Department of Biochemistry, University of Chicago, USA).

All cell lines were cultured at 37°C/5% CO_2 in the appropriate media at a density of 1 x 10^6/ml in 75 cm^2 tissue culture flasks (Greiner Bio-One Ltd, Stonehouse, Gloucestershire)

2.2.2 Cell Growth Media

RPMI-1640 medium (for Jurkat, THP1, EL4, Kelly, Paju cells) or DMEM (for T98G, HOG cells) supplemented with 5% heat inactivated foetal bovine serum (FBS), penicillin/streptomycin (50μg/ml), L-glutamine (2mg/ml), sodium pyruvate (1mg/ml), and fungizone (2.5μg/ml) (Invitrogen).

Freezing Cells

Cells (taken during the log phase of growth) were removed from tissue culture flasks, and pelleted by centrifugation at 800 rpm for 3 minutes. Pellets from each 75cm^2 flask were resuspended in 1 ml of freezing medium (FBS/10% DMSO), and placed in cryovials (Greiner Bio-One Ltd, Stonehouse, Gloucestershire, UK). Cells were brought to –80°C at a rate of 1°C/min by using a Cryo 1°C freezing container (Nalgene) which used isopropanol to control the rate of freezing. After freezing was complete, cells were quickly placed in a liquid nitrogen storage tank for long-term storage.

Thawing Cells

Cells were routinely kept in liquid nitrogen for long-term storage. Once removed from liquid nitrogen, cells were rapidly thawed using warm medium appropriate to the cell line in question. Cells were then resuspended in 20ml of medium prior to centrifuging at 1000 g for 3 minutes. This step pelleted the cells and allowed any potentially toxic excess DMSO (used as a cryopreservant) to be removed.

The pellet was resuspended in 20 ml of the appropriate medium containing FBS and additives, and cells were placed in a 37°C incubator with 5% CO_2.

Counting Cells

All cells were counted using a haemocytometer. Cells (20µl) were pipetted into the counting chamber, which has a maximum displaceable volume of 10µl. Cells were counted under an inverted phase contrast microscope by counting within a 5 x 5 square grid corresponding to 0.1µl, which gives a cell number by 10^4/ml.

2.3 Reagents and Antibodies Used in Study

2.3.1 Apoptotic Induction Reagents

Camptothecin 5µg/ml (Sigma, stock dissolved in DMSO at 1mg/ml)

Anti-Fas mAb (80ng/ml) (Clone CH11, Upstate signalling solutions)

Necrotic Induction Reagents

Hydrogen peroxide (H_2O_2) 0.01M (Sigma, stock solution at 37%)

2.3.2 Primary Antibodies Used in this Study

Table 2.1. Monoclonal Antibodies Used in the Screening for Markers on Apoptotic Cells:

CD No.	Isotype	Concentration used in PBS/BSA	Obtained from
CD2 MEM65	IgG1	1/100	V. Horejsi
CD3 MEM57	IgG2a	1/100	V. Horejsi
CD4 MEM115	IgG2a	1/100	V. Horejsi
CD5 MEM32	IgG1	1/100	V. Horejsi
CD6 MEM98	IgG1	1/100	V. Horejsi
CD8 MEM87	IgG1	1/100	V. Horejsi
CD9 MEM62	IgG1	1/100	V. Horejsi
CD10 MEM78	IgG1	1/100	V. Horejsi
CD11a MEM95	IgG1	1/100	V. Horejsi
CD13 BF10	Not determined	1/100	Neomarkers
CD29 MEM101a	IgG1	1/100	V. Horejsi
CD31 Mem05	IgG1	1/100	V. Horejsi
CD34 BIRMAk3	IgG1	1/50	IBGRL
CD35 4D2	Not determined	1/100	T cell Sci
CD41 5B12	IgG1	1/50	DAKO
CD43 Mem 59	IgG1	1/100	BD Pharmingen
CD44 MEM85	IgG1	1/100	V. Horejsi
CD45RA	IgG2b	1/100	V. Horejsi
CD46 Mem258	IgG1	1/100	J.P. Atkinson
CD46 GB24	IgG1	1/100	J.P. Atkinson
CD46 Tra2.10	IgG1	1/100	J.P. Atkinson
CD46 IIC5	IgG1	1/100	E. Rubenstein
CD46 J4.48	IgG1	1/100	Serotec
CD47 Bric 126	IgG2b	1/100	IBGRL
CD53 MEM53	IgG1	1/100	V. Horejsi
CD54 MEM111	IgG2a	1/100	V. Horejsi
CD55 Bric 216	IgG1	1/50	IBRGL
CD56 Mem 188	IgG2b	1/100	V. Horejsi
CD57 Leu7	IgM	1/100	BD Pharmingen
CD59 Bric 229	IgG2a	1/50	IBGRL
CD81 Mem38	Not determined	1/100	V. Horejsi
CD90 Thy-1 K117	IgG1	1/100	ATCC
CD94 HP-3D9	IgG1	1/100	BD Pharmingen
CD95 Fas Lob 3/11	Not determined	1/100	M. Glennie
CD100 A8	IgG1	1/200	BD Pharmingen
CD230 (Prion) Pri08	Not determined	1/200	CEA
HLA I w6/32	IgG2a	1/20	ATCC

Table 2.2. Anti-Complement Antibodies Used in this Study

Anti-complement antibodies	**Concentration used in PBS/BSA**	**Obtained from**
Rb α hu C1q	1/200 ICC, F 1/1000 WB	Dade Behring
Rb α C2	1/200 ICC	Nordic
Rb α hu C3 L440	1/200 F	In house
Murine α hu iC3b	1/1000 F	Quidel
Mo α hu C3b (C3/30)	1/20 F	Nordic
Rb α hu C4	1/200 F	Dade Behring
Mo α hu C5b9 (B7)	1/50 F	Binding Site
Rb α hu CD46	1/200 ICC, 1/1000 WB	In house
Rb α hu CD55	1/200 ICC, 1/1000 WB	In house
Rb α hu CD59	1/200 ICC, 1/1000 WB	In house
Mo α hu CD59 B229	1/200 WB	IBGRL

F – Flow Cytometry, ICC – Immunocytochemistry, WB – Western Blot

Table 2.3. Antibodies Used in the Screening for Phagocytic Receptors

Antibody	**Isotype**	**Concentration used in PBS/BSA**	**Obtained from**
Mo α CR3 (CD11b) Mem 170	IgG1	1/200 F	V. Horejsi
Mo α CR4 (CD11c) 3.9	IgG1	1/200 F	N. Hogg
Rb α PSR		1/500 F	Sigma
Mo α CD91	IgG1	1/200 F	BD Pharmingen
Rb α CRT		1/200 F	Chemicon

F = Flow cytometry

Table 2.4. Secondary Antibody Conjugates Used in this Study

Antibody	**Working dilution**	***Supplied conc.***	**Source**
rPE conjugated goat anti mouse IgG	1/200 F	1mg/ml	DAKO
rPE conjugated goat anti rabbit IgG	1/200 F	0.3 mg/ml	SIGMA
ALEXA GREEN (488nm) conjugated goat anti mouse	1/1000 ICC	2 mg/ml	Invitrogen
ALEXA RED (594nm) conjugated goat anti mouse	1/1000 ICC	2 mg/ml	Invitrogen
ALEXA GREEN (488nm) conjugated goat anti rabbit	1/1000 ICC	2 mg/ml	Invitrogen
ALEXA RED (594nm) conjugated goat anti rabbit	1/1000 ICC	2 mg/ml	Invitrogen
HRPO conjugated goat anti-mouse IgG	1/1000 WB	1.5 mg/ml	Biorad
HRPO conjugated goat anti-rabbit IgG	1/1000 WB	1 mg/ml	Biorad

F – Flow Cytometry, ICC – Immunocytochemistry, WB – Western Blot

Table 2.5. Antibodies and Reagents Used to Monitor Apoptosis and Necrosis

Antibody	**Working dilution**	***Supplied condition***	**Source**
Annexin V FITC	1/50 F, ICC	25 tests, conc. not given	Biosource
Propidium Iodide	1/200 F, ICC	1mg/ml	Sigma
Anti Cleaved Caspase 3 (Asp 175) rabbit pAb	1/100 ICC	10 mM	Cell Signaling
Anti PARP p85 fragment rabbit pAb	1/100 ICC, 1/1000 WB	50 μl, conc. not given	Promega
TMRM (Tetramethylrhodamine)	1/100 F	25 mg	Molecular Probes
DAPi	1/1000 ICC	10 mg/ml	Sigma
General Caspase inhibitor (Z-VAD-FMK)	1/500	10 mM	BD Pharmingen

2.4 Immunocytochemistry (ICC)

2.4.1 Preparation of Cell 'Cytospins' for Immunocytochemistry

Cells were isolated from culture, washed in RPMI-1640 (without FBS) and resuspended at a density of $5x10^5$ cells/ml. A cell suspension (50µl) was 'cytospun' onto poly-L-lysine coated glass slides (Surgipath Europe Ltd, Peterborough, UK) at 200 rpm for 5 minutes using a Cytofuge (Thermo Shandon, Cheshire, UK). Slides were allowed to air dry for 10 minutes prior to use or storage at –20°C.

2.4.2 Fluorescent Immunocytochemistry

Cytospins were fixed in acetone for 30 seconds, dried for 10 minutes and re-hydrated in PBS for 5 minutes. Non-specific protein binding sites were blocked by incubating slides in PBS/BSA (1% w/v BSA) for 30 minutes at room temperature. Cytospins were incubated with primary antibody at appropriate concentrations in RPMI (no FBS) for 1 hour at room temperature or overnight at 4°C in humid chambers (See Tables 2.1, 2.2, 2.4). Slides were washed 3 times in PBS prior to a further blocking step in PBS/BSA. Secondary Fluorescein Isothiocyanate (FITC)/Alexa Green or Tetramethylrhodamine Isothiocyanate (TRITC)/Alexa Red conjugated antibodies with specificity for the primary antibody, were added at an appropriate concentration (Table 2.3) with DAPi nuclear stain (100ng/ml final concentration) in RPMI (no FBS) for 1 hour at room temperature. Slides were washed 3 times in PBS and mounted with

'Vectashield' (Vector Laboratories, Peterborough, UK) which prolonged fluorochrome half-life under illumination.

2.4.3 Microscopy

Fluorescent microscopy was performed using an upright Leica DM LB light microscope (Leica UK, Milton Keynes, UK). Fluorochrome excitation was effected by a mercury lamp and specific emission spectra were separated using filters for Fluorescein Isothiocyanate (FITC)/Alexa Green (L4 filter) and Rhodamine (TRITC)/Alexa Red (N2.1 filter). Images were observed using a x100-0.50 oil objective lense, captured with a digital camera, and all images were analysed using the Openlab v3.09 software package (Openlab/Improvision UK Ltd, Coventry, UK) (Gasque 1998)

2.5 Flow Cytometry

2.5.1 Preparation of Cells for Flow Cytometry

Cells growing in suspension were harvested and centrifuged at 800 rpm for 3 minutes to pellet cells and separate any cell debris. Cells were washed 3 times in flow cytometry buffer by consecutively pelleting and resuspending cells in 20ml of buffer. Cells were commonly resuspended to a final cell density of 5×10^5 cells/ml in flow cytometry buffer.

2.5.2 Single Colour Flow Cytometry

Cells were prepared from culture as described above, and 10^5 cells/well were aliquoted into round-bottomed 96 well plates. The cells were resuspended in 100µl flow cytometry buffer containing an appropriate concentration of primary antibody (Table 2.1) and incubated at 4°C for 1 hour with 15minute intermittent resuspension of cells by shaking. Cells were then washed 3 times in 100µl flow cytometry buffer prior to addition of a secondary FITC or Red Phycoerythrin (rPE) conjugated antibody, diluted in flow cytometry buffer, with specificity for the primary antibody (Table 2.3). Plates were again incubated at 4°C for 1 hour with intermittent resuspension, and cells were finally washed 3 times in flow cytometry buffer and resuspended in 200µl of flow cytometry buffer prior to analysis on a FACScan flow cytometer (Becton Dickinson UK Ltd, Oxford, UK).

2.5.3 Two-Colour Flow Cytometry

For two-colour analysis, cells were stained and washed as described above. To assess specific expression of molecules by apoptotic cells, the cells were first incubated with monoclonal antibodies, followed by Annexin V FITC (1/50) and phycoerythrin (PE) conjugated $F(ab)_2$ goat antibody to mouse IgG.

2.6 Western Blot Analysis

Western blotting was used to detect the presence of specific proteins with antibodies.

2.6.1 Preparation of Cell Lysates

Cells (10^7) were pelleted by centrifugation at 1000 rpm for 3 minutes and lysed with 1ml lysis buffer. The cells were then vigorously vortexed and incubated for 30 minutes at room temperature. Lysates were transferred to a 1.5 ml microfuge tube and centrifuged at room temperature for 15 minutes at 13,000 rpm. The supernatant containing total proteins was isolated. Samples were stored at –20°C or diluted 1:1 with Laemmli buffer for immediate use.

2.6.2 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins can be separated by SDS-PAGE according to their size. All SDS-PAGE were performed using the Hoefer Mighty Small apparatus (Hoefer Scientific Instruments, Newcastle-under-Lyme, Staffordshire, UK) as per manufacturer's instructions (Hoefer Scientific Instruments). Resolving gels were poured to a suitable acrylamide concentration for the protein under investigation (50-110 KDa proteins were analysed on 10% gels), and overlaid with 70% methanol until set. The methanol was washed away with distilled water before pouring the 4% stacking gel. Combs were inserted to create ten sample-loading wells of 20µl capacity each.

After complete polymerisation the gels were connected to the Hoefer electrophoresis unit as per manufacturer's instructions, and electophoresis (running) buffer was added. Protein samples were mixed 1:1 with non-reducing Laemmli buffer. 20μl of sample was loaded per lane and prestained molecular weight standards of known size were included in first lane of each gel. (See Blue prestained markers, Invitrogen). Gels were run under a constant current of 30milliamps (mA) per gel using a 'PowerPac 300' power source (Bio-Rad) until the dye front had reached the bottom of the gel (approx 30 min). Gels were kept cool by a controlled flow of cold tap water through the system.

2.6.3 Western Blotting

Following separation of proteins by SDS-PAGE the gel was removed from the electrophoresis apparatus and proteins were transferred from the gel to a nitrocellulose (0.45 μm pore size, Hybond-C Extra, Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, UK) membrane by electroblotting. Electroblotting was performed by placing the gel onto a nitrocellulose membrane, which was then sandwiched between two sheets of filter paper followed by two sponges. The 'sandwich' was then placed in a plastic frame, and immersed into an electroblotting tank containing transfer buffer, with the nitrocellulose facing the anode. Proteins were blotted at a constant voltage of 60 Volts for 2 hours.

2.6.4 Protein Detection

The nitrocellulose blot was removed from the sandwich post electroblotting, placed in a 50 ml tube, and 20 ml of PBS milk added for 30 minutes with gentle rotation to block the proteins. The blot was incubated with primary antibody to the protein of interest, diluted in PBS milk (10 ml) for 1 hour at room temperature (RT), or overnight at 4°C with gentle rotation. The blot was washed 3 times for 10 minutes with PBS Tween (10 ml), proteins blocked with PBS milk for 30 minutes, followed by incubation with the appropriate secondary antibody diluted in PBS milk for 2 hours at RT. The blot was then washed for a further 3 times prior to protein analysis.

2.6.5 Chemiluminescent Revelation of Western Blot (ECL)

Washed blots were placed in a clear plastic bag, Super Signal Reagent (1ml, Pierce, Chester, UK) added, and the bag sealed. The blot was exposed to X-OMAT UV film (Kodak, Harrow, UK) for a period of time (range from 30 seconds to 2 minutes), which yielded maximum resolution of bands. The exposed blot was developed using a Compact X2 developer (X-Ograph Ltd, Malmesbury, Wiltshire).

2.7 RT-PCR Analysis

2.7.1 Total RNA Extraction

Total ribonucleic acid (RNA) was extracted from 5 x 10^6 THP1 cells (human monocyte cell line). The cells were harvested from culture and washed 3 times with NaCl at 1200 rpm for 3 minutes to remove FBS. RNA was extracted from the cells using 1ml of Ultraspec RNA isolation reagent (Biotecx Laboratories, Houston, Texas, USA). The resulting mixture was transferred to a microtube and incubated on ice for 5 minutes to allow dissociation of nucleoprotein complexes. Chloroform (200µl) was added to the cells, vortexed for 15 seconds and incubated on ice for a further 5 minutes. The tube was then centrifuged at 13,000 rpm at 4°C for 15 minutes using a bench top centrifuge. The upper aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was added. The tube was vortexed and incubated on ice for 10 minutes to precipitate the RNA. The precipitate was pelleted by centrifugation at 12,000 rpm for 15 minutes, washed with 1ml of cold 75% (w/v) ethanol, left to air dry then dissolved in 100µl sterile water.

2.7.2 Reverse Transcription for cDNA Synthesis

Reverse Transcription (RT) produces a deoxyribonucleic acid (DNA) strand from an RNA template in a reverse transcriptase catalysed reaction. The RT was performed using 4µg of total RNA in the presence of 10mM dTT (1.5µl, Promega), 10mM dNTPs (3µl, Bioline Ltd, London, UK), 60/U RNasin (1.5µl,

Promega), 250 pmol random hexamer primers (pdN6, 2.5µl, Amersham Biosciences), in 5 x RT buffer (6µl, Invitrogen) and dH_20 (6µl). The mix was incubated for 5 minutes at 65°C, followed by 5 minutes on ice. 400 U Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (2µl, Invitrogen) was added and the tube centrifuged at 13,000 rpm for 1 minute. The RT reaction was then performed at 37°C for 2 hours. At the end of the reaction the cDNA produced was incubated at 95°C for 2 minutes to denature any secondary structures.

2.7.3 Polymerase Chain Reaction (PCR)

2.7.3.1 Theory

The PCR technique was devised by Kary Mullis in the mid-1980s and has revolutionised molecular genetics by making possible a whole new approach to the study and analysis of genes (Mullis, et al. 1986). It can produce enormous numbers of copies of a specified DNA sequence without resorting to cloning. DNA polymerase, 2 primers, and a mixture of all 4 deoxynucleotide precursors (dNTPs) are added to a tube of DNA. DNA polymerase uses a single stranded DNA as a template for the synthesis of a complementary new strand. Simply heating double-stranded DNA to a temperature near boiling can produce these single-stranded DNA templates. A primer can direct DNA polymerase to synthesise a specific region of DNA, as the primer flanks the region of DNA that needs to be amplified. Both DNA strands can act as templates provided an oligonucleotide primer is annealed to each strand. The reaction mixture is again

heated to separate the original and newly synthesised strands, which are available for further cycles. At the end of 'n' cycles, 2^n double-stranded DNA molecules are produced which are copies of the DNA sequence between the primers. A DNA polymerase from the bacterium Thermus aquaticus called Taq polymerase is used. It has a temperature optimum of 72°C and is stable at 94°C. It can be added just once at the start of a reaction and will remain active through a complete set of amplification cycles.

2.7.3.2 Method

The PCR mixture contained 3µl of cDNA, 2mM dNTPs (5µl), 1x PCR buffer (5µl), upper primer (5µl) and lower primer (5µl) specific to the sequence to be amplified, dH_20 (21.5µl), and Taq polymerase (0.5µl Promega). β actin primers were used to confirm adequate mRNA isolation and cDNA synthesis. The samples were incubated in a Touchgene PCR machine (Techne, Duxford, Cambridge). The PCR cycling parameters consisted of 94°C for 4 minutes (denaturation of DNA), 58°C for 40 seconds (annealing primers), 72°C for 50 seconds (synthesis of DNA) for a total of 30 cycles, followed by a final extension of the DNA for 72°C for 10 minutes.

2.7.4 Agarose Gel Electrophoresis of DNA

RT-PCR products were resolved on a 0.7 % (w/v) agarose gel in 1x TAE buffer. Agarose (Invitrogen) was dissolved in TAE buffer by boiling in a microwave oven. After cooling, Ethidium Bromide (EtBr, 2µl, Sigma) was added to a final

concentration of 100ng/ml gel mixture, mixed thoroughly and poured into a gel-casting tray. A gel comb was placed in an appropriate position to form sample wells and the agarose was left to set at 4°C. The comb was removed, the gel transferred to a gel tank, and TAE buffer added at a suitable amount to cover gel. RT-PCR products (20µl) were mixed with 2µl DNA loading buffer and loaded into gel wells. A DNA Ladder (1Kb Smart ladder, Eurogentec, Hampshire, UK) was used as a marker of DNA size. Samples were run at 100 V until complete separation of the DNA fragments, and the gel was analysed in a UV box.

Chapter 3

Characterisation of the Different Apoptotic and Necrotic Models

3.1 Introduction

Apoptosis stands in sharp contrast to necrosis. Apoptosis being a non-phlogistic programmed form of cell death proceeding in an orderly and controlled manner, and necrosis a pro-inflammatory, passive and pathological form of cell dying (Kerr, *et al.* 1972, Kuan and Passaro 1998). An important stage in the apoptotic process, distinct from necrosis, involves specific recognition and safe phagocytosis of apoptotic cells before secondary necrosis and lysis ensues, but the exact mechanisms as to how this occurs is still unclear (Savill and Fadok 2000). Therefore, in order to obtain further information on the cellular and molecular mechanisms that mediate the safe clearance of apoptotic cells compared to the mechanisms resulting in necrosis, the development of *in vitro* models of apoptosis and necrosis were paramount.

3.1.1 Model Systems

3.1.1.1 Apoptotic Model Systems

The methods adopted to specifically induce apoptosis (γ irradiation, camptothecin, anti-Fas) have already been well described in the literature (Attali, *et al.* 2004, Boesen-de Cock, *et al.* 1999, Gershov, *et al.* 2000, Johnson, *et al.* 1997, Matsui, *et al.* 1994, Mevorach, *et al.* 1998, Nauta, *et al.* 2003, Verbovetski, *et al.* 2002), and the human Jurkat T lymphocyte cell line is a well-established cellular model for use in apoptotic studies (Attali, *et al.* 2004, Gershov, *et al.*

2000, Kim, *et al.* 2002, Matsui, *et al.* 1994, Nauta, *et al.* 2003, Nauta, *et al.* 2002, Ogden, *et al.* 2001, Takizawa, *et al.* 1996, Taylor, *et al.* 2000, Verbovetski, *et al.* 2002).

3.1.1.1.1 γ Irradiation

γ Irradiation is able to trigger the apoptotic process in cells by delivering DNA damage (double strand breaks) directly and initiating p53 directed *de novo* synthesis of the Bcl-2 antagonist Bax, leading to activation of the mitochondria apoptosis pathway (Hengartner 2000) (Figure 3.1). DNA fragmentation, a distinct morphological change in the apoptotic process will be evident (Boesen-de Cock, *et al.* 1999).

3.1.1.1.2 Camptothecin

Camptothecin, a quinoline-based alkaloid found in the barks of Chinese camptotheca trees, is a specific DNA topoisomerase I inhibitor binding reversibly to the enzyme-DNA complex causing complete cessation of DNA synthesis (Johnson, *et al.* 1997). Camptothecin and related compounds are highly cytotoxic, and provide potent therapeutics against cancer.

3.1.1.1.3 Anti-Fas

Fas and its natural ligand (Fas L) play an important role in the apoptotic elimination of cells. The anti-Fas antibody recognises the cell surface antigen Fas (type 1 transmembrane protein of TNF receptor family). Upon Fas binding

to the receptor, the receptor trimerises and induces apoptosis via a caspase dependent pathway (Bennett, *et al.* 1998).

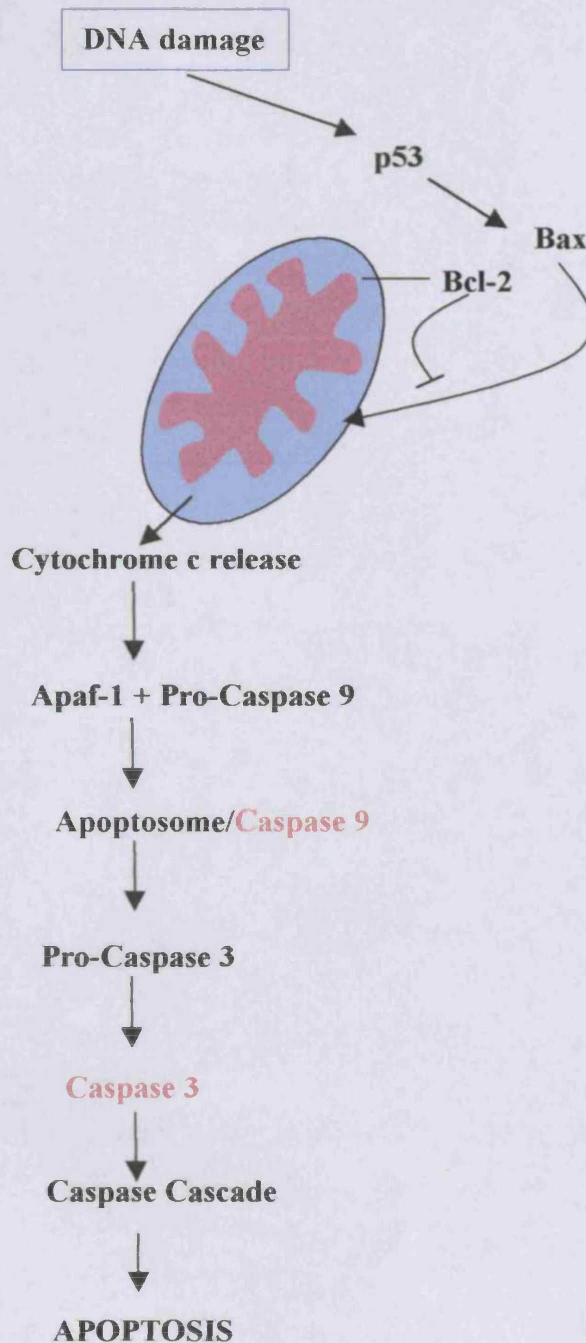


Figure 3.1 Activation of the Mitochondrial Apoptotic Pathway as a Result of DNA Damage.
The stress signal initiates p53 directed de novo synthesis of the Bcl-2 antagonist Bax, leading to activation of the mitochondrial apoptosis pathway. The commitment to cell death is determined by pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) members of the Bcl-2 family, which decide whether the stress signal is strong enough to condemn the cell to death. If the pro-apoptotic signal is adopted, mitochondrial membrane integrity is damaged leading to the release of cytochrome C that in turn binds to apoptotic protease-activating factor 1,Apaf-1 and Caspase 9 to form the apoptosome. Caspase 9 becomes activated by the apoptosome and can then cleave Pro-caspase 3 to form activated Caspase 3 to initiate the execution phase of apoptosis.

3.1.1.2 Necrotic Model System

Hydrogen peroxide (H_2O_2) induction of necrosis has been adopted as a suitable method in a number of reports (Antunes and Cadenas 2001, Chiaramonte, et al. 2001, Hampton and Orrenius 1997, Teramoto, *et al.* 1999). The reactive oxygen species (ROS), H_2O_2, is formed during normal metabolism and is also produced at sites of inflammation by phagocytic cells (Hyslop, *et al.* 1995). Previous evidence has indicated that the degree of oxidative stress determines whether apoptosis or necrosis is triggered, i.e. high levels of H_2O_2 induced necrosis (1mM-10mM), while lower levels resulted in apoptosis (10μM-100μM) (Teramoto, *et al.* 1999). Studies by Hampton *et al* have also shown that at high H_2O_2 concentrations caspase activity was not triggered and necrosis ensued, whereas at lower concentrations a long lag phase was evident before caspase activation. Therefore, hydrogen peroxide has two distinct effects. It initially inhibits the caspases and delays apoptosis. Then, depending on the degree of the initial oxidative stress, the caspases are activated and the cells die by apoptosis, or they remain inactive by oxidative inhibition and necrosis occurs (Hampton and Orrenius 1997).

3.1.2 Detection Methods

The induction of apoptosis and necrosis was confirmed by several immunocytochemistry analyses, using various well-established apoptosis detection reagents, as discussed below.

3.1.2.1 Annexin V FITC and Propidium Iodide

Annexin V is a 35-36 kDa Ca^{2+}-dependent phospholipid-binding protein that binds with high affinity to phosphatidylserine (PS), which becomes translocated from the internal cell membrane surface to the external surface during the early stages of apoptosis (Figure 3.2). This event precedes nuclear breakdown, DNA fragmentation and the appearance of most apoptosis-associated molecules. Annexin V FITC is therefore a useful marker for detection of apoptosis. Propidium iodide (PI) labels cellular DNA in necrotic cells where the cell membrane has been totally compromised. The exposure of test cells to the combination of Annexin V FITC and PI allows the differentiation among early apoptotic cells (Annexin V positive, PI negative), necrotic cells (Annexin V positive, PI positive) and viable cells (Annexin V negative, PI negative) (Vermes, *et al.* 1995).

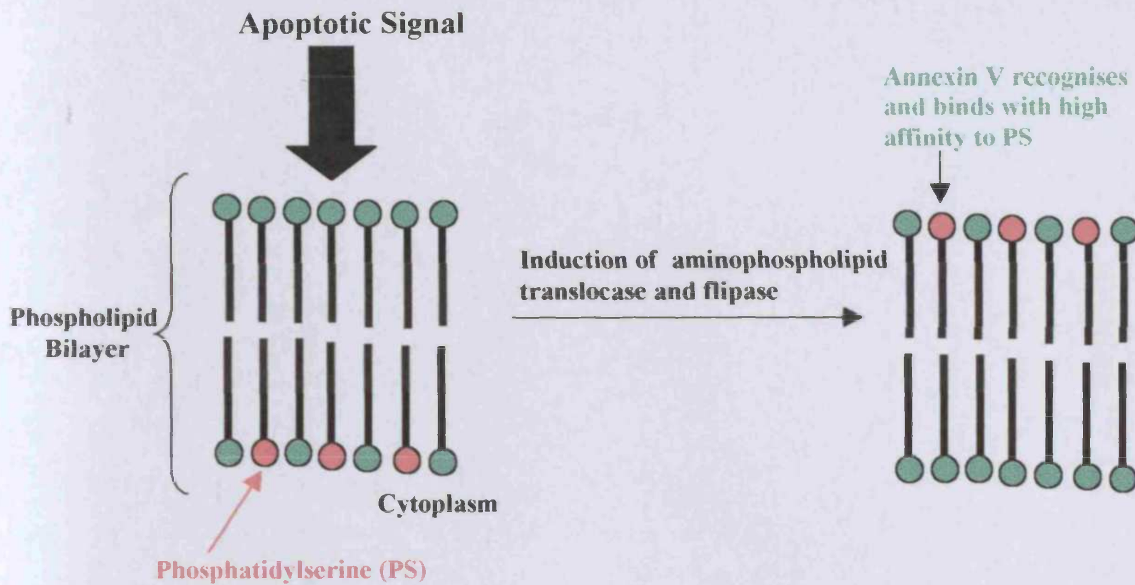


Figure 3.2 A Schematic of Phosphatidylserine Exposure on Early Apoptotic Cells and Detection by Annexin V. Following an apoptotic induction signal (i.e. external or internal insult) the cell is committed to an apoptotic cell death, which is characterised by stereotypic morphological and biochemical changes. One of these changes includes modifications on the surface membrane. Phosphatidylserine exposure is a well-characterised cell surface alteration, which is translocated to the external surface of the plasma membrane as opposed to its usual position on the inner membrane by the activation of an aminophospholipid translocase and flipase. Annexin V is a phospholipid binding protein that binds with high affinity to PS, therefore exposure of cells to Annexin V FITC allows the identification of the apoptotic cell population.

3.1.2.2 Trypan Blue

Trypan Blue (Sigma) staining of unpermeabilised cells allows the distinction between apoptotic and necrotic cells. Necrotic cells will stain blue due to cell membrane damage enabling uptake of the dye into cells, whereas apoptotic cells will remain unstained due to conservation of membrane integrity (Ren, *et al.* 2003, Suzuki, *et al.* 2004).

3.1.2.3 TetraMethylrhodamine Methyl Ester Perchlorate (TMRM)

TMRM (Molecular probes) is a mitochondrial membrane-potential sensor. The mitochondrial membrane-potential is known to decrease during apoptosis, allowing the accumulation of TMRM in the mitochondria during depolarisation of the membrane, resulting in diminished fluorescence due to self-quenching (Loew, *et al.* 1994).

3.1.2.4 Cleaved Caspase 3

Caspase 3, an effector caspase, and a key executioner of apoptosis, is responsible partially or totally for proteolytic cleavage of many key proteins. These include the DNA repair enzymes PARP and lamin, the p21-activated protein kinase 2 (once cleaved triggers plasma membrane blebbing), and the caspase-activated DNAse (CAD) (inactivated by binding to iCAD and cleavage of iCAD results in release of the active endonuclease, which produces internucleosomal DNA cleavage) (Figure 3.2) (Amarante-Mendes and Green 1999, Strasser, *et al.* 2000).

Activation of caspase 3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 subunits (Nicholson, *et al.* 1995). The cleaved-caspase-3 antibody (Cell Signaling Technology) specifically recognises the large fragment (17 kDa) of activated caspase 3 that is formed after the cleavage of its inactive zymogen residue adjacent to Aspartate 175 (Figure 3.3) (Fernandes-Alnemri, *et al.* 1994, Nicholson, *et al.* 1995).

3.1.2.5 Poly (ADP-ribose) Polymerase (PARP)

The anti-PARP p85 fragment polyclonal antibody (Promega) is directed against the 85-kDa fragment of PARP that results from caspase 3 cleavage. Once cleaved, PARP no longer supports the enzymatic DNA repair function, thereby contributing to the irreversibility of the apoptotic mechanism (Figure 3.4) (Smulson, *et al.* 1998).

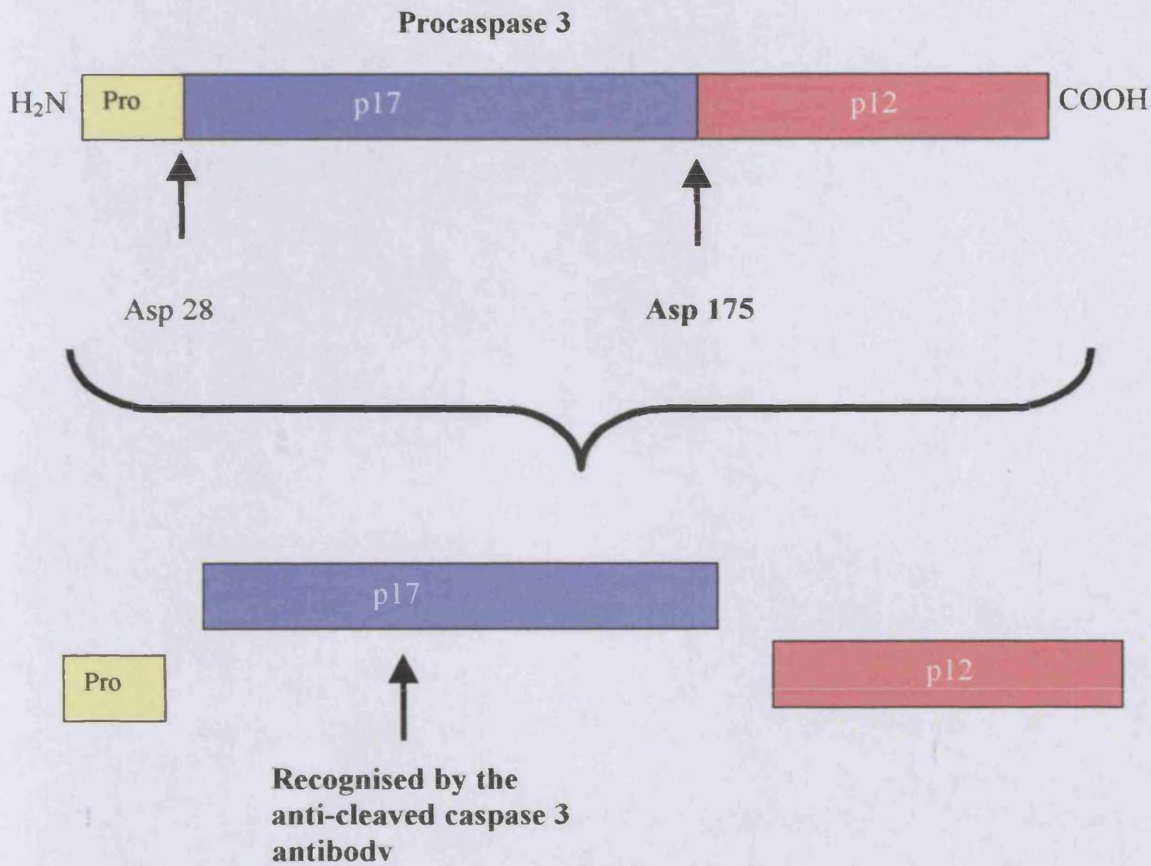


Figure 3.3 Cleavage of Caspase 3

Caspase 3 is constitutively expressed as a zymogen consisting of a N-terminal pro-domain, a large subunit in the middle of the molecule (17 kDa) and a C-terminal small subunit (12 kDa). Two caspase consensus sites separate these three distinct domains - one responsible for the cleavage of the pro-domain (cleavage at Aspartate 28) and the other for the separation of the two subunits (cleavage at Aspartate 175) into their activated subunits. This proteolytic activation is achieved either by auto processing or cleavage by another member of the caspases (Caspase 8, 9).

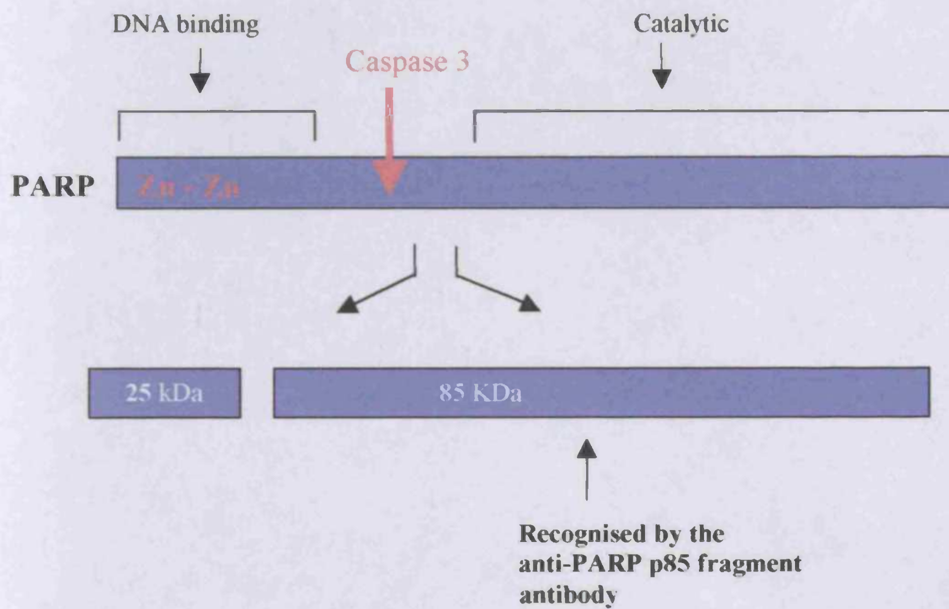


Figure 3.4 Schematic of PARP Cleavage
PARP is possibly the best-characterised proteolytic substrate of caspases, being cleaved in the execution phase of apoptosis in many systems. Intact PARP is cleaved to 25 kDa and 85 kDa fragments, representing the N-terminal DNA binding domain and the C-terminal catalytic domain of the enzyme respectively. PARP is cleaved at the sequence DEVD▼G by caspase 3.

3.2 Methods

3.2.1 Development of *In vitro* Models of Apoptosis and Necrosis

3.2.1.1 *In vitro* Models of Apoptosis

3.2.1.1.1 Camptothecin and anti-Fas

Jurkat cells (human T lymphocyte cell line, ECACC) were harvested from culture and resuspended at a density of 10^6/ml in RPMI 1640 without Foetal Bovine Serum (FBS). Apoptosis was induced in 10ml of cell suspension (1×10^7 cells) in 25 cm^2 flasks maintained in an incubator at 37°C, 5% CO_2. Cells were exposed to doses in the range of 1 - 10 μg/ml camptothecin, or 10 – 100 ng/ml anti-Fas mAb for various time points (2, 4, 6, 8, 16 hrs) to determine the optimum percentage of cells undergoing apoptosis. Control cells for the camptothecin experiment were exposed to DMSO for identical range of time points.

3.2.1.1.2 γ Irradiation

Jurkat cells were harvested from culture and resuspended at a density of 10^6/ml in RPMI 1640 with 5% FBS. Induction of apoptosis was tested in 10ml of cells in a 25 cm^2 flask for various time points (2, 4, 6, 8, 16 hours) at 37°C, 5% CO_2 following various doses of γ irradiation (in house facility) – 1000cGy (3 min), 2000cGy (6 min), 3000cGy (9 min), 4000cGy (12 min), 5000cGy (15 min) to determine the optimum percentage of cells undergoing apoptosis.

3.2.1.2 *In vitro* Model of Necrosis

3.2.1.2.1 H_2O_2

Jurkat cells were harvested from culture and resuspended at a density of 10^6/ml in RPMI 1640 without FBS. Necrosis was induced in 10ml of cells in a 25cm^2 flask by incubation for 4 hours, at 37°C, 5% CO_2 with various doses in the range of 1 - 10 mM hydrogen peroxide (H_2O_2) to determine the optimum percentage of cells undergoing necrosis.

3.2.2 Morphological Assessment of Different Cell Lines for their Susceptibility to Undergo Apoptosis

Light microscopy (x200 magnification) was used to assess cell morphology of Jurkat cells in culture post camptothecin treatment (4 hours) and gamma irradiation (6 hours). Other cell models were also assessed for their ability to undergo apoptosis namely THP1 (human monocyte) and EL4 (mouse T lymphocyte).

3.2.3 Dual Fluorescence Flow Cytometry for Detection of Apoptosis/Necrosis

At the end of the apoptosis and necrosis induction periods, cells were centrifuged at 1000 g for 3 minutes to pellet cells. Cells were washed 3 times in 10ml RPMI 1640 without FBS, resuspended to a final density of 5 x 10^5 cells, and 1 x 10^5 cells aliquoted into 5ml tubes. Cells were incubated with FITC conjugated Annexin V (1/50) and Propidium Iodide (PI, 5μg/ml) diluted to an appropriate concentration in 100μl RPMI 1640 (without FBS) for 30minutes at 4°C. Cells

were washed 3 times in RPMI 1640 and resuspended in 100μl RPMI 1640 prior to analysis on a FACScan flow cytometer.

Table 3.1 Antibodies and Reagents Used to Monitor Apoptosis and Necrosis

Process identified	*Antibody*	*Working dilution*	*Source*
Apoptosis	Annexin V FITC	1/50 F, ICC	Biosource
Necrosis	Propidium Iodide	1/200 F, ICC	Sigma
Apoptosis	Cleaved Caspase 3 (Asp 175) rabbit polyclonal Ab	1/100 ICC	Cell Signaling
Apoptosis	Anti PARP p85 fragment rabbit pAb	1/100 ICC, 1/1000 WB	Promega
Apoptosis	TMRM (Tetramethylrhodamine)	50nM F	Molecular Probes
Apoptosis/Necrosis	DAPi	1/1000 ICC	Sigma
Apoptosis/Necrosis	Trypan Blue	1:1 ICC, on cells	Sigma

3.3 Results

3.3.1 Optimal Dosage of Camptothecin, α Fas and γ Irradiation Required For Induction of Apoptosis and H_2O_2 for Induction of Necrosis.

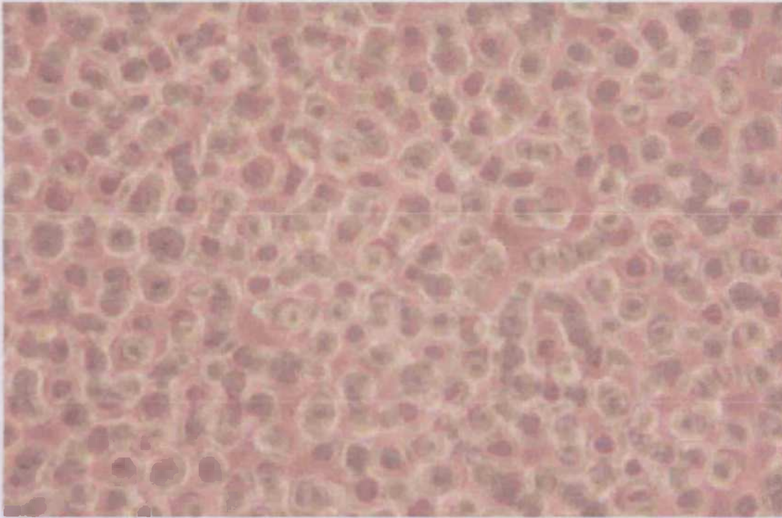
The dose and time point required to obtain the optimal percentage of apoptotic cells was determined to be 5μg/ml Camptothecin and 80ng/ml α-Fas for 4 hours, and 4000 cGy γ-irradiation followed by 6 hours culture at 37°C. The dose and time point required to obtain the optimal percentage of necrotic cells using H_2O_2 was 10 mM for 4 hours. Please refer to the following sections for details on how this was achieved.

3.3.2 Detection of Apoptosis/Necrosis by Morphological Examination of Cells in Culture Post Treatment

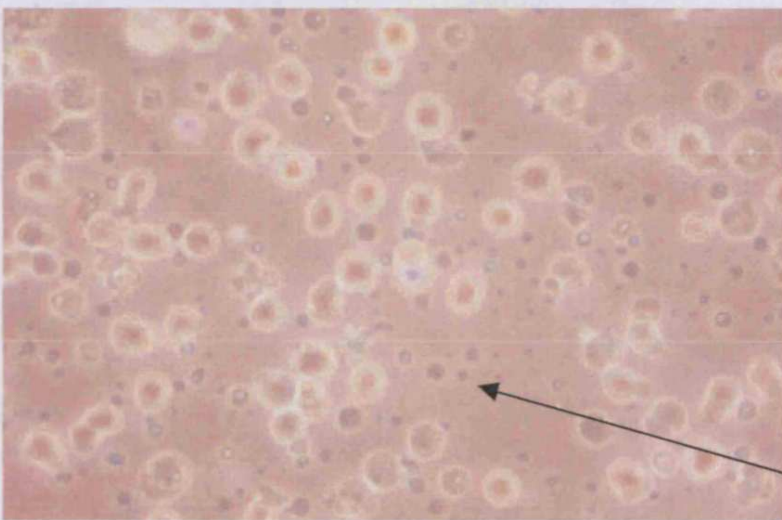
Morphological examination of cells by light microscopy was performed 6 hours post γ irradiation (4000cGy) and 4 hours camptothecin (5μg/ml) treatment to observe gross morphological changes on cells undergoing apoptosis/necrosis. THP1 cells appeared highly susceptible to camptothecin treatment and γ irradiation resulting in necrosis of the cells as shown by the presence of necrotic debris devoid of nuclei and plasma membranes (Figure 3.5a). Jurkat cells also seemed susceptible to both treatments, but in comparison to THP1 the cells underwent apoptosis - observed by cell shrinkage and formation of apoptotic bodies (Figure 3.5b). EL4 remained largely unaffected by both treatments

(Figure 3.5c) with no necrosis or apoptotic blebbing observed after 4 hours treatment. Only the Jurkat cell model was further characterised.

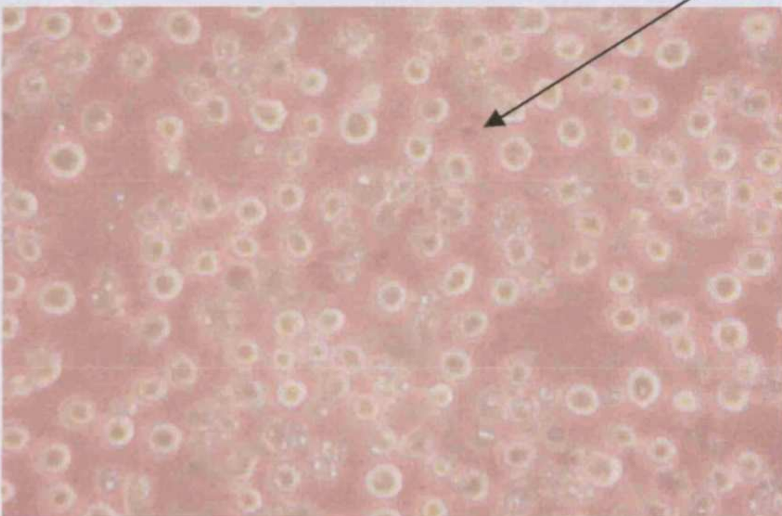
A Untreated



B γ irradiated



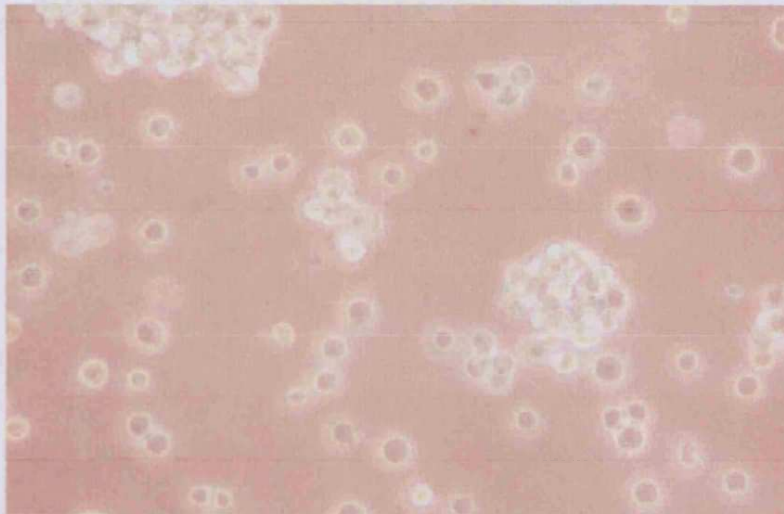
C Camptothecin treatment



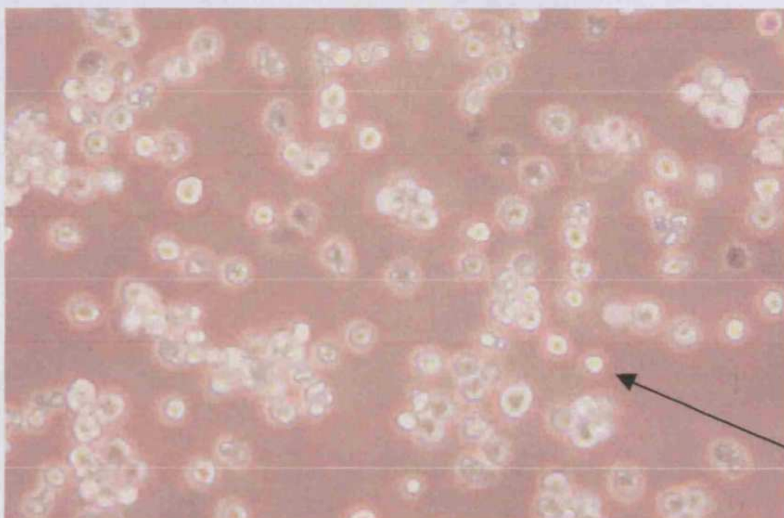
Necrotic cell debri

Figure 3.5a THP1 (human monocyte cell line) cells in culture post γ irradiation (4000cGy, 6 h) (B) and camptothecin (5μg/ml, 4 h) treatment were analysed by light microscopy (x200 magnification) for morphological changes distinct from normal cells (A, untreated) and characteristic of cell toxicity. Necrotic cell debri, characteristically devoid of nuclear material are observed in B and C.

A Untreated



B γ irradiation



C Camptothecin

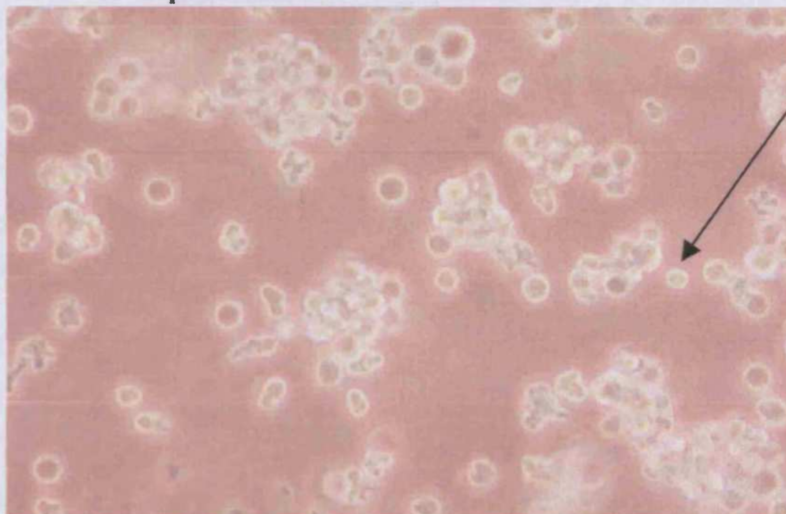
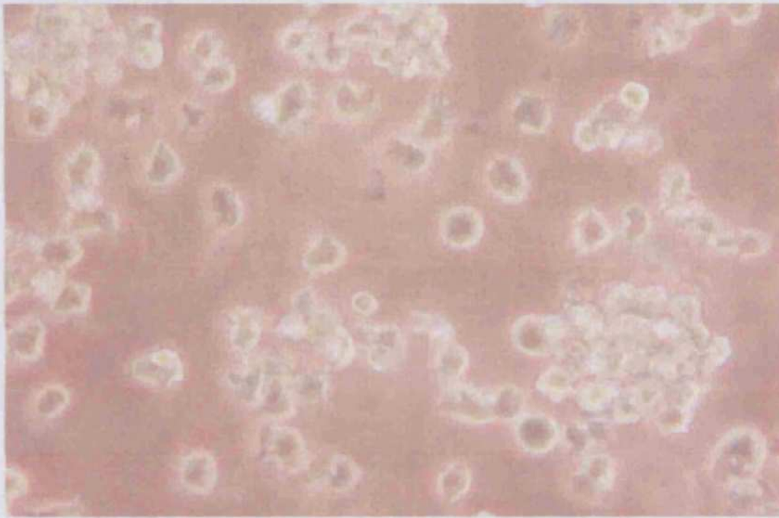
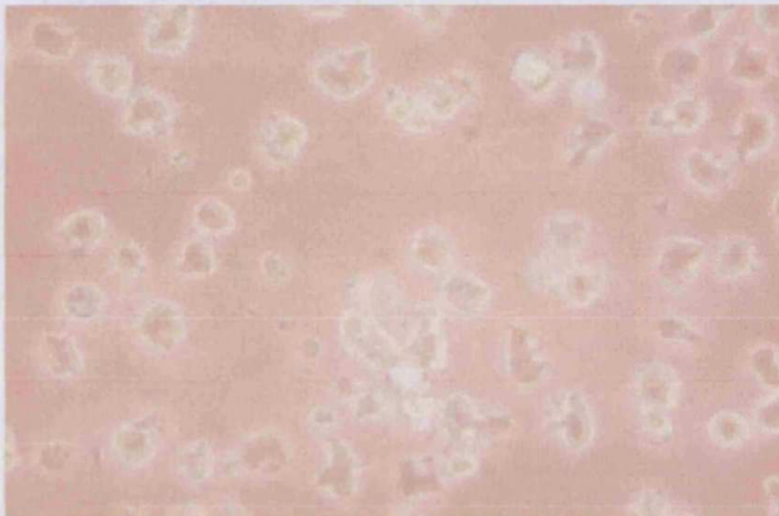


Figure 3.5b Jurkat (human T lymphocyte cell line) cells in culture post γ irradiation (4000cGy, 6 h) (B) and camptothecin (5μg/ml, 4 h) treatment were analysed by light microscopy (x200 magnification) for morphological changes distinct from normal cells (A, untreated) and characteristic of cell toxicity. Apoptotic bodies were observed post γ irradiation and camptothecin (B, C).

A Untreated



B γ irradiation



C Camptothecin

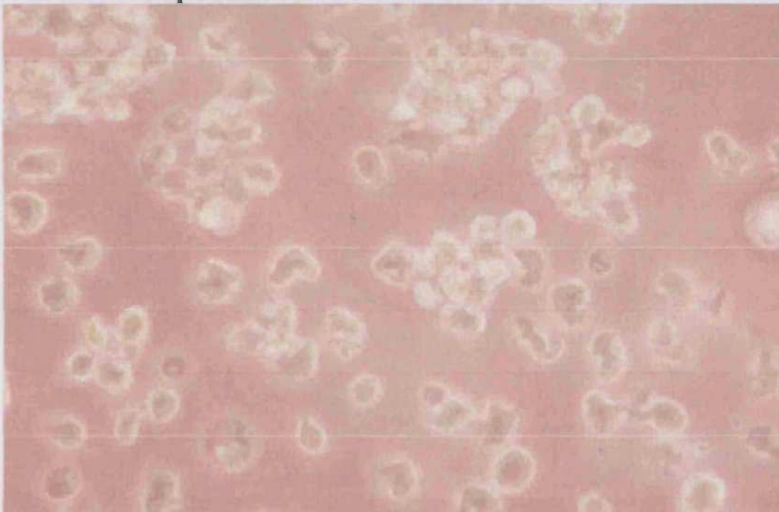


Figure 3.5c EL4 (mouse T lymphocyte cell line) cells in culture post γ irradiation (4000cGy, 6 h) (B) and camptothecin (5μg/ml, 4 h) treatment were analysed by light microscopy (x200 magnification) for morphological changes distinct from normal cells (A, untreated) and characteristic of cell toxicity. No changes in cell morphology were observed post treatment compared to untreated cells, i.e. no necrosis nor apoptotic blebbing were observed after 4 hours post treatment.

3.3.3 Detection of Apoptosis/Necrosis by Flow Cytometry Staining with FITC labelled Annexin V and PI

3.3.3.1 Camptothecin

The progression of apoptosis with respect to time (30 minutes - 16 hours) using various concentrations between 1 – 10 μg/ml camptothecin was determined by Annexin V/PI staining and subsequent analysis by flow cytometry. The dose required to obtain the optimal percentage of apoptotic cells was determined to be 5μg/ml. The results obtained after 2, 4, 6, 8 and 16 hours camptothecin treatment are depicted in Figure 3.6. Three distinct cell populations were identified: viable cells (Annexin V and PI negative), cells stained with PI but not with Annexin (apoptotic I) and cells stained with PI and Annexin V (apoptotic II). The optimal percentage of cells that underwent apoptosis was seen at 4 hours with ~30% of cells stained highly for Annexin V. A similar percentage of apoptotic cells were seen at 6 & 8 hours. Therefore for future experiments the 4 hours incubation was adopted. Secondary necrosis of cells was observed after 16 hours with ~95% of cells strongly stained for Annexin V and PI. The results were shown to be reproducible (depicted in Table 3.2), whereby the mean percentage of cells stained for Annexin V and PI over time were calculated from three experiments. These time course experiments revealed nucleic acid (NA) exposure (PI dim staining) was shown as early as 30 minutes, long before the exposure of PS (Annexin V staining, peak at 4 hours).

3.3.3.2 γ Irradiation

The progression of apoptosis was determined with respect to time (2, 4, 6, 8, 16 hours) after various doses of γ irradiation (1000, 2000, 3000, 4000, 5000cGy). The dose required to obtain the optimal percentage of apoptotic cells was determined to be 4000cGy and the results are depicted in Figure 3.6. Untreated cells did not stain strongly for Annexin V or PI, indicating they were viable. Treated cells showed three distinct cell populations: viable cells (Annexin V and PI negative), cells stained with PI but not with Annexin (apoptotic I) and cells stained with PI and Annexin V (apoptotic II). The optimal percentage of cells that underwent apoptosis for γ irradiated cells was seen 6 hours post irradiation, with 40% of the cells staining for Annexin V. All apoptotic cells eventually underwent secondary necrosis overnight (16 hours) displaying a strong PI and Annexin V staining. Therefore for future experiments the 6 hours incubation was adopted.

3.3.3.3 α Fas

The progression of apoptosis was determined with respect to time (2, 4, 6, 8, 16 hours) using various concentrations between 10 – 100ng/ml α Fas. The dose required to obtain the optimal percentage of apoptotic cells was determined to be 80ng/ml and the results are depicted in Figure 3.7. The optimal percentage of cells that underwent apoptosis was seen at 4 hours with ~ 40% of cells highly stained for Annexin V. Secondary necrosis of cells was observed after 16 hours with ~90% of cells strongly stained for Annexin V and PI. Similar levels of

apoptotic cells were seen for 6 & 8 hours. Therefore for future experiments the 4 hours incubation was adopted.

Of note, the three-apoptotic models that were developed were all successful in producing approximately 30-40% apoptotic cells (as shown by Annexin V/PI staining by flow cytometry), and thus all three models were used in future experiments in this chapter to further clarify the induction of features of apoptosis. For each figure only the result from one apoptotic induction method was shown, however a similar observation was confirmed for the other two models (data not shown).

3.3.3.4 Hydrogen Peroxide (H_2O_2)

The progression of necrosis was determined with respect to time (30 min – 16 hours) using various concentrations between 1 – 10 mM. In order to compare the process of necrosis with apoptosis the time point of 4 hours was adopted, and the amount of H_2O_2 needed to obtain an optimal percentage of cells undergoing necrosis after 4 hours was determined by Annexin V/PI staining and analysis by flow cytometry (Figure 3.8). The optimal percentage of necrotic cells at 4 hours with ~90% of the cells being PI positive was achieved with a H_2O_2 concentration of 10 mM. The results were shown to be reproducible (depicted in Table 3.2, experiment was performed by P. Gasque and published in the JBC paper, Elward *et al 2003*), whereby the mean percentage of cells stained for Annexin V and PI over time were calculated from three experiments (Table 3.2). Even after 30 minutes of H_2O_2 treatment the cells were stained bright for PI.

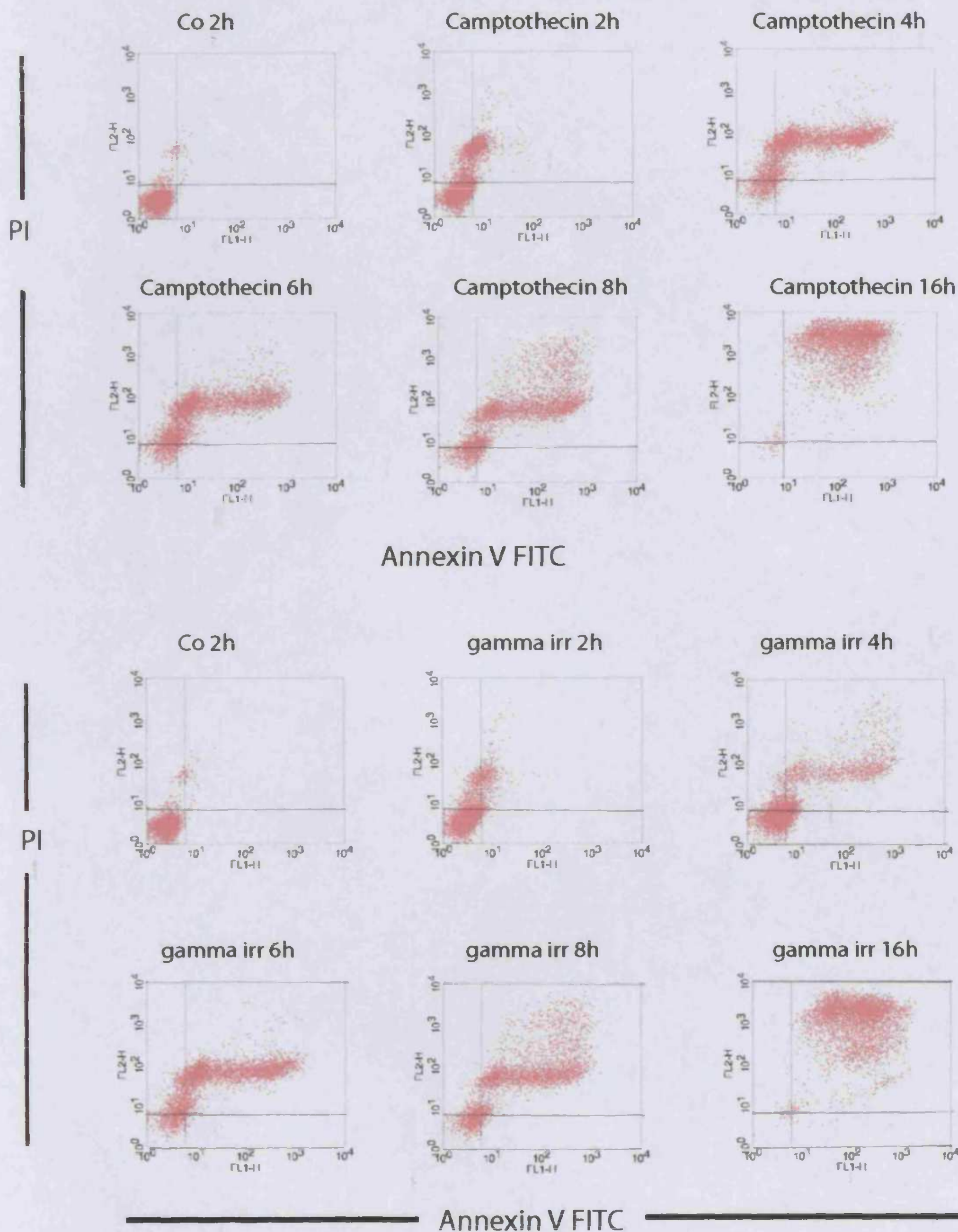


Figure 3.6 The process of apoptosis in time of Jurkat cells during various time periods (2,4,6,8,16 hrs) after Camptothecin treatment (5ug/ml) and gamma irradiation (4000cGY). Untreated Jurkat cells were incubated in DMSO for the same time course. Cells were incubated with FITC conjugated Annexin V FITC (1/50) and propidium iodide (1/200) for 30 mins at 4 degrees centrigrade. Cells were washed and resuspended in 100ul RPMI prior to analysis of a FACS flow cytometer. In Camptothecin treated cells An-/PI- became An+/PI- after 4 hours and for gamma irradiated cells after 6 hours and developed strong PI positivity (An+/PI+) overnight

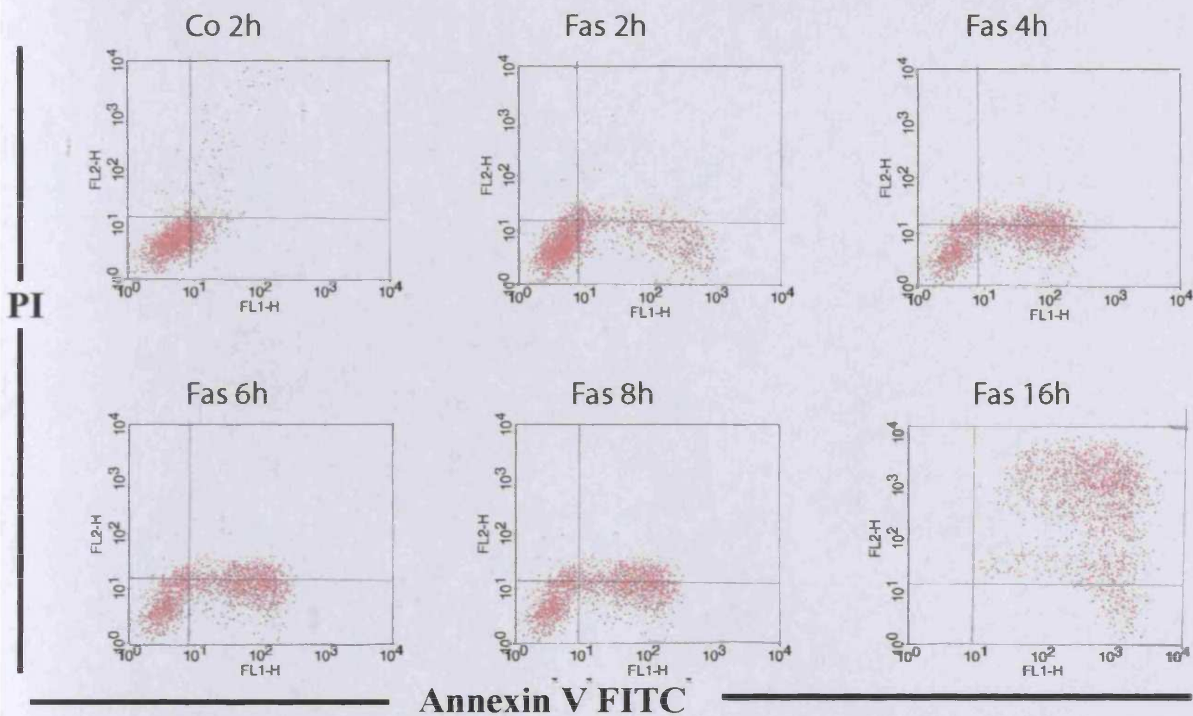


Figure 3.7 The process of apoptosis in time of Jurkat cells during various time periods (2,4,6, 8, 16 hrs) after anti-Fas (Clone CH11) treatment (80ng/ml) in RPMI (no FBS). Control cells were incubated in RPMI (no FBS) for the same time course. Cells were incubated with FITC conjugated Annexin V (1/50) and propidium iodide (1/200) for 30 mins at 4 degrees centigrade. Cells were washed and resuspended in 100ul RPMI prior to analysis on a FACS flow cytometer.
In anti-Fas treated cells optimal levels of apoptosis were seen after 4 hours with 40% of the cells being Annexin V FITC +ve. After 16 hours necrotic cells were strongly stained with PI and Annexin V FITC

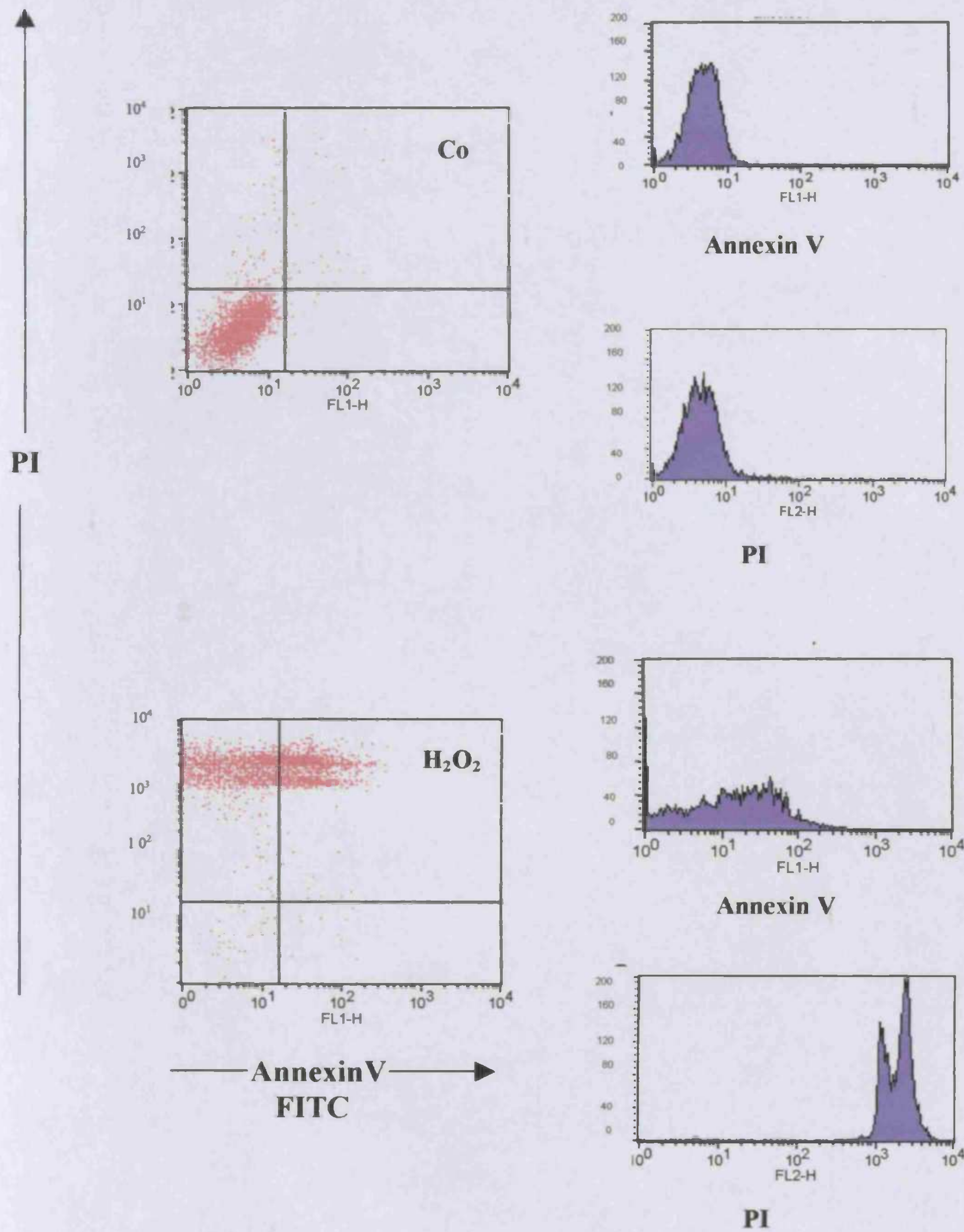


Figure 3.8 Necrosis of Jurkat cells after 4hr H_2O_2 treatment (10 mM) in RPMI (no FBS). Control cells (Co) were incubated in RPMI (no FBS) for the same time course. Cells were incubated with FITC conjugated Annexin V (1/50) and propidium iodide (1/200) for 30 mins at 4°C. Cells were washed and resuspended in 100µl RPMI prior to analysis on a FACS flow cytometer. 95% of the cells were PI positive after 4 hours treatment.

(Camptothecin, inhibitor topoisomerase I, - treated cells)

Time course	*Co*	*30 min*	*1h*	*2h*	*4h*	*8h*	*16h*
PI ^{dim} cells %	-	**12**	**20**	**24**	**45**	42	-
PI ^{bright} cells %	-	<1	<1	<1	<2	15	>95
PS ^{bright} cells %	-	-	-	5	35	60	>95

(Oxidative Necrosis: H_20_2 treated cells)

Time course	*Co*	*30 min*	*1h*	*2h*	*4h*	*8h*	*16h*
PI ^{dim} cells %	-	-	<10	-	-	-	-
PI ^{bright} cells %	-	**21**	**62**	**76**	**82**	**>95**	**>95**
PS ^{bright} cells %	-	19	64	65	76	>95	>95

Table 3.2
Key Hallmarks of Cell Death Ascertained by Flow Cytometry Analysis on Camptothecin and H_2O_2 Treated Jurkat Cells (n=3) Stained with Annexin V and PI.
Cells were either untreated (control) or treated with Camptothecin (5μg/ml) and H_2O_2 (10 mM) for various time periods (30 minutes to 16 hours), and stained with Annexin V FITC (1/50) and PI (5μg/ml) for 30 minutes at room temperature. Data are expressed as mean of FL2 intensity. The optimum time point to achieve apoptosis was shown to be 4 hours (shaded) with 35% of the cells PS bright and 45% of the cells PI dim. In comparison, after 4 h H_2O_2 treatment 82% of the cells were PI bright and 76% of the cells were PS bright. Camptothecin treated cells revealed NA exposure (PI staining) to be prominent as early as 30 minutes, long before exposure of PS (Annexin V staining) where peak is seen at 4 hours.
(Experiment performed by P. Gasque, and published in the JBC paper Elward *et al* 2003)

3.3.4 Detection of Apoptosis by Flow Cytometry Staining with TetraMethylRhodamine Methyl Ester Perchlorate (TMRM)

Untreated and camptothecin/α Fas treated cells (10^5) were incubated with TMRM (Molecular Probes, 50nM) post apoptosis induction for 30 minutes at room temperature, washed 3 times in RPMI 1640 and analysed by flow cytometry. Camptothecin and α Fas treated cells were confirmed to be apoptotic with a decreased expression of TMRM compared to untreated cells (Figure 3.9).

3.3.5 Immunofluorescent Microscopy

3.3.5.1 Annexin V/Propidium Iodide Dual Fluorescence Staining

Annexin V/PI immunofluorescent staining of unpermeabilised Jurkat cells 6 hours post γ irradiation is shown in Figure 3.10. Cells (10^5) were incubated with FITC conjugated Annexin V (1/50) and PI (5μg/ml) for 30 minutes at 4°C, placed directly onto microscope slides and analysed by fluorescent microscopy. Different stages of the apoptotic process leading to necrosis were observed. Early apoptotic cells with their plasma membrane intact were stained for Annexin V and weakly stained for PI at the membrane, as depicted by the flow cytometry data (Figure 3.6). Late apoptotic cells/early necrotic cells with damaged plasma membranes were also stained for annexin V and showed increased PI staining as it entered the cell. Necrotic cells having lost their plasma membrane were strongly stained for PI (nuclei staining) and no Annexin V FITC staining was observed due to destruction of the cell membrane.

Further microscopy analysis of Jurkat cells induced to undergo apoptosis by 4 hours camptothecin treatment and necrosis by 4 hours H_2O_2 treatment and stained with either trypan blue, propidium iodide or Annexin V FITC revealed 30 % of the cells were shown weakly positive for PI (membrane staining) and strongly stained for Annexin V following camptothecin treatment, whereas following H_2O_2 treatment the necrotic cells were membrane-permeable and strongly stained with trypan blue and PI nuclei stained (>95%) (Table 3.3, experiment performed by P. Gasque and published in the JBC paper, Elward *et al 2003*). Of note, 50-60% of the cells were strongly stained for Annexin V, indicating they were in the early stages of necrosis before the membrane was totally lost.

3.3.5.2 Trypan Blue Staining

Trypan Blue staining of unpermeabilised Jurkat cells post camptothecin treatment (4 hours) and H_2O_2 treatment (4 hours) is shown in Figure 3.11. Cells (100 μl) were incubated with an equal volume of Trypan blue dye and placed on a glass slide (cells not fixed) for analysis under a light microscope. Following camptothecin treatment the cells excluded trypan blue, whereas following H_2O_2 treatment the cells were membrane permeable and strongly stained with trypan blue (Figure 3.11 and Table 3.3).

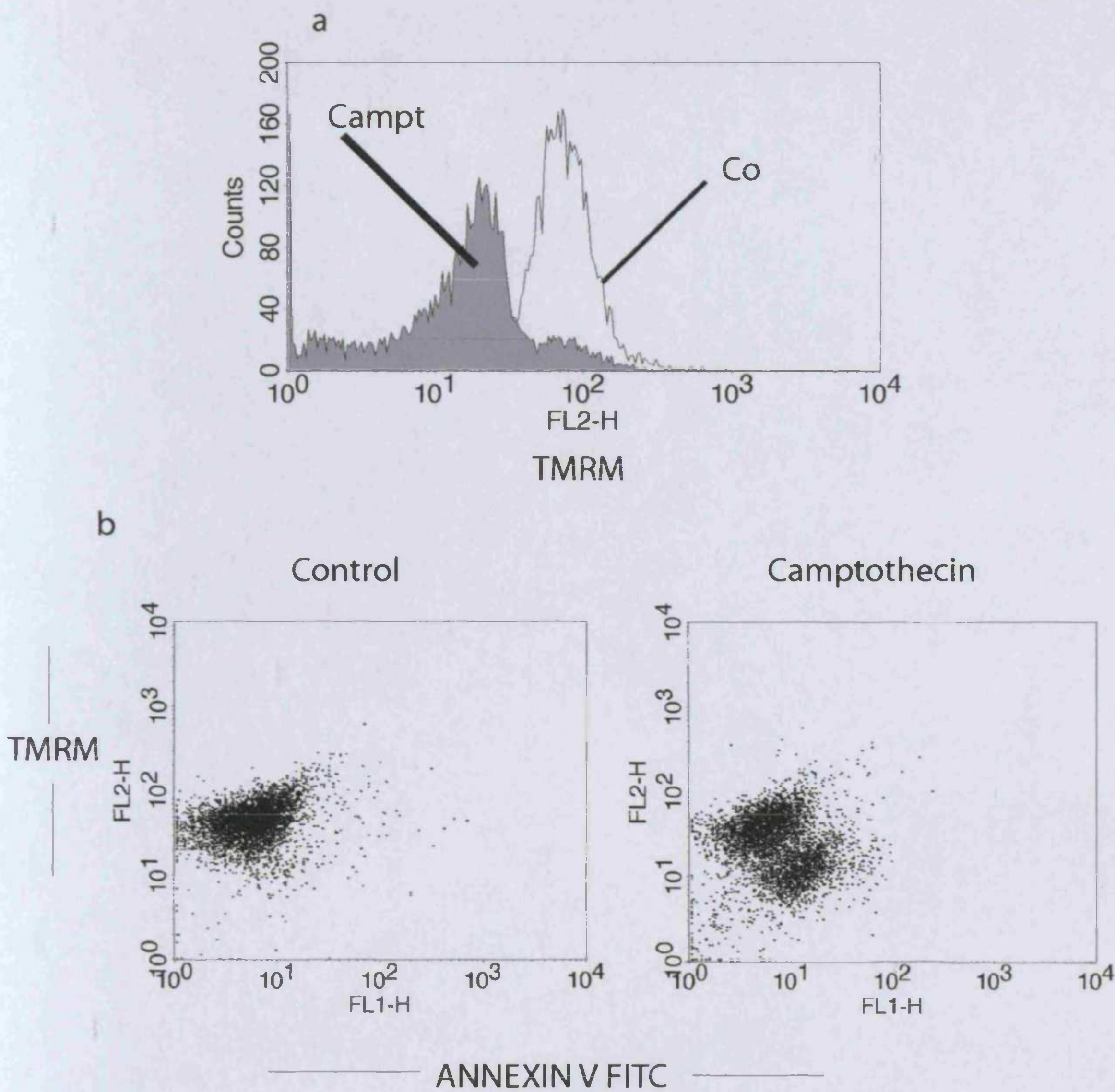


Figure 3.9 Flow cytometry TMRM staining of camptothecin (5ug/ml, 4h) treated Jurkat cells. Control cells were incubated in DMSO for the same time course. Cells were incubated with TMRM post apoptotic induction for 30 minutes at room temperature, washed in RPMI and analysed by flow cytometry.
a. Camptothecin treated cells (grey filled) showed a decreased expression of TMRM signal compared to control cells (black line). b. Dualfluorescence staining after camptothecin treatment with TMRM and annexin V FITC showed a decreased expression of TMRM on annexin V positive cells.

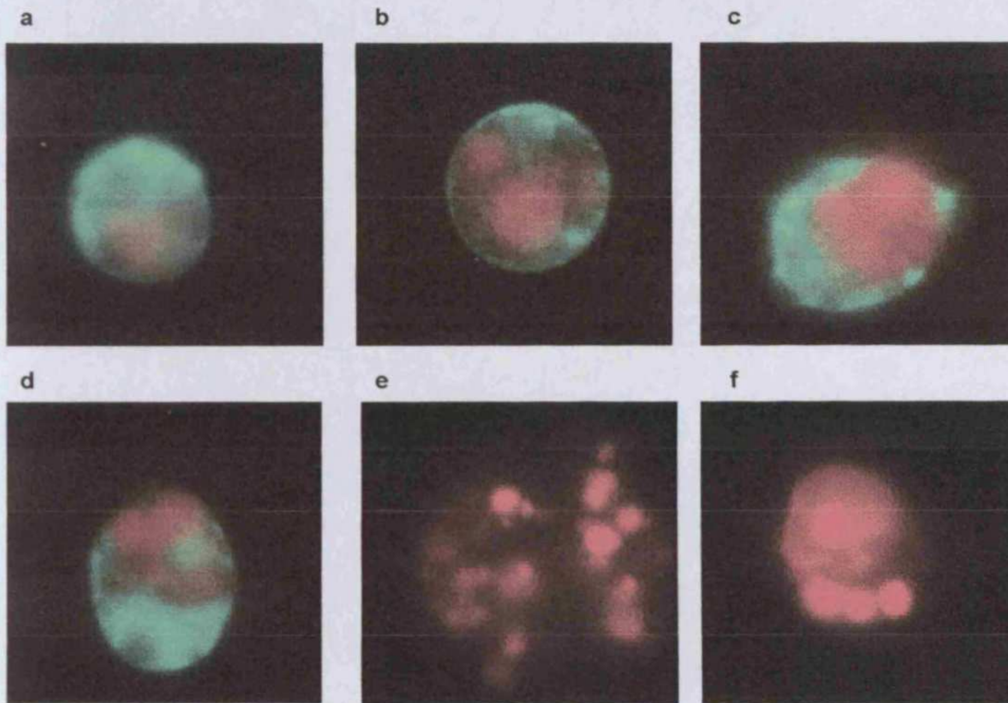


Figure 3.10. Immunofluorescent microscopic images of Jurkat cells post 6h γ irradiation (4000cGy). Cells were incubated with FITC conjugated Annexin V FITC (1/50) and PI (1/200) for 30 minutes at 4°C, placed directly onto microscopic slides, were not fixed but analysed directly by fluorescent microscopy. Different stages of the apoptotic process leading to necrosis were observed and depicted in: a-b early apoptotic cells with membrane intact - Annexin V FITC staining and weak PI staining observed at membrane; c-d late apoptotic cells/early necrotic cells with damaged cell membrane - increased PI staining as it enters the cell; e-f necrotic cells - no cell membrane detected and only nuclei are strongly PI positive. No Annexin V FITC staining due to destruction of cell membrane. Magnification x 1000

Treatments:	Camptothecin-treated cells		H_2O_2-treated cells	
	Staining:	%	Staining:	%
Trypan blue	Negative	~90-95	Positive	>95
Prop. Iodide	Weak (memb)	~40-50	Strong (nucl)	>95
Annexin V-FITC	Strong (memb)	~30-35	Strong (memb)	~50-60%

Table 3.3. Hallmarks of Necrosis and Apoptosis (microscopy analysis, n=3) Jurkat cells were induced to undergo apoptosis (camptothecin-treated cells, 4h) and necrosis (H_2O_2, 4h) and the hallmarks of programmed cell death and necrosis were confirmed by staining with either trypan blue, propidium iodide or Annexin V-FITC on unfixed cells cells followed by microscopy analysis. Annexin V positive apoptotic cells excluded trypan blue confirming membrane integrity and were weakly stained for PI indicating the exposure of nucleic acids at the cell membrane (memb). Strong nucleic acid staining with PI was localised to nuclei (nucl) in necrotic cells and Annexin V strongly stained damaged membranes. Experiment performed by P. Gasque and published in the JBC paper, Elward *et al 2003.*

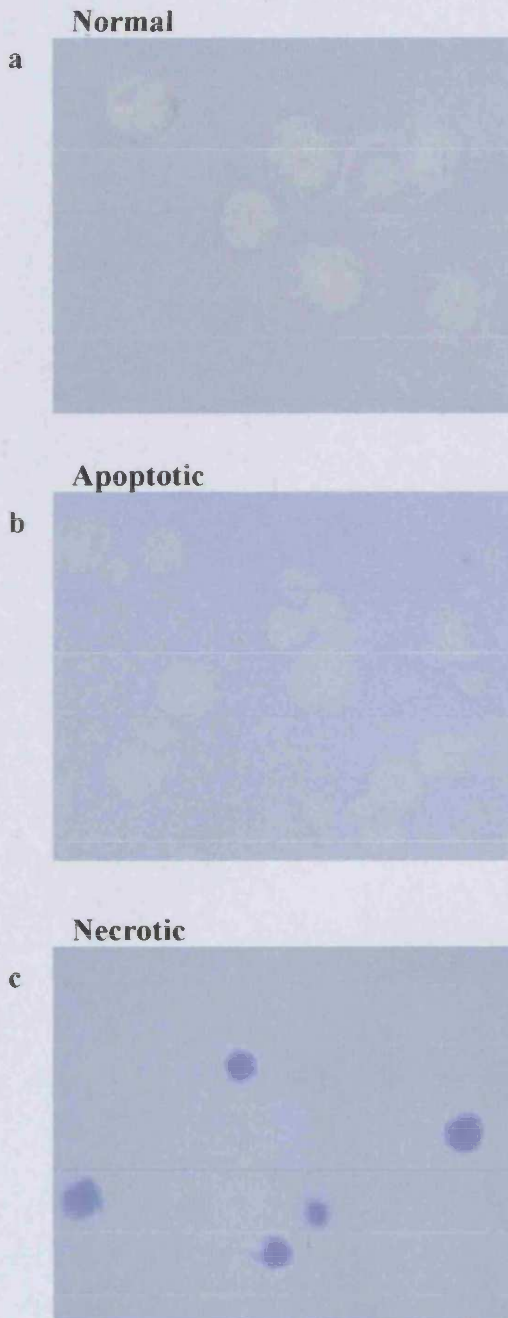


Figure 3.11 Bright Field Imaging of Unpermeabilised/Unfixed Jurkat Cells Stained with Trypan Blue

Cells (100μl) that were untreated , treated with camptothecin (5μg/ml, 4 h 37°C) to induce apoptosis or treated with H_2O_2 (10mM, 4 h 37°C) to induce necrosis were incubated with an equal volume of Trypan blue dye and analysed immediately under a light microscope without fixation or permeabilisation. Necrotic cells (c) stained with Trypan blue as the membrane became damaged allowing the dye to leak into the cell. Normal (a) and apoptotic cells (b) showed no staining as membranes remained intact (Magnification x400).

3.3.5.3 4'-6-Diamidino-2-Phenylindole (DAPi) Staining

DAPi is known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for AT, AU and AC base clusters (Matsumoto, et al. 1981). Immunofluorescent staining with DAPi nuclear stain of normal and apoptotic (4 hours, camptothecin treatment) Jurkat cytospins (fixed in acetone) is shown in Figure 3.12 a and b. Cytospins were incubated with DAPi (100ng/ml) for 30 minutes at room temperature. Cells were confirmed to be apoptotic when DAPi stained fragmented nuclear material. Of note, at high exposure a ring of nuclear material was observed around apoptotic cells but not normal cells.

3.3.5.4 Cleaved Caspase 3 pAb and anti-PARP p85 Fragment pAb Immunofluorescence Staining Untreated and camptothecin treated Jurkat cytospins were incubated with anti- cleaved caspase 3 pAb (1/100, Cell signalling) or anti-PARP p85 fragment pAb (1/100, Promega) for 1 hour at room temperature, followed by secondary Alexa Red conjugated goat anti-mouse (1/1000) antibody for 1 hour at room temperature. Camptothecin treated Jurkat cytospins revealed cytoplasmic localisation of cleaved caspase 3 (Figure 3.12 c) whereas PARP staining was restricted around the nucleus (Figure 3.12 d).

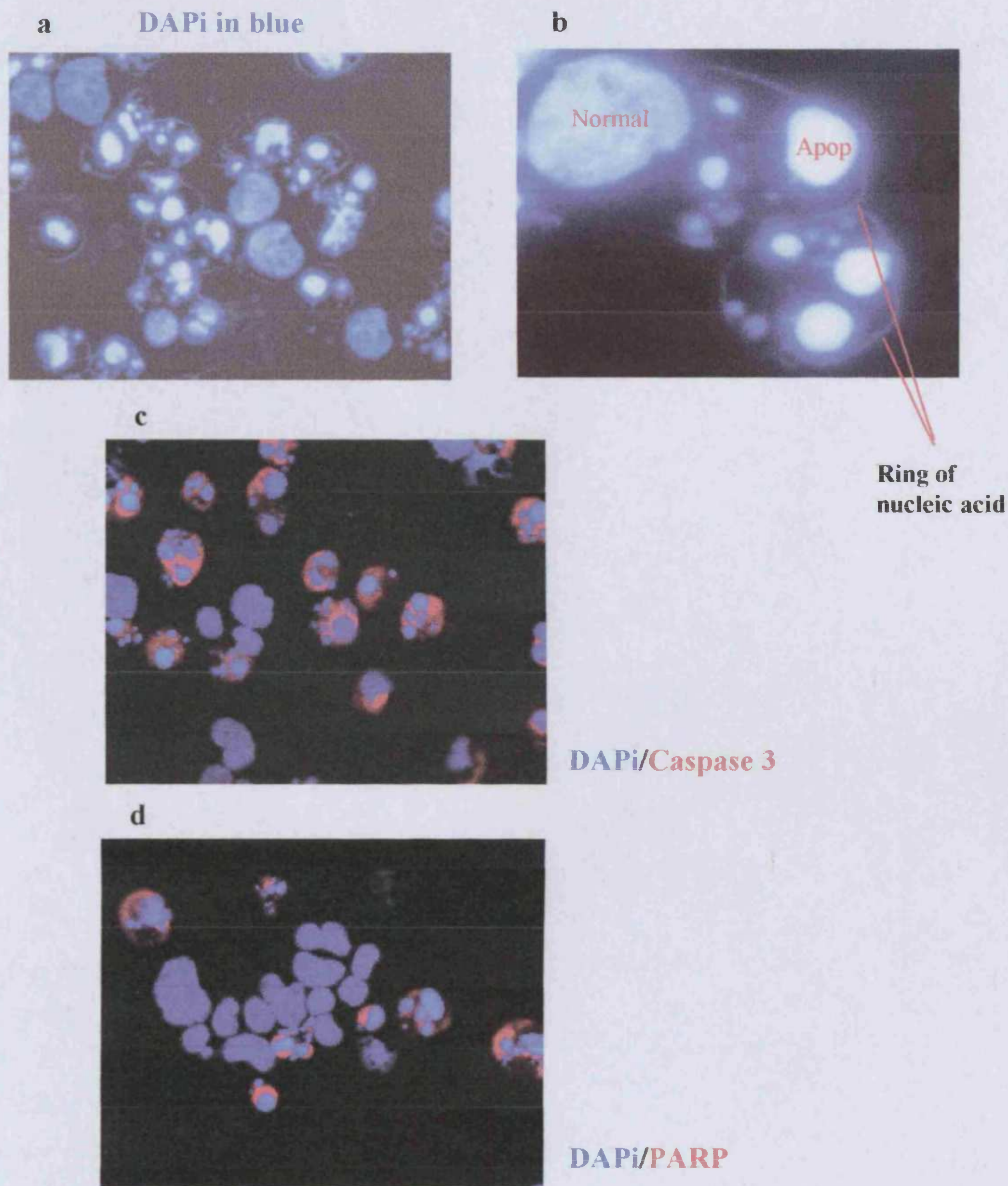


Figure 3.12. Immunofluorescent microscopic images of Jurkat cytospins post Camptothecin treatment (5μg/ml, 4h). **a,b**. Cells stained with DAPi (100ng/ml) for 30minutes at RT and image captured at high exposure to reveal ring of nuclear material around apoptotic cells. Image captured at x40 (a) and x100 (b) magnification; **c**. Cells stained with cleaved Caspase 3 pAb (1/100, cell signaling) for 1 hour at RT, followed by secondary Alexa Red conjugated antibody and DAPi for 1 hour at RT (x40); **d.** Cells stained with anti-PARP p85 fragment pAb (x40)(1/100, Promega) for 1 hour at room RT, followed by secondary Alexa Red conjugated antibody and DAPi for 1 hour at RT (x40).

3.3.6 Western Blotting for Cleaved PARP Expression during apoptotic induction

Western blot analysis of anti-Fas and camptothecin treated Jurkat cell lysates at various time points during treatment (30 min, 1h, 2h, 4h, 8h and 16h) was performed to determine cleaved PARP (p85 fragment) expression. Camptothecin and anti-Fas treated cells were confirmed to be apoptotic when a signal for PARP cleavage was present at 85 kDa (Figure 3.13). In the two different apoptotic models cleaved PARP expression was strong even after 2 hours treatment. Equivalent cell numbers were used and equal protein loading confirmed by using the anti-tubulin antibody.

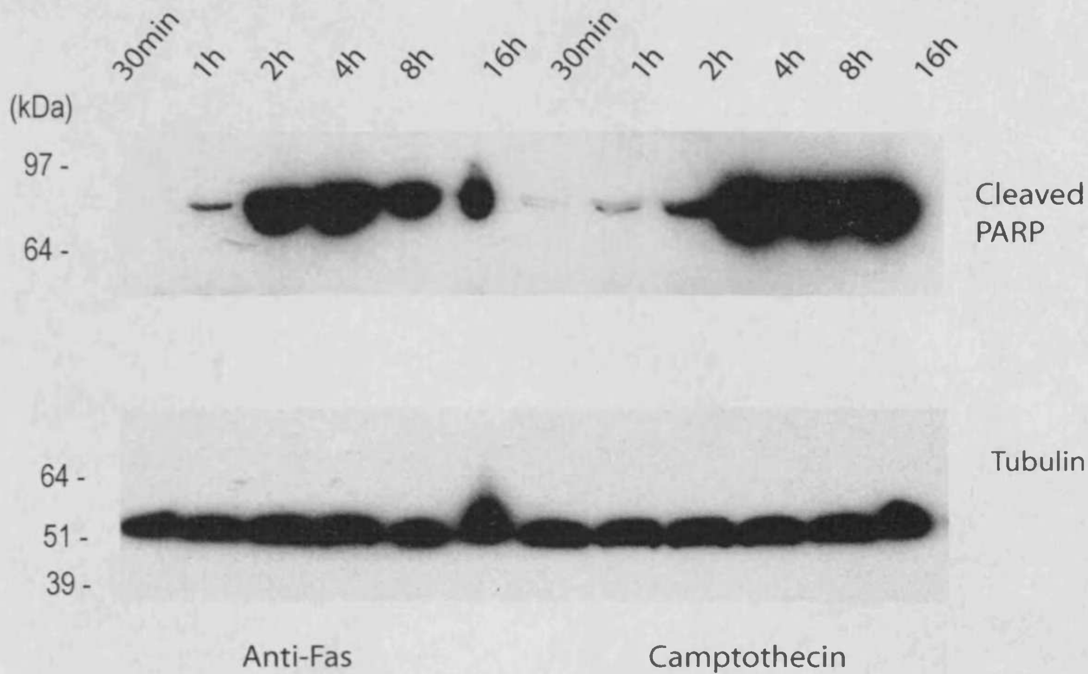


Figure 3.13. Kinetics of Cleaved PARP Expression using Two Different Apoptotic Models

Cell lysates of treated Jurkat cells (80 ng.ml anti-Fas or 5ug/ml camptothecin) were prepared at various time points during treatment (30 min, 1 h, 2 h, 4 h, 8 h and 16 h) and analysed by Western blot immunostaining (n=4) for cleaved PARP expression using a polyclonal rabbit anti-PARP (1/1000). Equivalent cell numbers were used and equal protein loading was confirmed by the anti-tubulin antibody

3.4 Discussion

Initial experiments focused on the development of *in vitro* models of apoptosis and necrosis to enable further assessment of the mechanisms of safe clearance of apoptotic versus necrotic T cells by phagocytes. Studying the apoptosis of T cells in relation to CNS pathology is paramount as during brain inflammation apoptotic cell death of infiltrating autoreactive T cells, and their subsequent safe clearance, is vital before secondary necrosis ensues. Insufficient clearance of autoreactive T cells can lead to pathology, an example of which is multiple sclerosis, where the pro-inflammatory response leads to damage of the myelin sheath and the underlying axon (Chan, *et al.* 2003).

From the results it can be seen that the initial goal of setting up well-validated apoptotic (γ irradiation, Camptothecin or α Fas antibody) and necrotic models (H_2O_2) in the Jurkat T lymphocyte cell line through dose titration experiments over time was successfully achieved. The γ irradiation model, which delivers DNA damage (double strand breaks) and triggers death by apoptosis via the mitochondrial pathway, seemed a very efficient model producing the optimal level of apoptosis (40% of cells) after 6 hours post 4000cGy treatment. Treatment with camptothecin, a specific DNA Topoisomerase I inhibitor and α Fas antibody also seemed appropriate models since optimal apoptosis (30-40%) of Jurkat cells was reached in just 4 hours. To use as a comparison to the apoptotic models in future experiments the dose and time point required to obtain

the optimal percentage (~90%) of necrotic cells after 4 hours using H_2O_2 was 10 mM.

During apoptosis, a key alteration to the cell membrane is the translocation of the negatively charged phospholipid, PS, from the inner to the outer surface membrane. Annexin V binds to PS on the cell surface in a calcium dependent manner. Flow cytometry analysis revealed after 4 hours camptothecin/α-Fas treatment that 35% of the cells were 'PS bright' and 45% of the cells were 'PI dim'. In comparison, after 4 hours H_2O_2 treatment 82% of the cells were 'PI bright' and 76% of the cells were 'PS bright'. The staining of PS in H_2O_2 treated cells with Annexin V FITC suggested these cells were in the process of early necrosis and had not lost total membrane integrity.

In summary after 4 hours camptothecin/α-Fas treatment or post 6 hours γ irradiation three distinct cell populations were identified: viable cells (Annexin V and PI negative), cells stained with PI but not with Annexin (apoptotic I) and cells stained with PI and Annexin V (apoptotic II). In contrast after 4 hours H_2O_2 cells in the early stages of necrosis were stained strongly for PI and Annexin V.

The findings from the present study correlated with previous evidence on the development of apoptotic models, however, conflicting observations were also revealed. The present study induced apoptosis in 40% of cells 6 hours post γ irradiation in the presence of 5% FBS, which is a similar percentage obtained in a study by Mevorach *et al* who obtained 20-30% Annexin V positive cells 6 hours post γ irradiation in the absence of serum (Mevorach, *et al.* 1998). In these

conditions greater than 95% of the cells did not take up PI, which the present study also conveyed whereby Annexin V positive apoptotic cells were only weakly stained for PI at the cell membrane. The observed high susceptibility to apoptosis for Jurkat cells within 4 hours of exposure to camptothecin correlates with previous studies (Johnson, *et al.* 1997), whereby morphological signs of nuclear changes were observed between 4 and 5 hours exposure, with no change in membrane integrity. The results in the present study for anti-Fas induction (80ng/ml) also demonstrated the susceptibility of Jurkat cells to undergo apoptosis within a short time (4 hours). The percentage of apoptotic cells obtained in this study after 4 hours (RPMI without FBS) never exceeded 40% before secondary necrosis ensued, which was also evident in a study by Attali *et al* at a concentration of 100ng/ml of the α Fas in RPMI with 10% FBS (Attali, *et al.* 2004). However, this was in contrast to a study by Gershov *et al* who portrayed 70-80% after 4 hours incubation with 50ng/ml α Fas in the presence of 10% FBS. Whereas the present studies only detected early apoptotic cells at 4 hours, their studies involved the detection of early and late apoptotic cells in order to achieve such a high percentage (Gershov, *et al.* 2000). Secondary necrosis has been referred to as the final stage of apoptotic cell death where many of the cells are unable to maintain membrane integrity and consecutively become permeable for ions and pro-inflammatory molecules. It is known that PS exposure on apoptotic cells is required for efficient uptake of apoptotic cells by phagocytes (Fadok, *et al.* 1992) . However, if this clearance does not occur in a sufficient time, as in this study's *in vitro* experiments, with no addition of

phagocytes, secondary necrosis will ensue within a few hours of initiating apoptosis.

In the present study a necrotic cell model was also set up in the Jurkat cells by incubation with H_2O_2 (10mM, 4 h). The percentage of necrotic cells (~90%) obtained correlated with previous evidence, whereby high concentrations of H_2O_2 (1-10 mM) triggered a cell death exhibited by necrosis on ~85% of the cell population of human lung fibroblasts (Teramoto, *et al.* 1999). Also, evidence by Hampton and Orrenius have shown that H_2O_2 has two distinct effects on the activity of caspases, which became activated during apoptosis, in Jurkat T lymphocytes. While lower concentrations of H_2O_2 (10 μM to 100 μM) activated the caspases and caused apoptosis in the T lymphocytes, higher concentrations (1 mM to 10 mM) as used in the present studies did not trigger caspase activity and the cells died by necrosis (Hampton and Orrenius 1997).

In addition to the development of apoptotic models in the Jurkat T lymphocyte cell line a number of different cell lines were analysed for their susceptibility to under apoptosis following exposure to camptothecin, α Fas, and γ irradiation. Whereas the Jurkat cells showed a distinctive morphology of apoptosis, THP1 (human monocyte cell line) and EL4 (mouse T lymphocyte cell line) exposed to the same treatments (camptothecin, γ irradiation) failed to develop a reliable apoptotic phenotype. Therefore, further studies concentrated on using the Jurkat

cell line, which has also been well described in the literature as a suitable model for apoptotic studies.

Even though cells can undergo apoptosis in response to a broad range of stimuli, including receptor stimulation (α Fas), treatment with cytotoxic drugs (Camptothecin) and γ irradiation, the execution phase responsible for the dramatic stereotypic morphological and biochemical changes leading to apoptotic cell death is believed to remain shared (i.e. caspase mediated) (Boesen-de Cock, *et al.* 1999).

To confirm the observed morphological changes were caused by induction of apoptosis, a wide range of well established apoptotic detection markers, in addition to Annexin V and PI were used in the analysis. These included DAPi, TMRM, anti-cleaved caspase 3, anti-cleaved PARP, and trypan blue. The execution phase involves signal transduction pathways to the effectors (caspases) of apoptosis (TMRM mitochondrial membrane potential sensor indicating route *via* mitochondrial pathway, anti-caspase 3 staining), followed by the effectors of apoptosis (i.e. caspase 3) acting on key proteins of the cell (anti-PARP staining), which results in specific morphological changes. These are characterised firstly by condensation of chromatin into typical dense crescents at the periphery of the nucleus adjacent to the nuclear membrane (DAPi staining), followed by modifications on the surface membrane (Annexin V binding to exposed PS). The cell begins to shrink with the compaction of cytoplasmic organelles,

fragmentation of nuclear chromatin continues (DAPi staining) and plasma membrane integrity remains (Trypan blue exclusion). Following camptothecin treatment, Annexin V positive apoptotic cells excluded trypan blue confirming membrane integrity and were weakly stained for PI indicating the exposure of nucleic acids at the cell membrane. Camptothecin treated cells revealed NA exposure (PI staining) to be prominent as early as 30 minutes, long before exposure of PS (Annexin V staining) where a peak is seen at 4 hours. DAPi staining of unpermeabilised camptothecin treated cells also confirmed nucleic acid exposure at the cell membrane of apoptotic but not normal cells, revealed by a distinct ring of nuclear material (DAPi staining) around the cell surface, which was not evident on normal cells. It is believed that apoptotic cells possess carbohydrate-containing polymers such as DNA on their surfaces, and accumulation of these cells, as well as excess free DNA, is known to cause inflammation. (McLachlan, *et al.* 2000, Schwartz, *et al.* 1997, Schwartz, *et al.* 1999a) However, as this ring appears early during apoptosis it could be hypothesised that DNA/RNA could have a role in clearance of apoptotic cells, but the mechanisms to how this could occur remain to be elucidated. Studies by Palaniyar *et al* have shown that certain members of the family of collectins (SP-D and not SP-A, MBL, C1q) bind carbohydrate (pentose sugar-based anionic phosphate) polymers such as free DNA and RNA as well as nucleic acids present on apoptotic cells *via* both their globular or collagen domains (Palaniyar, *et al.* 2004). The collectins have been recently characterised as pattern recognition proteins of the innate immune system, known for their ability to act as bridging

molecules between as yet unidentified collectin binding sites on apoptotic cells and the phagocyte calreticulin/CD91 complex to initiate clearance of apoptotic cells (Ogden, *et al.* 2001, Vandivier, *et al.* 2002). Therefore the binding of these proteins to surface exposed nucleic acids could result in the recognition and safe clearance of apoptotic cells by phagocytes.

The changes observed in the H_2O_2 necrotic model also correlated with known features of the hypothetical necrotic process (Antunes and Cadenas 2001, Hampton and Orrenius 1997, Teramoto, *et al.* 1999) – i.e. loss of membrane integrity whereby Annexin V strongly stained damaged membranes as early as 30 minutes into the H_2O_2 treatment, with trypan blue uptake into cells, and PI uptake with strong staining of nucleic acids also after just 30 minutes incubation with H_2O_2, resulting in a pathological form of cell dying, in contrast to apoptosis, releasing toxic contents including pro-inflammatory cytokines into the cellular environment.

Chapter 4

Apoptotic and Necrotic Cells Differentially Control the Complement System

4.1 Introduction

Over the past few years there has been growing evidence to confirm a now recognised role for complement in the safe clearance of apoptotic cells. Several studies have shown specific binding of complement proteins to apoptotic cells (Gershov, *et al.* 2000, Kim, *et al.* 2002, Korb and Ahearn 1997, Nauta, *et al.* 2002, Navratil, *et al.* 2001, Ogden, *et al.* 2001) (please refer to Table 1.1 in introduction). The complement system is tightly controlled by regulatory proteins (CD46, CD59, CD55) to prevent cell lysis, and a reduction in their cell surface expression during apoptosis has been shown (Hara, *et al.* 1996, Jones and Morgan 1995, Tsuji, *et al.* 1994). However, it is still unclear whether the regulatory proteins have a role in controlling and signalling in the apoptotic process, and if the observed down regulation is key to complement activation and initiation of the apoptotic cascade. Recently, reports have also revealed a role for complement in clearance of necrotic cells by phagocytes *via* a pro-inflammatory response (Gaipl, *et al.* 2004, Nauta, *et al.* 2003, Roos, *et al.* 2004).

This investigation assessed the role of complement opsonins (C1q, C3b) in correlation with complement inhibitors (CD46, CD55, CD59), which may mediate safe clearance of apoptotic Jurkat T cells, and to contrast these findings with complement activation on necrotic cells. Flow cytometry and

immunocytochemistry analyses, with antibodies raised specifically to complement activation products and complement regulatory proteins, were performed.

4.2 Methods

4.2.1 Complement Binding Assay on Normal/Apoptotic/Necrotic Jurkat Cells

As a source of complement, apoptotic and necrotic Jurkat cells were incubated with normal human serum (NHS) post apoptotic/necrotic induction.

4.2.2 Dilution of Serum on Cells and Incubation Time to Obtain Optimum Complement Activation.

Complement component	*Dilution of NHS*	*Incubation Time*
C1q	¼	15mins, RT
C3	¼	1 h. RT
C3b	¼	1 h, RT
iC3b	¼	1 h, RT
C5b9	¼	1 h, RT

4.2.3 Preparation of Normal Human Serum (NHS)

Whole blood from healthy donors was collected and allowed to clot for 30 minutes at room temperature, followed by 2 hours at 4°C. Serum was separated from the clot by centrifugation at 2,500 rpm for 10 minutes, and aliquoted into 500μl volumes for storage at –80°C until use.

4.2.4 Detection of Complement Opsonisation on NHS Treated Normal, Apoptotic, and Necrotic Jurkat Cells.

For the detection of bound complement components specifically on apoptotic and necrotic cells post incubation with NHS, dual immunofluorescence analysis of cells was performed where-by cells were incubated first with antibodies recognizing C1q (Rb α hu C1q, 1/200), C3 (Rb α hu C3 L440, 1/200), C3b (Mo α hu C3b (C3/30), 1/20), iC3b (Mo α hu iC3b, 1/1000), Membrane Attack Complex (MAC, Mo α hu C5b9 (B7) 1/50), followed by Annexin V FITC (1/50) and Phycoerythrin (PE) conjugated $F(ab)_2$ goat antibody to mouse or rabbit IgG (1/200). For all experiments apoptotic and necrotic induced cells in the absence of complement served as negative controls. Cells were analysed by dual fluorescence flow cytometry and in addition membrane localisation was deciphered by immunofluorescent microscopy of cytospins.

4.3 Results

4.3.1 Flow Cytometry Analysis to Determine Apoptotic Cell Opsonisation with Complement

To discriminate the level of complement activation between apoptotic and necrotic cells, cell opsonisation with C1q, C3, C3b, iC3b, and MAC was verified with co staining of Annexin V FITC. Flow cytometry analysis (Figure 4.1) revealed prominent staining of Annexin-V positive cells for C1q, C3b and iC3b following camptothecin treatment while the monoclonal anti-CD59 neoepitope did not detect the membrane attack complex (MAC) on apoptotic cells. This is in sharp contrast to necrotic cells, which displayed strong staining for complement opsonins and MAC (5 fold higher on necrotic compared to apoptotic cells). C1q staining was most prominent when cells were incubated only for 10-15 minutes with NHS, and clearly identified two populations of camptothecin-treated cells double stained (38%) or not (36%) for PS exposure. Interestingly, this pattern was reminiscent of the PI/Annexin V staining pattern depicted in the previous chapter (Figure 3.8). Control cells failed to activate the complement system, with only background staining observed. The results were shown to be reproducible, whereby the mean fluorescence (FL2) intensity for complement components was calculated from three experiments (Table 4.1 and 4.2, experiments performed by P. Gasque for inclusion in JBC paper, Elward *et al* 2003). Data indicated the mean fluorescence above background using either isotype control anti-mouse antibodies or non-immune control rabbit antiserum.

The classical pathway was confirmed to be the main route of complement activation on cells undergoing apoptosis (Table 4.1 and 4.2). Although necrotic cells bound C1q, activation of the alternative pathway by necrotic cells was also observed, with only a weak Bb staining on apoptotic cells compared to strong staining on necrotic cells (Table 4.1). Increased complement staining was shown on apoptotic and necrotic cells as the camptothecin and H_2O_2 incubation time increased (Table 4.2).

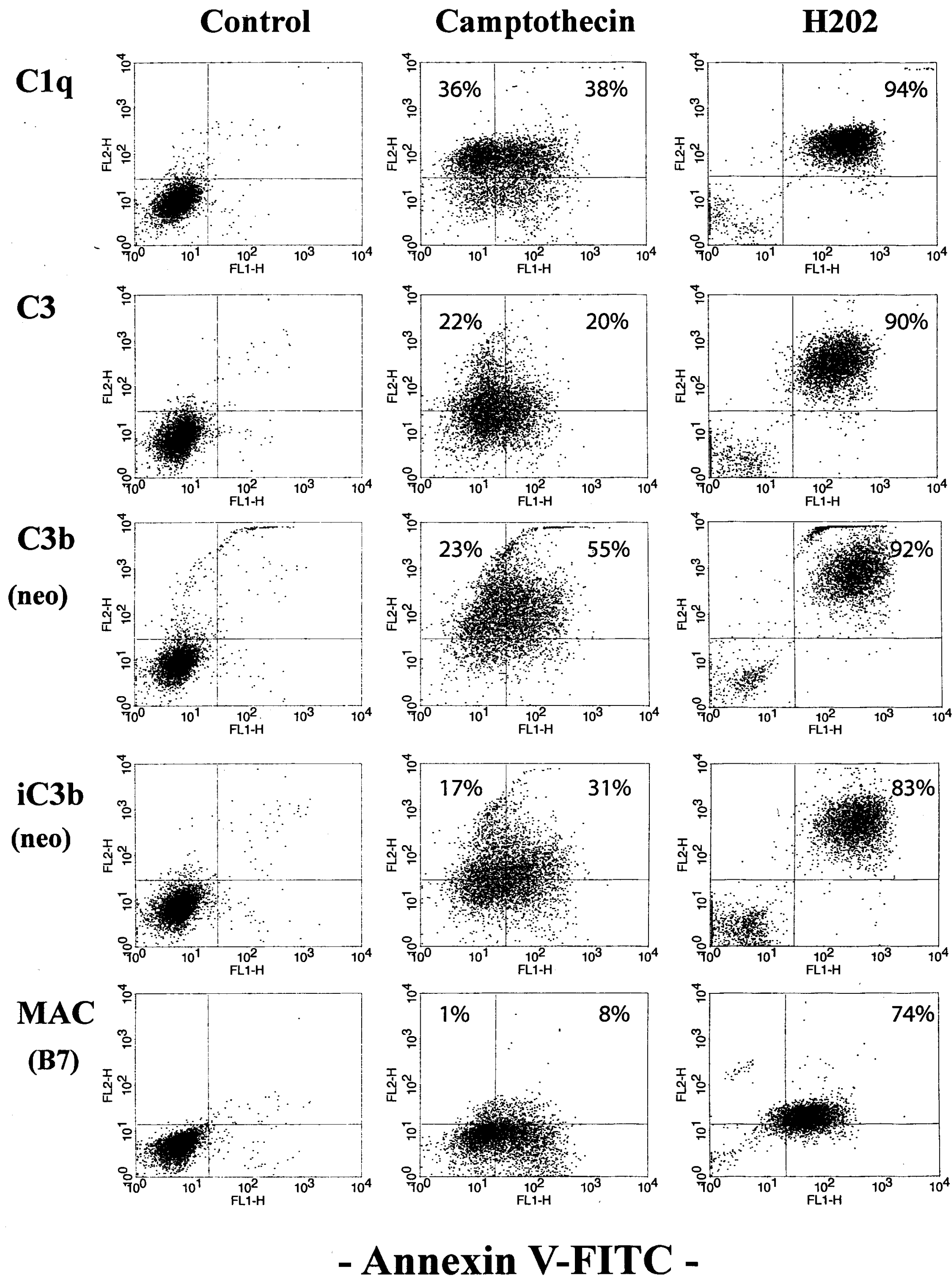


Figure 4.1 Complement Activation on Normal/Apoptotic/Necrotic Cells. Jurkat cells were induced to undergo apoposis (4h, 5ug/ml Camptothecin) and necrosis (4h, H2O2), incubated with 25% NHS for 15 minutes for C1q and 1h for C3, C3b, iC3b and MAC at RT, and analysed by two colour flow cytometry for complement proteins of the classical pathway and Annexin V FITC (marker of apoptosis). Data from one experiment with reproducible findings (n=4)

Immunostainings	Camptothecin Mean FL2	H_2O_2 Mean FL2
Mo α-C1q (12A5B7, ATCC)	4.3	19.7
Rb α C1q (Dako)	37	183.5
Gt α C1q (Calbiochem)	47.3	605.9
Gt α C4 (ATAB, Dr M. Fontaine)	104.3	1031.8
Gt α C4 (ICL, Dr M. Fontaine)	71.2	1187.8
Rb α C2 (Nordic)	25.6	251.3
Gt α C2 (Calbiochem)	73.4	371.2
Mo α C3 H1C3 (Dr M. Fontaine)	26.6	183.8
Rb α C3c (L440, Dr M. Fontaine)	17.13	230.7
Gt α C3 (Calbiochem)	11.7	1297.9
Mo α C3b neo (C3/30, Dr Taylor)	62.8	1345
Mo α iC3b (Quidel)	72.4	1030
Mo α C3d (BGRL11, IBGRL)	4.30	56.9
Rb α C3d (Dako)	25.50	712.6
Rb α C5 (Dr M. Fontaine)	2.31	56.6
Sh α C9 (Binding site)	3.6	158.7
Mo α C9 neo (B7, in house)	12.1	42.5
Mo α Bb (Prof. O. Gotze)	3.7	9
Rb α Bb (Prof. O. Gotze)	5.5	149.1
Rb α fH (L740, Dr M. Fontaine)	26.5	81

Table 4.1 Routes of Complement Activation and Opsonisation on Apoptotic and Necrotic Jurkat Cells (n=3). The route and level of complement activation on apoptotic cells versus necrotic cells was ascertained by incubation of cells 4h post-treatment with NHS (25% in RPMI for 15 min for C1q staining and 30 min otherwise) followed by immunostaining using RPE-conjugated secondary antibodies and FACS analysis. Data indicate the mean fluorescence (FL2) above background using either isotype control anti-mouse antibodies or non-immune control rabbit antiserum. Of important note, control Jurkat cells incubated in the presence of NHS (25%) failed to activate the complement system and hence was not stained for complement fragments (data not shown, see Table 4.2 for detail). N.B. The experiment was performed by P. Gasque for inclusion in the JBC paper, Elward *et al 2003.*

(Apoptosis I: Camptothecin, treated cells, n=3)

Time course	*Co*	*30min*	*1h*	*2h*	*4h*	*8h*	*O/N*
Gt αC1q	-	**26**	**48**	**57**	**62**	123	125
Mo αC3b neo	<10	**15**	**34**	**42**	**185**	314	760
Mo α MAC neo	-	-	-	-	-	18	167

(Oxidative Necrosis: H_2O_2 treated cells, n=3)

Time course	*Co*	*30min*	*1h*	*2h*	*4h*	*8h*	*O/N*
Gt αC1q	-	**45**	**243**	**560**	**754**	Nd	Nd
Mo αC3b neo		**200**	**347**	**871**	**1560**	Nd	Nd
Mo α MAC neo	-	**34**	**30**	**45**	**87**	121	232

Table 4.2 Complement Opsonisation on Apoptotic and Necrotic Jurkat Cells: FACS Analysis (n=3).
The overall capacity to activate the complement system was ascertained in a model of apoptosis (5µg/ml Camptothecin, 4h) and necrosis (10 mM H_2O_2, 4h). Cells were either untreated (control) or treated for various time periods (30 min to overnight, O/N). Post-treatment cells were incubated with NHS (25% in RPMI for 15 min for C1q staining and 30 min otherwise) followed by immunostaining using RPE-conjugated secondary antibodies and FACS analysis. Data indicate the mean fluorescence (FL2). Optimal staining for C1q and C3 was seen after 4h camptothecin and H_2O_2 treatment (highlighted in grey), and strong staining for MAC was seen after 4h H_2O_2 treatment but was not present after camptothecin treatment until 8h.
N.B. The experiment was performed by P. Gasque for inclusion in the JBC paper, Elward *et al 2003.*

4.3.2 Immunocytochemistry for Detection of Complement Activation on Apoptotic Cells

Immunofluorescent staining of normal and apoptotic (6 hours post γ irradiation) Jurkat cytospins was performed for the detection and membrane localisation of complement components. Post apoptotic induction and prior to cytospin preparations, cells were incubated with NHS (25%) for 1 hour at room temperature and also for 15 minutes for C1q binding. Immunofluorescent staining with antibodies recognising specific complement components (C1q, C3, C2) followed by secondary Alexa Green or Alexa Red conjugated secondary antibody specific to the primary antibody (1/1000) and DAPi nuclear stain (1/1000). Normal cells showed no complement staining at the cell surface whereas complement components were shown assembled on the surface of apoptotic cells post 1hour incubation with NHS (C1q, C3, C2) and post 15 minutes incubation (C1q) (Figure 4.2). C1q binding was shown distributed evenly around the cell membrane after 15 minutes incubation with NHS, and was clustered to vesicle-like structures, which were blebbing out from the cell surface together with C3 and C2 after 1hour incubation.

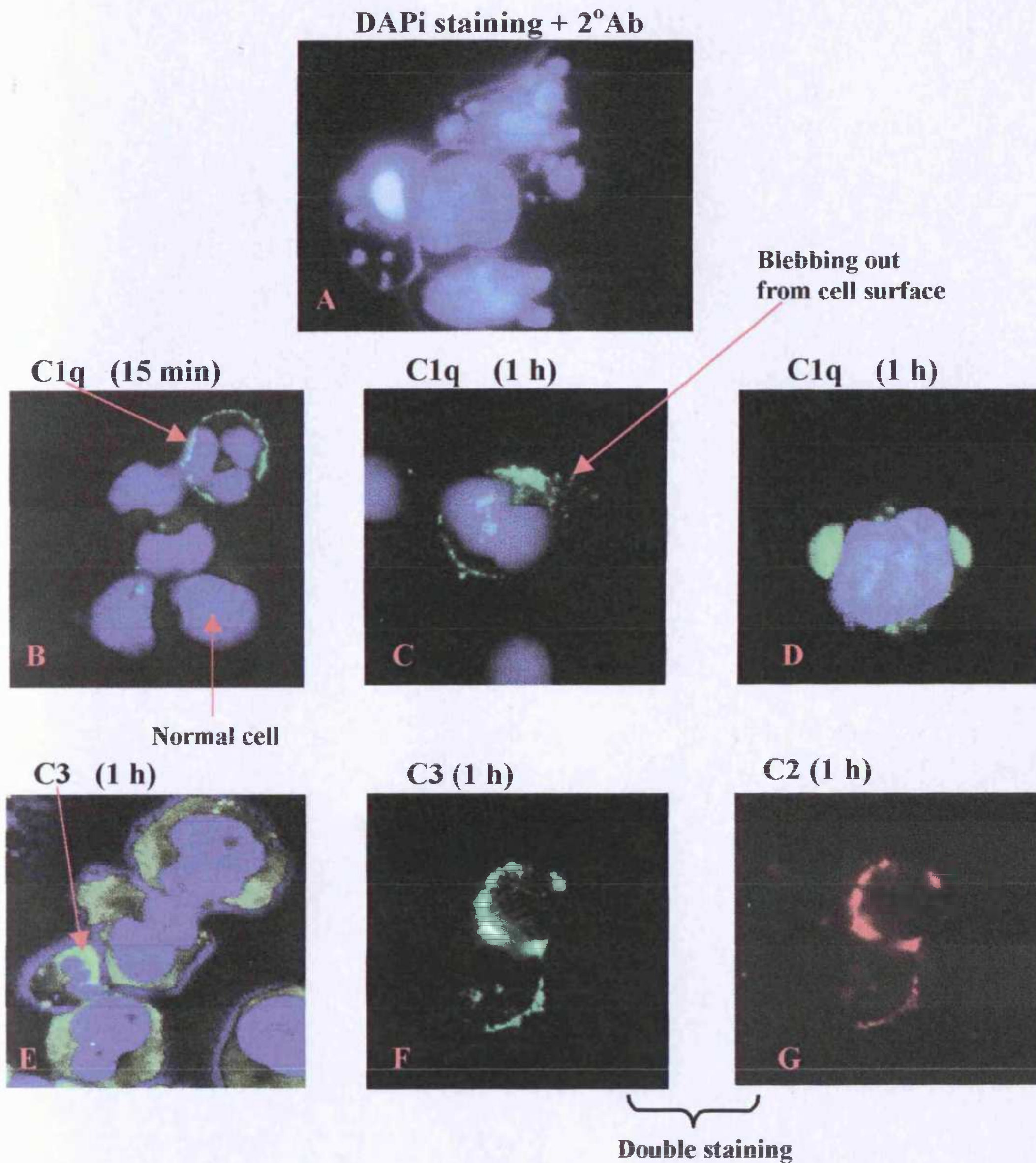


Figure 4.2 Serum Complement Assembles on the Surface of Apoptotic Cells.
Jurkat cells were induced to undergo apoptosis (6h post 4000cGy γ irradiation), incubated with either NHS (25%) for 15 min or 1h at RT and cytospins prepared. Cells were fixed with acetone and stained for C1q (B, C, D), and C3 (E) or double stained with goat α C3 (F) and rabbit α C2 (G) followed by specific secondary antibody and DAPi nuclei stain. Cytospins were stained with secondary antibody and DAPi as control (A). C1q staining was evenly distributed around the cell surface after 15 minutes incubation with NHS or displayed a granular pattern with vesicles detaching from the cells after 1h incubation with NHS. Normal cells failed to stain for C1q (B). C3 and C2 staining was evenly distributed around the surface of apoptotic cells (E, F, G).

4.3.3 Dual Flow Cytometry Analysis to Determine Nucleic Acid (NA) Exposure and Complement Activation on Apoptotic and Necrotic Cells

Results from the previous chapter revealed nucleic acid exposure at the cell membrane of apoptotic cells (Figure 3.12). Therefore, further experiments were performed to determine whether NA exposure at the cell membrane contributed to the initiation of the classical pathway given that DNA has long been known as a C1q binding molecule (Palaniyar, et al. 2004). Apoptotic and necrotic cells were treated with a cocktail of DNAse I/RNAse (Sigma, 10 μg/ml in RPMI, 30 min at 37°C) before incubation with NHS. DNAse/RNAse treated Jurkat cells remained viable and displayed markedly reduced levels of PI staining at the cell membrane (Table 4.3, experiment performed by P.Gasque for inclusion in JBC paper, Elward *et al 2003*). The removal of NA dramatically affected the activation of the classical pathway on apoptotic cells with reduced C1q binding and C3b levels at the cell surface ($p<0.005$). In contrast, only a 15-20% reduction of C1q binding was observed when necrotic cells were treated with DNAse/RNAse (data not shown).

(Mean FL2 ± SD)

	No pre-treatment	DNAse, RNAse treated	P values
Propidium iodide	41.25±8.4	8.75±3.4	0.002
Rb anti-C1q	52.2±8.9	4.7±0.9	0.001
Mo anti-C3b neo	157±35.5	41±6.7	0.005

Table 4.3 The Exposure of Nucleic Acids (NA) in the Outer Leaflet of the Plasma Membrane Promotes C1q Binding and Drives Complement Activation on Apoptotic Jurkat cells: Flow Cytometry Analysis (n=4).
Camptothecin treated Jurkat cells (4h) were incubated in either RPMI alone or RPMI with 10µg/ml of RNAse (Sigma, for 30 min at 37°C). 90-95% of the cells excluded trypan blue (data not shown) and confirming membrane integrity. Nucleic acid exposure was determined by propidium iodide (PI) staining and the level of complement opsonisation (C1q and C3b staining) was performed as described (Table 1) followed by FACS analysis. Data are expressed as mean of FL2 ± standard deviation. The P value from the t-test was calculated using Sigmaplot software (n=4).
N.B. The experiment was performed by P. Gasque for inclusion in the JBC paper, Elward *et al 2005.*

4.3.4 Flow Cytometry Analyses for Expression Profile of Complement Regulators and other Surface Markers on Apoptotic Cells

In order to define complement regulators (CD46, CD55, CD59) and other surface markers with a modulated expression during the course of apoptosis/necrosis, the profile of surface molecule expression was assessed by flow cytometry. Cells were stained using specific monoclonal antibodies to complement regulators and surface antigens followed by the appropriate rPE secondary conjugate diluted in PBS/BSA. Only selected profiles on apoptotic and necrotic cells for CD46, CD55, CD59, CD47, CD43 and Annexin I are shown in Figures 4.3 and 4.4. Annexin I protein has been reported to translocate to the cell surface from the cytoplasm on apoptotic cells and was used as an internal control (Arur, *et al.* 2003). CD43 is a highly sialylated membrane protein which is known to be rapidly shed from apoptotic cells (Eda, *et al.* 2004). The other antibodies did not reveal differences in profiles of staining between normal and apoptotic cells (See Graph Figure 4.5). Untreated Jurkat cells showed high levels of staining for all complement regulatory proteins (CD46, CD55, CD59) together with CD43, CD47 (Figure 4.4). Annexin I staining was detected at the cell surface only after apoptosis and necrosis. A significant loss of CD46 staining was observed on apoptotic (Figure 4.3) and necrotic (Figure 4.4) cells with a distinct bimodal distribution of expression apparent on cells post camptothecin treatment, where one population retained a level of expression similar to that of normal cells, while the second population expressed a reduced level. CD55 and CD59 staining was slightly reduced when cells were induced into apoptosis and necrosis. From

the staining for CD59 it can be seen that complement activation is under safe control, and limiting the formation of cytolytic and cytotoxic MAC formation but yet allowing opsonisation of apoptotic cells possibly to facilitate phagocytosis. For the other CD markers analysed, CD47 staining was not affected on apoptotic or necrotic cells and CD43 was lost from apoptotic and necrotic cells with a distinct bimodal distribution observed on apoptotic cells. Kinetics analyses on camptothecin and H_2O_2 treated cells revealed CD46 was lost over time, with a rapid loss seen from the apoptotic and necrotic cell surface at 4 hours (Table 4.4, experiment performed by P. Gasque for inclusion in the JBC paper, Elward *et al* 2005). The results were shown to be reproducible, whereby the mean fluorescence (FL2) intensity for complement components was calculated from three experiments.

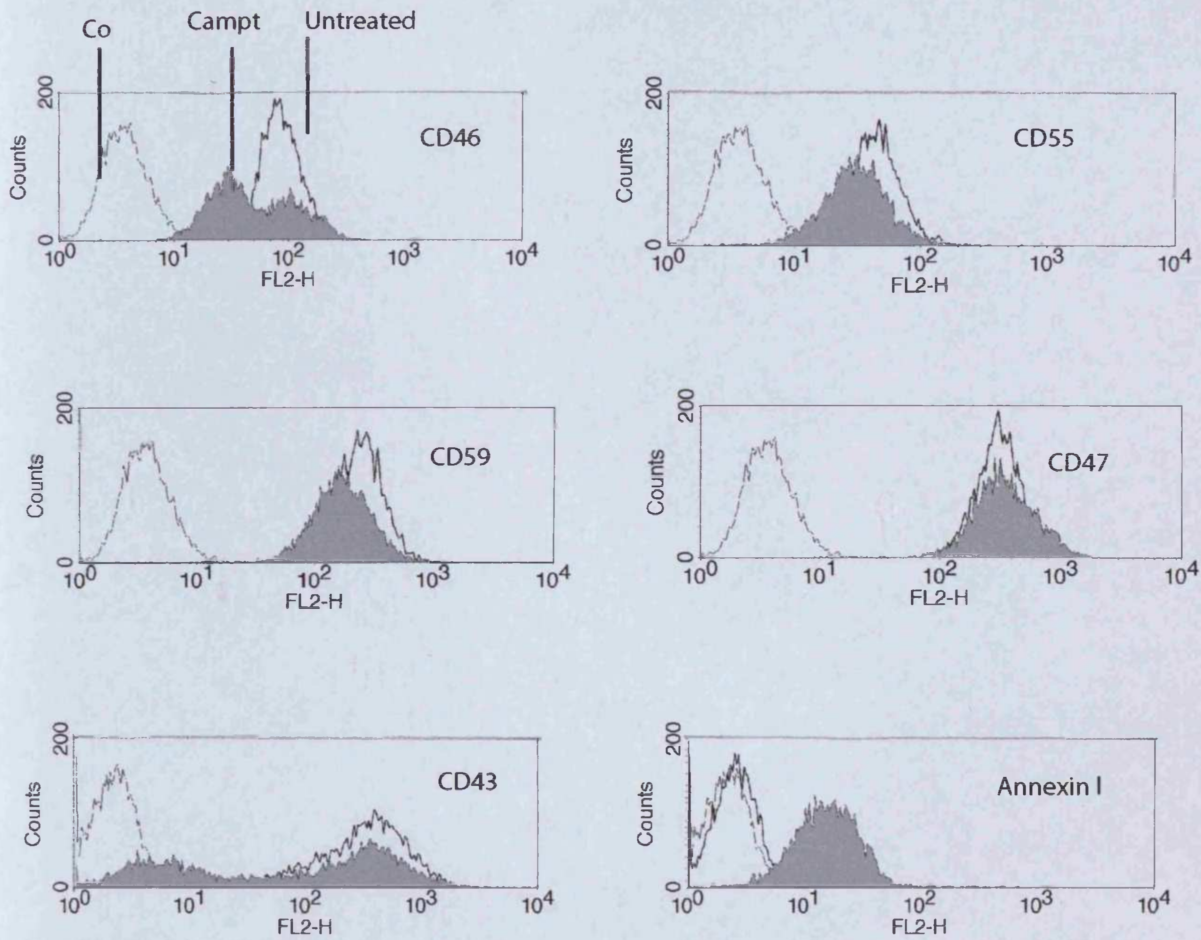


Figure 4.3 Flow cytometric analysis of Jurkat cells post camptothecin (5ug/ml, 4 h), labelled with antibodies to cell surface antigens followed by phycoerythrin conjugated F(ab)2 goat antibody to mouse IgG. Single parameter histograms are shown for each cell marker. Grey filled profiles represent treated Jurkat cells with primary antibody staining, light grey lines represent treated cells with no primary antibody, and black lines represent untreated cells stained with primary antibody.

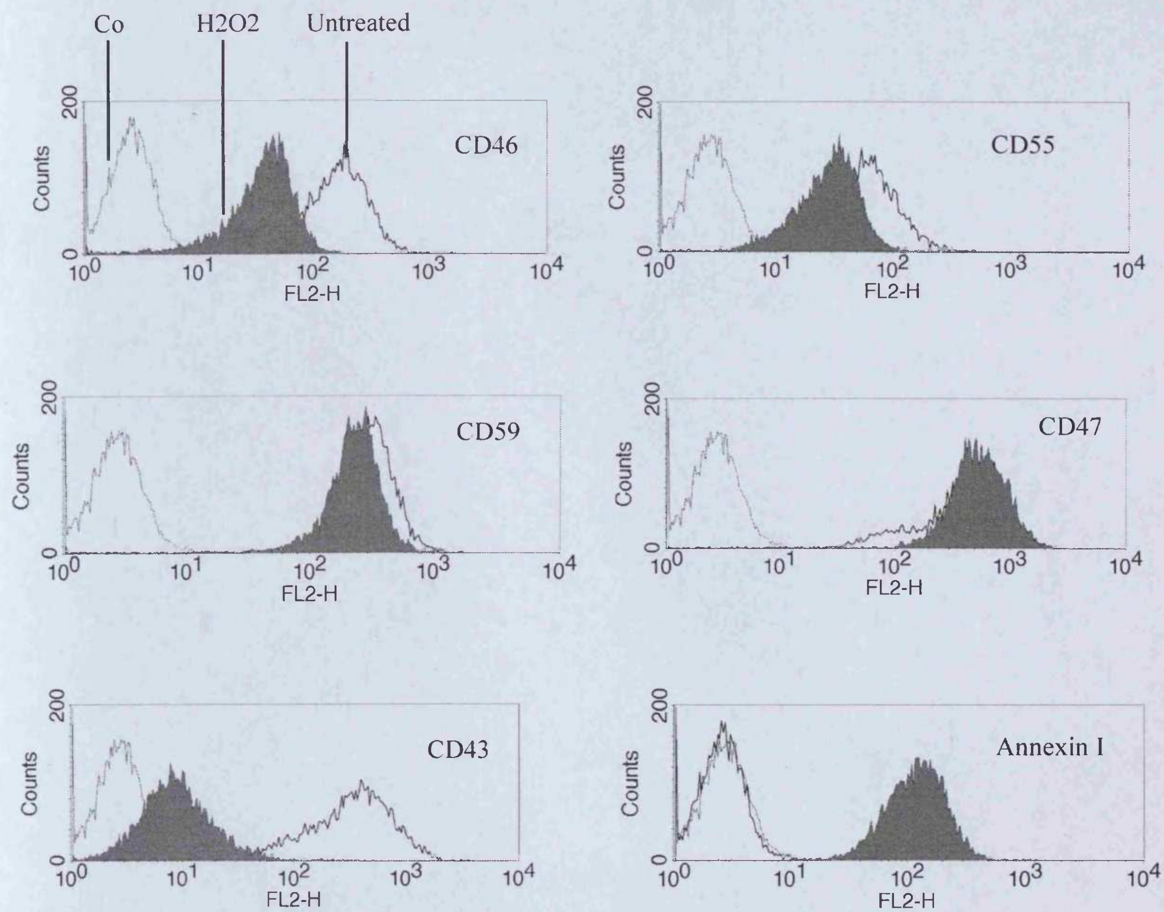


Figure 4.4 H2O2 treated cells (4 h, 0.01 M) were stained for the membrane regulators (CD46, CD55, CD59) and other known proteins (CD47, CD43, Annexin I) and analysed by single flow cytometry. Grey line represents treated cells with secondary antibody only, black line represents untreated cells with antibody and grey filled profiles represent treated cells with antibody

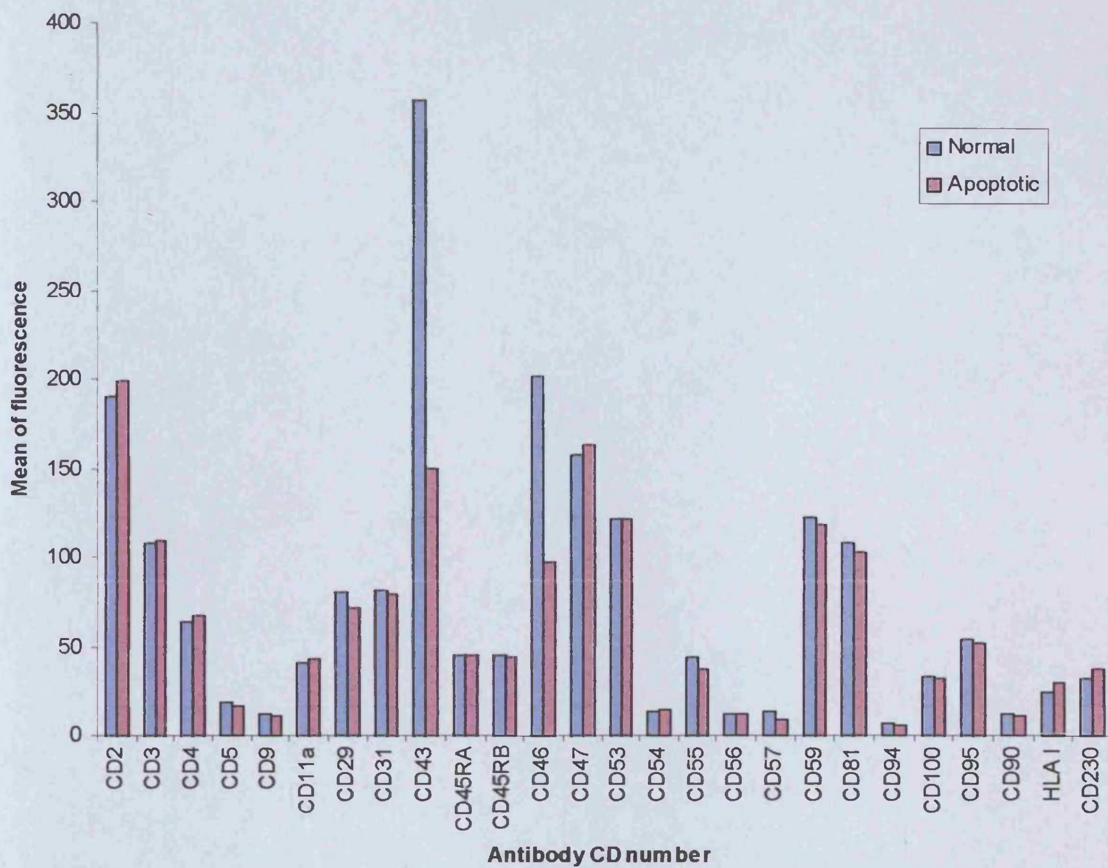


Figure 4.5 Graph to depict the expression of various surface markers on normal (blue) and apoptotic (purple) (post 6h γ irradiation) Jurkat cells. Unpermeabilised cells were stained for primary antibody (1h 4°C) followed by rPE conjugated secondary antibody (1h 4°C) and analysed by flow cytometry.

Apoptosis (Camptothecin treated cells)

Time course	*Co*	*30min*	*1h*	*2h*	*4h*	*8h*	*O/N*
CD46	110	**110**	**95**	**87**	**42**	35	32
CD55	68	61	58	58	59	55	46
CD59	364	354	345	360	325	329	330
CD43	90	84	54	16	7	4	3
CD47	439	411	459	489	511	554	527
Annexin I	<5	<5	8	10	37	56	111

Necrosis (H_2O_2 treated cells)

Time course	*Co*	*30min*	*1h*	*2h*	*4h*	*8h*	*O/N*
CD46	101	**86**	**47**	**28**	**22**	25	21
CD55	71	72	70	60	48	45	34
CD59	362	**321**	**305**	**287**	**240**	210	167
CD43	84	54	16	7	4	4	4
CD47	355	368	417	492	554	569	420
Annexin I	9	16	25	44	120	280	9

Table 4.4 Kinetics of expression of complement regulatory proteins on apoptotic and necrotic Jurkat cells.
Immunostainings for complement regulatory proteins (CD46, CD55, CD59) and other cell surface markers (CD43, CD47 and Annexin I) on apoptotic (camptothecin) and necrotic (H_2O_2) cell models. Cells were either untreated (control) or treated for various time periods (30 min to O/N) and stained using specific monoclonal antibodies to complement regulators and surface antigens followed by the appropriate rPE secondary conjugate, and analysed by flow cytometry. Data are expressed as mean of FL2 intensity (n =3)
N.B. The experiment was performed by P. Gasque for inclusion in the JBC paper, Elward *et al* 2005.

4.3.5 Immunodetection of Complement Regulatory Proteins on Normal, Apoptotic and Necrotic Cells by Western Blotting

To further clarify the loss of complement regulator (i.e. CD46) expression, cell lysates of normal, apoptotic (post 4 hours Camptothecin treatment) and necrotic (post 4 hours H_2O_2 treatment) Jurkat cells were prepared (Figure 4.6, n=4). Analysis of total cell lysate preparations confirmed CD55 expression to be unaffected after induction of apoptosis and necrosis. CD59 expression was reduced only when cells were treated with H_2O_2, while CD46 expression showed a profound difference between control and treated cells, with a greater than 50% loss of expression from apoptotic and necrotic cell lysates. CD43 expression was shown dramatically reduced from apoptotic cells while necrotic cells were largely CD43 free. β Tubulin was used as an internal control of equal protein loading although the signal was totally absent from necrotic cells indicative of the loss of the cytoskeleton along with other cellular contents when the membrane integrity was disrupted after H_2O_2 treatment.

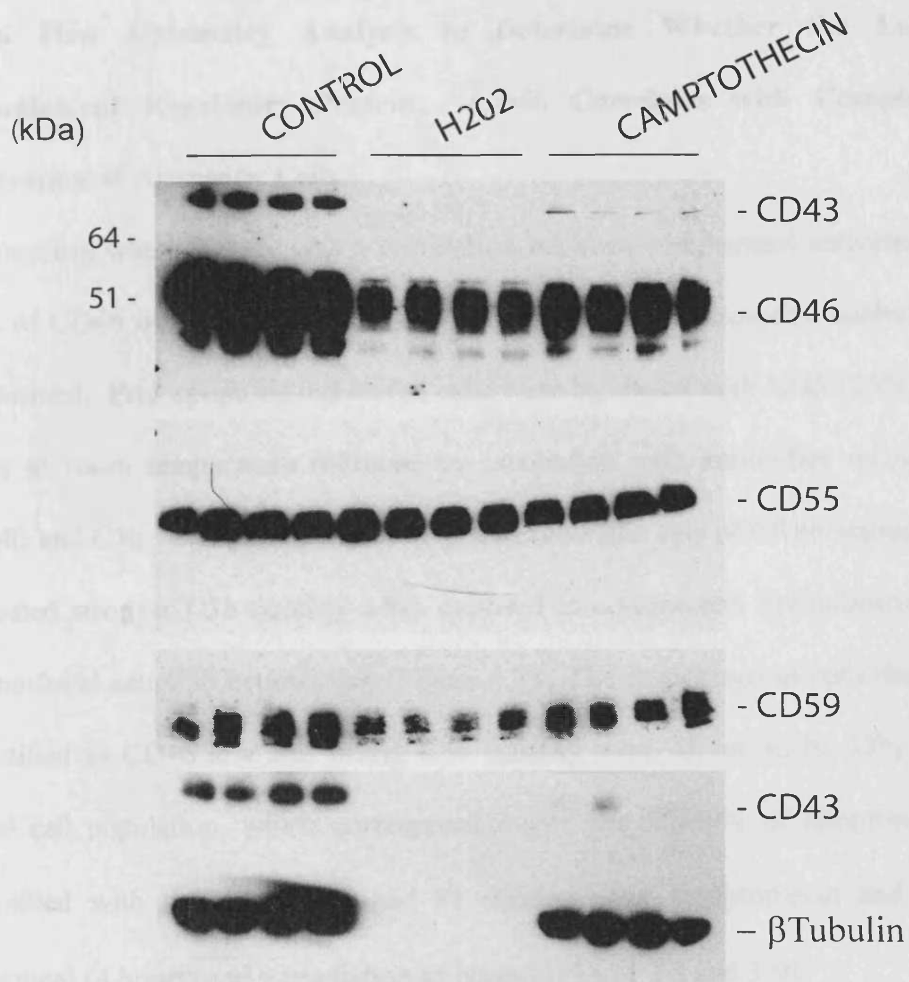


Figure 4.6. Western blot analysis of prepared cell lysates of normal, apoptotic (camptothecin 4 h) and necrotic (H2O2 4 h) Jurkat cells for expression of CD46, CD55, CD59 and CD43. Beta Tubulin was used as a control to determine equal loading amount. Data from four separate experiments

4.3.6 Flow Cytometry Analysis to Determine Whether the Loss of Complement Regulatory Protein - CD46 Correlates with Complement Activation of Apoptotic Cells

To confirm whether there was a correlation between complement activation and loss of CD46 on apoptotic cells, dual fluorescence flow cytometry analysis was performed. Post apoptotic induction, cells were incubated with NHS (25%) for 1 hour at room temperature followed by incubation with antibodies recognising CD46 and C3b. The population of cells that showed a loss of CD46 staining also revealed stronger C3b staining when exposed to complement (as indicated by a monoclonal anti-C3b neoepitope) (Figure 4.7). The percentage of cells that were identified as CD46 low and strong C3b staining were shown to be 33% of the total cell population, which corresponded with the 30-40% of apoptotic cells identified with the annexin V and PI staining after camptothecin and α-Fas treatment (4 hours) and γ irradiation (6 hours) (Figure 3.8 and 3.9).

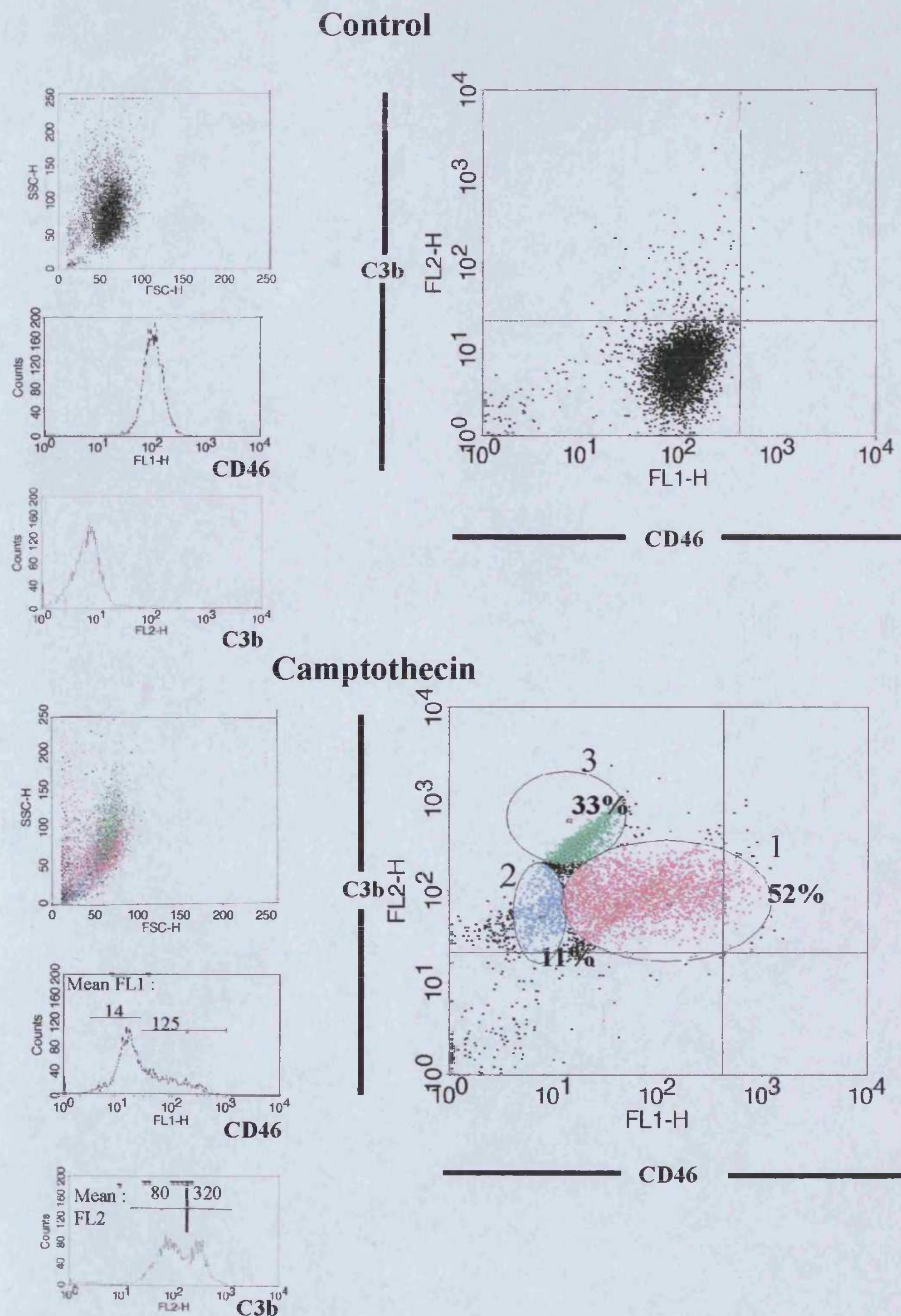


Figure 4.7. CD46 is lost from apoptotic cells and stronger C3b opsonisation is revealed on these cells when exposed to complement

Prior to (control cells, top panel) and post apoptotic induction (5µg/ml Camptothecin, 4 h, bottom panel), cells were incubated with NHS (25%) for 1 hour, stained using mouse anti-C3b neo antibody and rabbit anti-CD46. (A). Control cells expressed high levels of CD46 and failed to activate the complement systen as indicated by the lack of C3b staining. (B) Three distinct populations of cells were observed post camptothecin treatment; 1. CD46 high (52%) and weakly stained for C3b, 2. Early necrotic cells as indicated by FSC/SSC scatter plot and weakly stained for C3b (11%), and 3. Apoptotic cells with CD46 low and strong C3b opsonisation (33%).

4.4 Discussion

Analysis of complement regulator expression (CD46, CD55, CD59) has led to an important and novel finding concerning CD46 expression on apoptotic T Jurkat cells. Flow cytometry, and Western blot analysis of cell lysates, revealed a systematic loss of CD46 from apoptotic Jurkat cells. This remarkable loss of CD46 expression on apoptotic cells is in contrast with other membrane bound complement regulators such as CD55 and CD59, whose expression is largely unchanged between normal and apoptotic cells. There has been evidence to date showing a reduction in apoptotic cell surface expression of complement regulatory proteins (CRPs), however the results portrayed in this study contrast previous findings whereby other CRPs as well as CD46 were found reduced on apoptotic cells. Tsuji *et al* showed a reduction in both CD46 and CD55 expression on human umbilical vein endothelial cells (HUVEC) (Tsuji, *et al.* 1994), and Hara *et al* confirmed this on human lung adenocarcinoma cells (Hara, *et al.* 1996). Jones *et al* also revealed a reduction in CD55, and also for CD59 on aged polymorphonucleated cells, but no reduction in CD46 was observed.

The results in this study also revealed necrotic cells lose CD46 and CD59, and therefore are limited in their ability to control C3 opsonisation and MAC formation. This confirmed recent evidence whereby soluble CD46 was shown shed from necrotic tumour cells in response to matrix metalloproteases (Hakulinen, *et al.* 2004). CD46 is a widely distributed C3b/C4b-binding cell-surface glycoprotein of the complement activation pathway, acting as a cofactor

for the cleavage of C4b and C3b by Factor I into biologically inactive fragments C4d and iC3b, and in turn serves as an inhibitor of complement activation by inactivating the C3/C5 convertase enzyme. CD55 binds to and breaks up the classical and alternative C3/C5 convertase, and CD59 binds to C8 in the C5b-8 complex and blocks further incorporation of C9 to form the MAC. The MAC is likely to initiate a pro-inflammatory rapid cell death with classic necrotic morphology characteristic of swelling of mitochondria, dilation of rough endoplasmic reticulum, disruption of the golgi complex, plasma and nuclear membranes and heterochromatin disappearance (Fishelson, *et al.* 2001). In contrast, GPI-anchored CD55 and CD59 proteins remained largely on the cell surface during apoptotic cell death, and therefore supported the hypothesis that apoptotic cells can control the cytotoxic and cytolytic activities of the MAC. This also reiterates the importance of the complement regulators in controlling the type of cell death, which occurs, whether it is good (apoptotic) or bad (necrotic).

Analysis of complement activation between apoptotic and necrotic cells in the present study, showed it to be finely regulated on apoptotic cells compared to necrotic cells. Specific binding of complement proteins to apoptotic cells has been previously reported (See Table 1.1 in Introduction), however, a novel observation in the present study revealed the population of apoptotic cells (33%) that possessed decreased CD46 expression also had moderate and limited complement activation (C3b) on their cell surface. This is in sharp contrast to necrotic cells whereby high levels of complement activation were observed in

conjunction with the formation of the MAC. Immunofluorescent microscopy further indicated that complement C1q opsonised at the surface of apoptotic cells clustered to bleb structures from the cell surface together with C3 and C2. This supported previous findings whereby C1q was shown to bind directly to apoptotic cells and apoptotic blebs and released in C1q-enriched microparticles found in plasma (Nauta, *et al.* 2002). An additional aim involved determining whether the observed exposure of nucleic acid (NA) at the cell membrane contributed to the initiation of the classical pathway given that DNA has long been known as a C1q binding molecule. DNAse/RNAse treated camptothecin-induced apoptotic Jurkat cells displayed markedly reduced levels of PI staining (NA exposure) at the cell membrane. The removal of NA was also shown to affect the activation of the classical pathway on apoptotic cells with reduced C1q binding and C3 levels at the cell surface. In contrast only a 15-20% reduction of C1q binding was observed when necrotic cells were treated with DNAse/RNAse, which suggested that other mechanisms (e.g. cardiolipin exposed on mitochondria) were driving C1q binding and complement activation on necrotic cells. Therefore the present findings further conveyed a possible central role for C1q in the early detection of membrane bound NA on apoptotic cells (e.g. 30 minutes after camptothecin treatment) long before the exposure of the PS 'eat me' signal (peak at 4 hours). DNA has long been characterised as a non-immune activator of the complement pathway through its interaction with both the collagen-like region and the globular region of C1q (Jiang, *et al.* 1992) and recent evidence by Palaniyar *et al* highlighted that all major defence collagens

(SP-D, MBL, C1q but not SP-A) are involved in binding to carbohydrate polymers such as free DNA and RNA (Palaniyar, *et al.* 2004).

Therefore, *in vitro* evidence derived from this project so far has suggested that CD46, as well as being a regulator of complement activation, is lost from apoptotic cells, which are also opsonised by complement. Recently, apoptosis has been found accompanied by down regulation of a number of proposed 'Self Associated Molecular Patterns' (SAMPs), which may convey 'don't eat me' signals to specific 'Pattern Recognition Receptors' (PRRs) on phagocytes (for review refer to (Elward and Gasque 2003), (Grimsley and Ravichandran 2003)) . It could be proposed that CD46 has a role as a 'don't eat me signal' involved in innate immune recognition, and further experiments in this project aimed to analyse the loss of CD46 from apoptotic cells (Chapter 5).

In addition to the observed loss of CD46 from apoptotic and necrotic cells, studies in this project have also revealed a significant loss of CD43 and an increased surface expression of Annexin I from both apoptotic and necrotic Jurkat T cells. The observed Annexin I up regulation can be explained by its recent identification as one of the 'eat me signals' (ACAMPs) on apoptotic cells to be recognised and engulfed by phagocytes (Parente and Solito 2004). CD43, a known sialophorin/leukosialin, is involved in cell adhesion, and its shedding from neutrophil cell surfaces is known to enhance the ability of cell spreading in response to soluble, physiologic agonists (Nathan, *et al.* 1993). To date a small

array of possible SAMPS have been characterised and include sialic acids (i.e. CD43) binding to siglecs on macrophages. Lack of sialic acid expression on micro organisms, and in some cases on virally infected and transformed cells may be recognised as a missing SAMP (i.e. missing self signal) (Crocker and Varki 2001). Therefore, the results in this project may be analogous to these previous findings and could hypothesise that the loss of CD43 from apoptotic cells may have a role in signalling to phagocytes for engulfment.

CD47, an integrin associated protein, has recently been recognised as a classical SAMP, and known to interact with $\alpha V\beta 3$ (a known phagocyte receptor) and thrombospondin (a known bridging molecule) (for review see (Brown and Frazier 2001)). It has also been identified as a ligand for SIRPα, an ITIM (immunoreceptor tyrosine inhibitory motif)-containing inhibitory receptor expressed on macrophages (Oldenborg, *et al.* 2000). Senescent erythrocytes and infected cells have been shown to down regulate CD47 expression resulting in their phagocytosis and removal from the circulation (Blazar, *et al.* 2001). However, the present observations have shown CD47 expression not to be affected on apoptotic or necrotic cells. Therefore, the observations in this study and previous reports are consistent with the hypothesis, that a balance needs to exist between both "eat me" and "don't eat me" signals to allow the safe phagocytosis of apoptotic cells when required. It is also evident that there must be specific mechanisms in place to allow the switching off and on of certain

signals as required so that not all "eat me" or "don't eat me" signals are active at the same time, but how this occurs still needs to be elucidated.

Chapter 5

CD46 Signalling and Mechanism of Release from Apoptotic Cells

5.1 Introduction

The previous chapter demonstrated that the complement regulatory protein, CD46, was lost from apoptotic cells, which were also opsonised with complement (C3b). Future investigations focused solely on assessing the loss of CD46 from various developed apoptotic cells and necrotic cells using single and dual flow cytometry analysis. CD46 is a widely expressed type 1 transmembrane glycoprotein, being expressed on most cells with two distinct cytoplasmic tails of 16 (CYT-1) or 23 (CYT-2) amino acids (Figure 5.1). It is believed to have a role in intracellular processing and basolateral localisation through its cytoplasmic tails (Wang, *et al.* 2000).

To analyse the mechanism of cellular release of CD46 from apoptotic cells, nuclear and cytoplasmic/membrane cell extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents, and Western blotting performed for CD46 expression. To confirm that the loss of CD46 from apoptotic cells was either in apoptotic blebs or as a result of cleavage into its soluble form, Western blot analysis of bleb preparations and culture supernatant, and immunocytochemistry of unpermeabilised apoptotic cells was performed.

In order to establish further the mechanism of signalling of CD46 that results in its release from the apoptotic surface, investigations were performed to

determine if there was a correlation between CD46 release and signalling by caspases, and whether kinase signalling was involved. Cytoplasmic tails of CD46 encode putative signals for phosphorylation by kinases, and it is believed that the ability of these cytoplasmic sequences to couple with intracellular signalling pathways determines the nature of the cellular response (Liszewski, *et al*. 1994) (Figure 5.2).

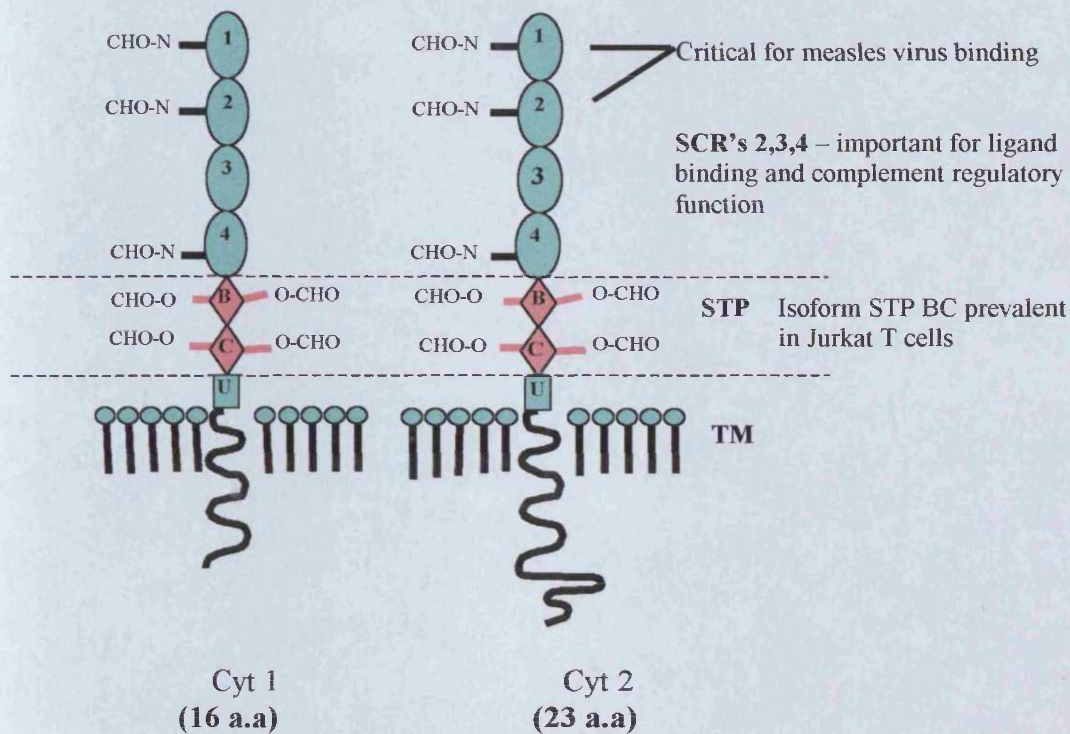


Figure 5.1 A Schematic of the Two Cytoplasmic Forms of CD46

CD46 possesses four Short Consensus Regions (SCR) from the amino terminus of the protein, with three potential sites for N-glycosylation (CHO-N). These are followed by a heavily O-glycosylated region (CHO-O), which is rich in Serine/Threonine/Proline residues, and hence termed the STP region. Three different STP regions have been identified in CD46 (A, B, C) with the STP BC form being the predominant in Jurkat cells. Between the STP region and the Transmembrane Domain (TM) is a short region of unknown function (U). There are also two different cytoplasmic tails - CYT-1 and CYT-2, which can exist with any of the STP variants.

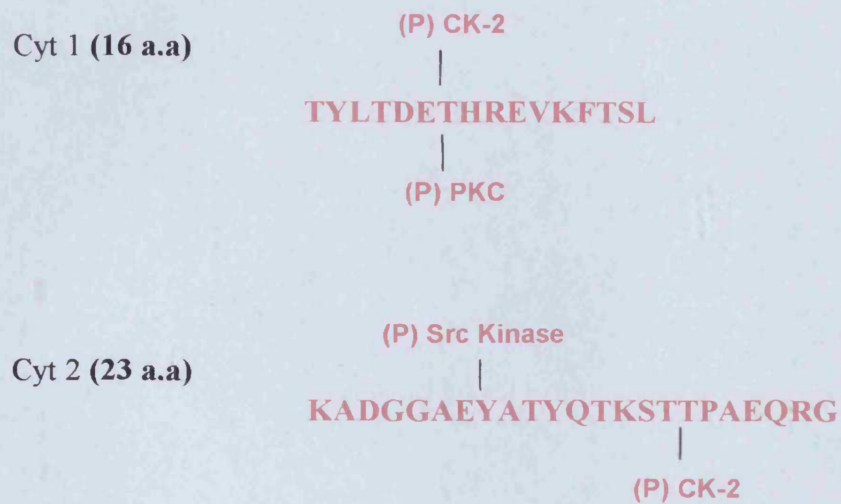


Figure 5.2 A Schematic Indicating the Phosphorylation Sites on CD46 Cytoplasmic Tails.

CYT-1 consists of 16aa and possesses putative signals for phosphorylation by casein kinase 2 (CK-2) and protein kinase C (PKC). CYT-2 consists of 23 aa and possesses putative signals for phosphorylation by src kinases and CK-2.

5.2 Methods

5.2.1 Preparation of Normal and Apoptotic Jurkat Cell Lysates and Blebs for CD46 Protein Analysis

At the end of the apoptosis induction period, 10^7 cells were pelleted by centrifugation at 1000 rpm for 3 minutes and lysed with 1ml lysis buffer. Cells were vigorously vortexed and incubated for 30 minutes at room temperature. Cell lysates were transferred to a 1.5 ml microfuge tube and centrifuged at room temperature for 15 minutes at 13,000 rpm. The supernatant containing proteins and cell membranes was isolated. Samples were stored at –20°C or diluted 1:1 with Laemmli buffer for immediate use.

The supernatant collected from the initial spin of cells post apoptotic induction was ultracentrifuged (100,000g, 1 hour, RT) in a Beckman TL-100 ultracentrifuge with a TLA 100.3 rotor. The isolated bleb/vesicle-enriched pellets were finally resolubilised in 100µl of Laemmli buffer. Lysate and bleb preparation samples were applied to 10% SDS PAGE gels for protein analysis.

5.2.2 Collection of Tissue Culture Supernatant from Normal, Apoptotic and Necrotic Cell Cultures for CD46 Protein Analysis

At the end of the apoptosis induction period, tissue culture supernatant was collected from the flasks of cells, and either filtered through a 0.2µm filter to eliminate blebs and concentrated by lyophilisation, or directly lyophilised without filtering before being analysed by Western blot.

5.2.3 Preparation of Cell Lysates and Blebs for Complement Protein Analysis

As a source of complement, apoptotic Jurkat cells were incubated with normal human serum (NHS) (25%, 1 h RT) post apoptotic induction (4h Camptothecin). Cell lysates and blebs were prepared as described in Section 5.2.1

5.2.4 Preparation of Nuclear and Cytoplasmic Extracts of Normal and Apoptotic Jurkat Cells: NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (#78833 kit, Pierce Biotechnology)

5.2.4.1 Background Information

According to the manufacturers' instructions, NE-PER Nuclear and Cytoplasmic Extraction Reagents enable stepwise separation and preparation of cytoplasmic/membrane and nuclear extracts from mammalian cultured cells or tissues. Non-denatured, active proteins are purified in less than two hours. Addition of the first two reagents (Cytoplasmic Extraction Reagent I and II (CER I and II)) to a cell pellet causes disruption of cell membranes and release of cytoplasmic and membrane contents. The subsequent supernatant isolated from centrifugation post addition of CER I and II will contain both membrane and cytoplasmic fractions. After recovering the intact nuclei from the cytoplasmic extract by centrifugation, the nuclei are lysed with a third reagent (Nuclear Extraction Reagent (NER) to yield the nuclear extract.

5.2.4.2 Method

Jurkat cells were harvested from culture and resuspended at a density of 10^6/ml in RPMI 1640 without FBS. Apoptosis was induced in 10ml of cells with 5μg/ml camptothecin for 4 hours at 37°C, 5% CO_2. Nuclear and cytoplasmic cell extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce).

20μl (40mg) of packed cell volume of cells was used (2 x 10^6). Cells were centrifuged in 1.5ml micro centrifuge tubes at 500g for 2-3 minutes. Using a pipette, the supernatant was carefully removed and discarded, leaving the pellet as dry as possible. 1 ml of ice cold Cytoplasmic Extraction Reagent 1 (CER I) was added to the cell pellet, the tube vortexed vigorously for 15 seconds to fully resuspend the cell pellet, and incubated for 10 minutes on ice. 55μl of ice-cold Cytoplasmic Extraction Reagent 2 (CER II) was added, the tube vortexed for 5 seconds, and incubated for 1 minute on ice. The cells were centrifuged at 13,000g for 5 minutes and supernatant containing the cytoplasmic extract was immediately transferred to a clean pre-chilled tube, and placed on ice until use. The pellet was resuspended in 500μl of ice-cold Nuclear Extraction Reagent (NER), vortexed for 15 seconds, returned to ice and vortexed at 10-minute intervals for 15 seconds over a 40-minute period. The tube was centrifuged at 16,000g for 10 minutes and supernatant containing the nuclear extract was transferred to a clean pre chilled tube and stored at –80°C until further use. Western blotting was used to detect the presence of specific complement regulatory proteins using antibodies.

5.2.4.2 Method

Jurkat cells were harvested from culture and resuspended at a density of 10^6/ml in RPMI 1640 without FBS. Apoptosis was induced in 10ml of cells with 5μg/ml camptothecin for 4 hours at 37°C, 5% CO_2. Nuclear and cytoplasmic cell extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce).

20μl (40mg) of packed cell volume of cells was used (2 x 10^6). Cells were centrifuged in 1.5ml micro centrifuge tubes at 500g for 2-3 minutes. Using a pipette, the supernatant was carefully removed and discarded, leaving the pellet as dry as possible. 1 ml of ice cold Cytoplasmic Extraction Reagent 1 (CER I) was added to the cell pellet, the tube vortexed vigorously for 15 seconds to fully resuspend the cell pellet, and incubated for 10 minutes on ice. 55μl of ice-cold Cytoplasmic Extraction Reagent 2 (CER II) was added, the tube vortexed for 5 seconds, and incubated for 1 minute on ice. The cells were centrifuged at 13,000g for 5 minutes and supernatant containing the cytoplasmic extract was immediately transferred to a clean pre-chilled tube, and placed on ice until use. The pellet was resuspended in 500μl of ice-cold Nuclear Extraction Reagent (NER), vortexed for 15 seconds, returned to ice and vortexed at 10-minute intervals for 15 seconds over a 40-minute period. The tube was centrifuged at 16,000g for 10 minutes and supernatant containing the nuclear extract was transferred to a clean pre chilled tube and stored at –80°C until further use. Western blotting was used to detect the presence of specific complement regulatory proteins using antibodies.

5.2.5 Caspase Inhibition on Jurkat Cells

5.2.5.1 General Caspase Inhibitor (Z-VAD-FMK)

Jurkat cells were harvested from culture and resuspended at a density of 10^6/ml in RPMI 1640 without FBS. Cells were incubated with a general caspase inhibitor (Z-VAD-FMK, 20μM) for 30 minutes at 37°C, 5% CO_2 prior to apoptosis induction (as described previously).

5.2.6 Treatment of Jurkat Cells with Protein Kinase Inhibitors

5.2.6.1 Src Kinase Inhibitor (PP2)

Jurkat cells were incubated with a selective inhibitor of the src family of protein tyrosine kinases (1μM PP2 – 4-Amino-5- (4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine, Calbiochem) (Hanke, et al. 1996) for 1 hour at 37°C, 5% CO_2 prior to apoptosis induction. At the end of the apoptosis induction period, cells were confirmed to be apoptotic by dual fluorescence analysis with Annexin V FITC and PI. To assess specific expression of CD46 on normal and apoptotic cells in the presence or absence of inhibitor, dual immunofluorescence was performed whereby cells were incubated first with monoclonal antibody to CD46 (IIC5) followed by Annexin V FITC (1/50) and phycoerythrin (PE) conjugated F $(ab)_2$ goat antibody to mouse IgG.

5.2.6.2 Protein Kinase C Inhibitor

Jurkat cells were incubated with a specific inhibitor of protein kinase C (PKC) (10µM Myr-N-FARKGALRQ-NH_2, Calbiochem) (Eichholtz, et al. 1993) for 1 hour at 37°C, 5% CO_2 prior to apoptosis induction. The inhibitor consisted of N-myristoylated synthetic peptides corresponding to the pseudosubstrate domains of protein kinase C (PKC) (N-Myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln).

5.2.6.3 Casein Kinase II Inhibitor

Jurkat cells were incubated with a cell-permeable benzotriazolo compound that acts as a highly selective, ATP/GTP-competitive inhibitor of casein kinase II (CK II) (1.6µM 4,5,6,7-Tetrabromobenzotriazole (TBBt), Calbiochem) (Borowski, et al. 2003) for 1 hour at 37°C, 5% CO_2 prior to apoptosis induction.

5.3 Results

5.3.1 Confirmation of Decreased Level of CD46 on Different Apoptotic Models

In addition to the profiles seen by flow cytometry analysis for the camptothecin apoptotic model described in Chapter 4, a gamma irradiated model (optimal level of apoptosis, 6 hours), and a α -Fas model (optimal level of apoptosis, 4 hours) was used to confirm the loss of CD46. The distinct bimodal distribution of CD46 expression with a population of decreased expression compared to normal was confirmed in these two new models (Figure 5.3).

5.3.2 Dual Fluorescence Analysis to Confirm the Decreased Level of CD46 Correlates with Apoptotic Cells

To ascertain whether the loss of CD46 membrane antigen staining was restricted to apoptotic cells, dual fluorescence analysis was performed post camptothecin treatment (4 hours), whereby monoclonal antibody staining to CD46 was followed by Annexin V FITC and rPE-conjugated goat anti mouse immunoglobulin (Figure 5.4). Untreated Jurkat cells were stained for comparison. Following camptothecin treatment two distinct cell populations were evident – 46% of the total number of cells were stained CD46 high, Annexin low, and 33% of the cells were stained CD46 low, Annexin high. The mean of fluorescence for CD46 (FL2) staining was determined and revealed CD46 high cells had a mean FL2 of 351 and CD46 low had a mean FL2 of 51.

Therefore a 7-fold decrease in expression of CD46 was observed between the two cell populations. Untreated cells revealed a single population of CD46 positive cells.

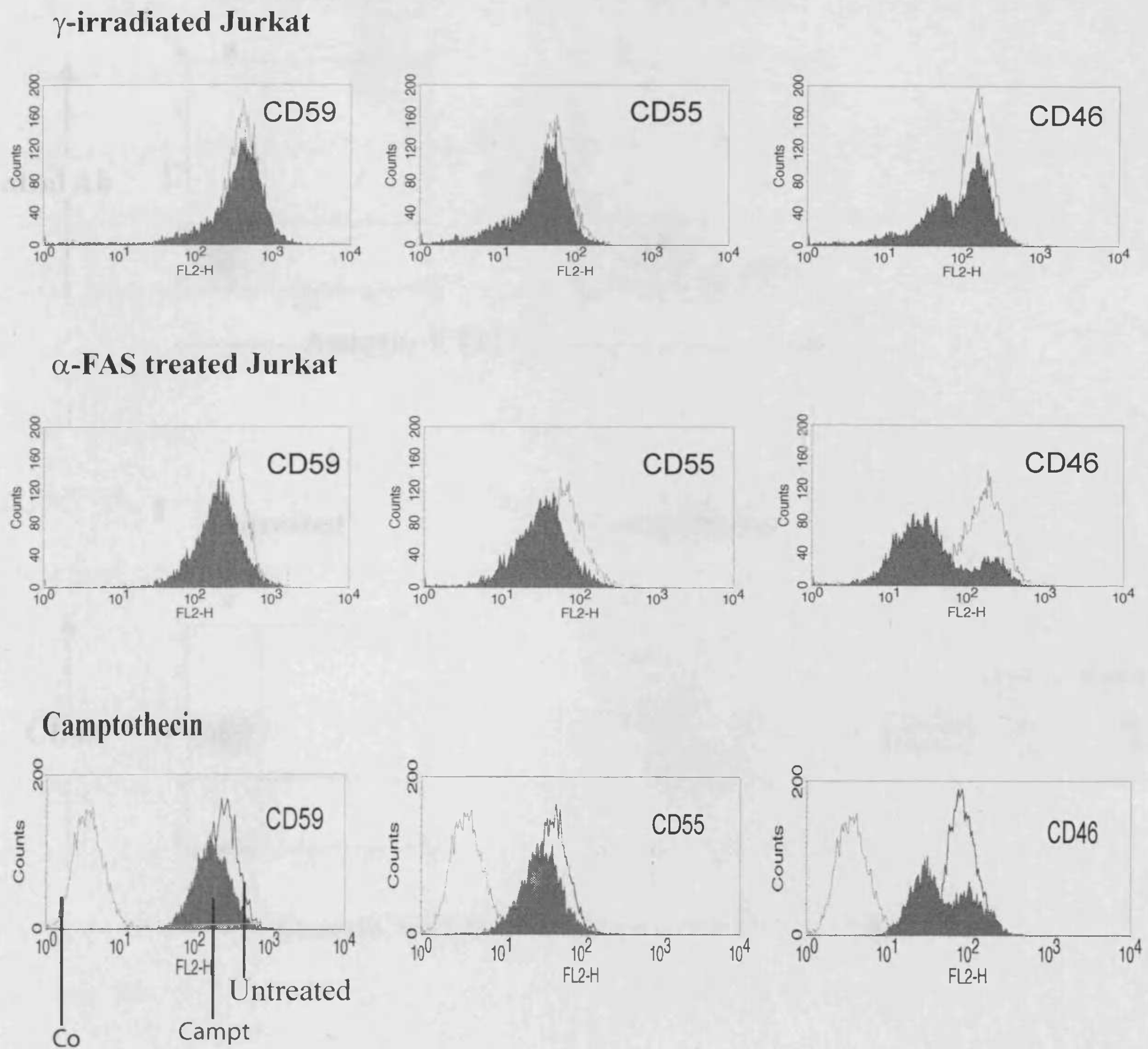


Figure 5.3 Flow cytometry analysis of Jurkat cells post gamma irradiation (4000cGy, 6h), anti-Fas (80ng/ml, 4h) and camptothecin (5μg/ml) labelled with antibodies to cell surface antigens followed by PE conjugated F(ab)2 goat antibody to mouse IgG. Single parameter histograms are shown for each cell marker. Grey lines represent untreated cells, and grey filled profiles represent treated cells

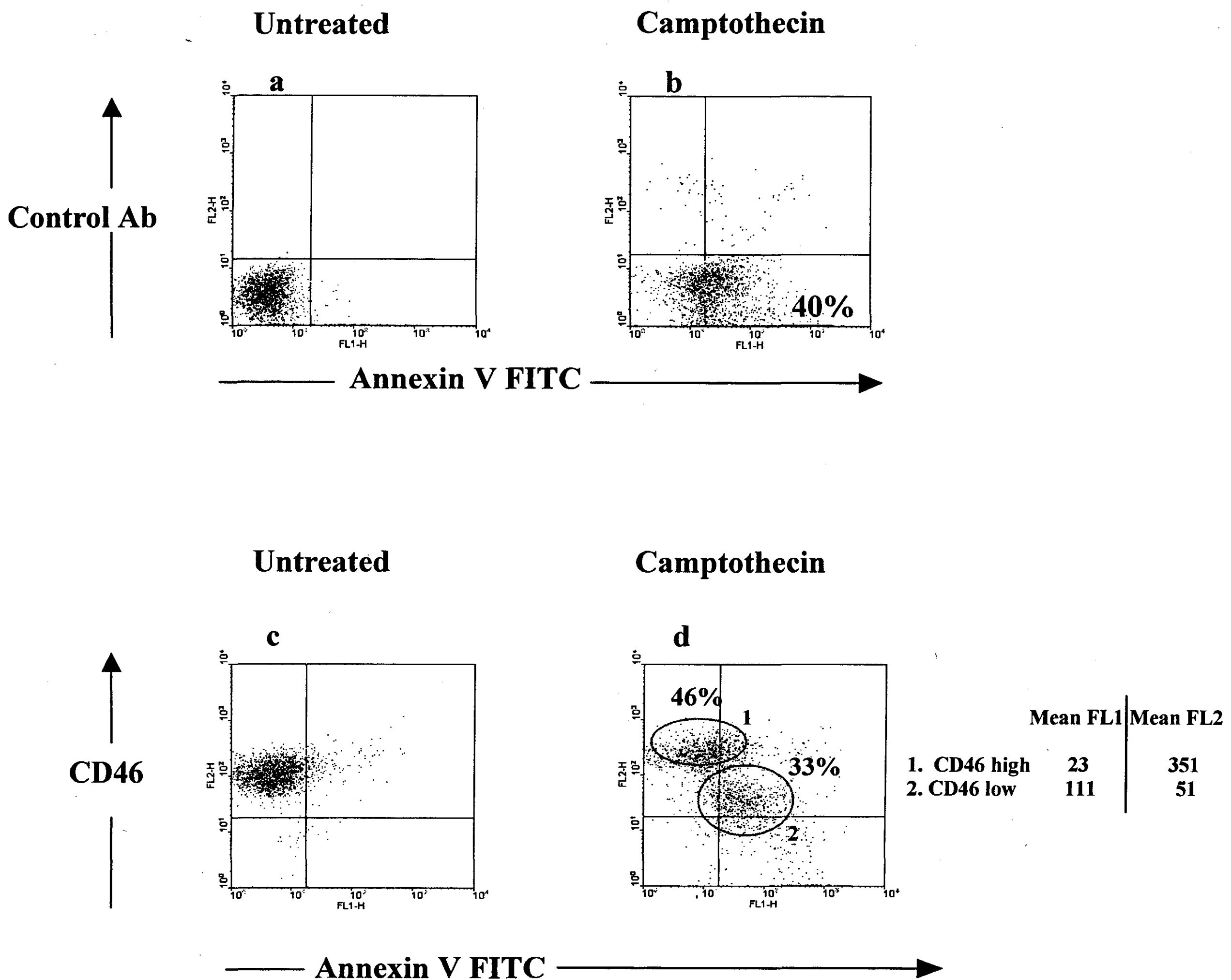


Figure 5.4 Untreated Jurkat (c) and Camptothecin treated (5µg/ml) Jurkat cells (d) were incubated with hu CD46 monoclonal antibody, followed by Annexin V FITC and Phycoerythrin (PE) – conjugated $F(ab)_2$ goat antibody to mouse IgG. Untreated Jurkat (a) and treated (b) cells were used as negative controls with no primary antibody staining. Following camptothecin treatment two distinct cell populations were evident: 1. CD46 high, Annexin low, and 2. CD46 low, Annexin high (d). Untreated cells revealed a single population of CD46 positive cells

5.3.3 Cell Sorting and Immunodetection of Complement Regulatory Proteins by Western Blotting

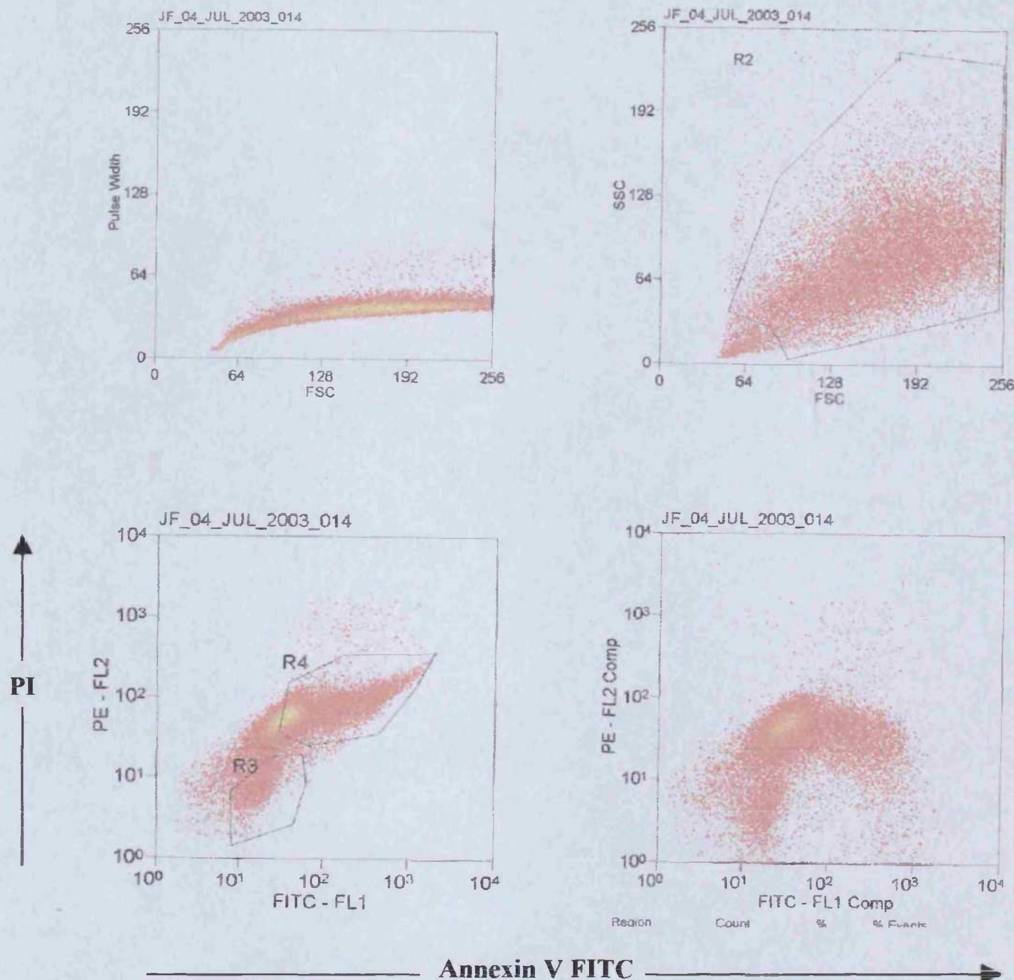
To obtain pure cell populations of normal and apoptotic Jurkat T lymphocytes for further confirmation that loss of CD46 is evident only on apoptotic cells, cells were sorted on the basis of expression of Annexin V (i.e. normal cells, Annexin V –ve and apoptotic cells, Annexin V +ve) using the Cytomation MoFlo high speed cell sorter (The Flow Cytometry Facility, Central Biotechnology Services, Cardiff University) (Figure 5.5). Western blot analysis of cell lysates was performed and revealed total CD46 expression was reduced on apoptotic (Annexin V +ve) cells by a factor of three to four fold compared to normal (Annexin V –ve) cells, whereas CD55 levels were confirmed to remain the same (Figure 5.6). Equal protein gel loading was confirmed by using the anti-Tubulin antibody and equivalent cell numbers. Given that the cell surface expression of CD46 on apoptotic cells was reduced by at least 7 fold (flow cytometry), the Western blot data (3 to 4 fold decrease) suggested that shedding and protein internalisation may together be responsible for the rapid and dramatic loss of CD46 at the cell surface of apoptotic Jurkat cells.

5.3.4 Mechanisms of Release of CD46 from Apoptotic Cells

5.3.4.1 Immunodetection of CD46 Expression on Bleb Preparations by Western Blotting

To determine whether CD46 was present in blebs shed from the surface of apoptotic cells, cell lysates, and bleb preparations (post ultra centrifugation) of

normal and apoptotic Jurkat cells (post 4 h camptothecin treatment) were analysed for CD46 protein expression by Western blotting (Figure 5.7). The experiment was normalised by using equal cell numbers, however, Tubulin could not be used to confirm the equal gel loading of protein, as it is not present in blebs. CD46 expression was confirmed reduced in apoptotic total cell lysates (n=2) and as a result of being shed in the form of blebs from the apoptotic cells.



Region	Count	%	% Events
Total	20000	100.00	2.00
R3	2428	12.14	0.24
R4	10845	54.23	1.08

Region	Count	%	% Events
Total	20000	100.00	2.00

Figure 5.5. Cell Sorting of Jurkat T Cells
To obtain pure cell populations of normal and apoptotic Jurkat T lymphocytes, cells were sorted on the basis of expression of Annexin V using a Cytomation MoFlo high speed cell sorter. Post 4 hour camptothecin treatment cells were stained with Annexin V FITC (1/50) and PI (1/200) for 30 minutes at 4°C, washed in RPMI 1640 and added to the cell sorter. Annexin V +ve/PI +ve cells (R4) were considered apoptotic and Annexin V/PI –ve cells (R3) were considered normal.

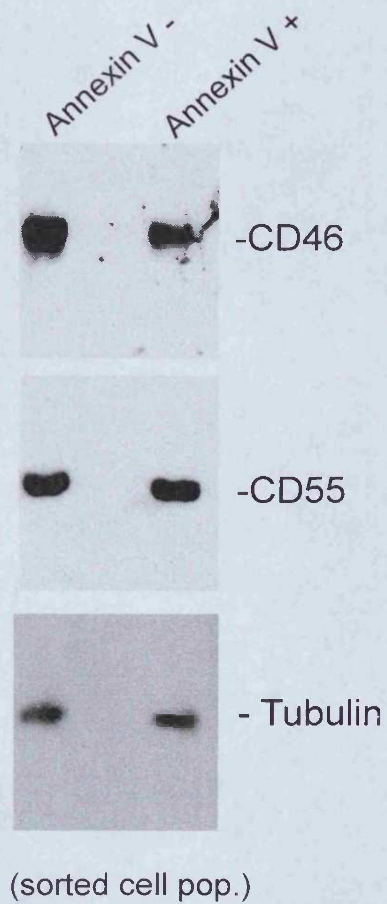


Figure 5.6. Loss of CD46 on apoptotic cells
Western blot analysis for CD46 and CD55 expression on camptothecin-treated cell lysates following cell sorting in Annexin V negative and Annexin V posititve cell fractions. Equivalent cell numbers were used and equal protein gel loading was confirmed using the anti-tubulin antibody.

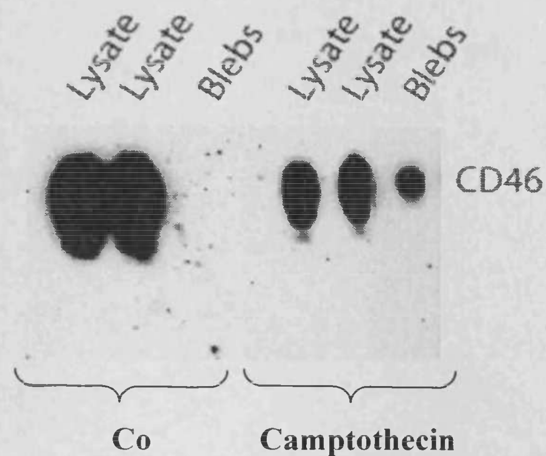


Figure 5.7 CD46 is Clustered to Apoptotic Blebs (n=2)
Total cell lysates and bleb cell lysates (isolated by ultra-centrifugation) were prepared from control and apoptotic Jurkat cells (Camptothecin, 4 h) and analysed by western blot for CD46 expression using the Rb α CD46 pAb (1/1000). CD46 was reduced on apoptotic total cell lysates compared to control as a result of being shed in the form of blebs from the apoptotic cells. The experiment was normalised by using equal cell numbers, but tubulin could not be used to confirm equal protein gel loading as it is not present in blebs.

5.3.4.2 Immunodetection of CD46 Expression in Tissue Culture Supernatant Post Apoptotic and Necrotic Induction by Western Blotting

To investigate the mechanism of release of CD46, supernatants from four separate experiments were collected, filtered using a 0.2μm filter to eliminate the contribution of blebs (0.5-1μm) and concentrated by lyophilisation before being analysed by Western blot. The experiment was normalised by using equal cell numbers. Interestingly, control cells (incubated in FCS-free medium) were shown to release soluble forms of CD46 (sCD46), CD55 (sCD55) but not CD59 (sCD59). Necrotic cell supernatants expressed very high levels of sCD46, sCD55 and sCD59 (Figure 5.8 A). However, it was surprising that only low levels of sCD46 expression were observed in apoptotic cell supernatant compared to control cell supernatant given that the previous flow cytometry (Figure 5.4) and Western blot (Figure 5.6, 5.7) analysis had shown CD46 dramatically lost from apoptotic cells. To determine further whether CD46 was released from apoptotic cells in the form of microparticles (microblebs) as part of the membrane blebbing, supernatants were lyophilised without filtering and analysed by Western blot (Figure 5.8 B, experiment performed by P. Gasque for inclusion in JBC paper, Elward et al 2005). Kinetics studies clearly indicated a strong CD46 signal accumulating during the course of apoptosis, along with complement regulator factor H (fH) and CD55. CD46 accumulating in the supernatants of apoptotic cells did not undergo proteolysis, although the accumulation of a band at 35 kDa was evident particularly in 8 hours and O/N samples (late apoptotic cells).

5.3.4.3 Immunodetection of Opsonisation of Apoptotic Cells and Blebs with Complement by Western Blotting

To determine whether complement deposition was concentrated to apoptotic blebs as was the case for CD46, cell lysates, and bleb lysates (post ultra centrifugation) were prepared of normal and apoptotic Jurkat cells (post camptothecin treatment), incubated with NHS for 1 h and analysed for C1q protein expression by Western blotting. The experiment was normalised by using equal cell numbers. A strong C1q signal was shown associated with the apoptotic bleb preparation (Figure 5.9).

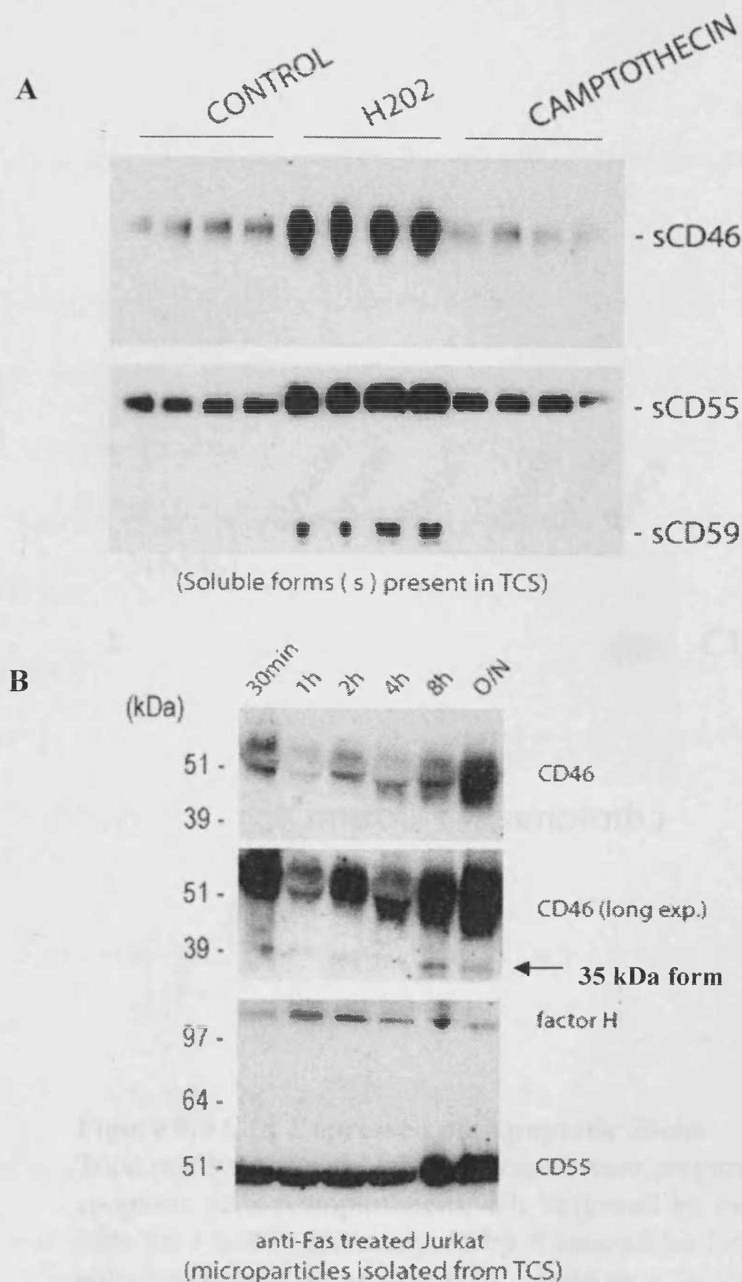


Figure 5.8. Apoptotic Jurkat Cells Released CD46 in Microparticle-like Structures during the Time Course of Programmed Cell Death.
Jurkat cell culture supernatants were separated into soluble (A) and vesicle-enriched fractions (B). Soluble fractions were prepared by filtering tissue culture supernatant using a 0.2 μm filter and concentrated by lyophilisation. Microparticle fractions were prepared by lyophilisation of culture supernatant without filtering. The fractions were analysed by Western blot using affinity purified rabbit polyclonal antibodies against CD46, CD55 and factor H, and monoclonal BRIC229 mouse anti-human CD59. The experiment was normalised by using equal cell numbers, but tubulin could not be used to confirm equal protein gel loading as it is not present in blebs.
Please note, experiment B was performed by P. Gasque for inclusion in the JBC paper (Elward *et al,2005)*

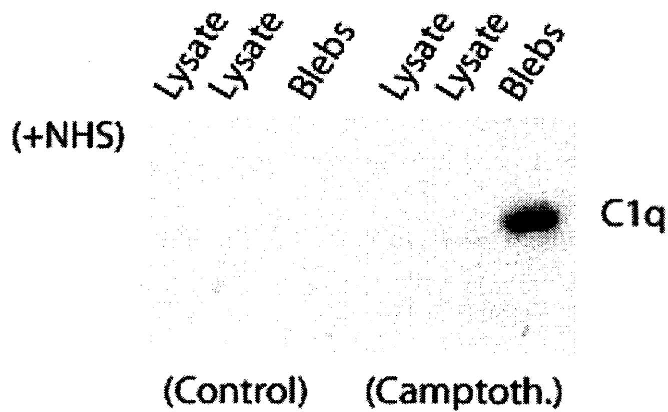


Figure 5.9 C1q Expressed on Apoptotic Blebs
Total cell lysates and bleb cell lysates were prepared from control and apoptotic cells (camptothecin, 4 h, followed by incubation with NHS 25% for 1 h RT) and analysed by Western blot for C1q using a rabbit polyclonal antibody against C1q. A strong C1q signal was associated with the bleb preparation. The experiment was normalised by using equal cell numbers, but tubulin could not be used to confirm equal protein gel loading as it is not present in blebs.

5.3.4.4 Immunofluorescent Staining of CD46 by Microscopy of Apoptotic Cells

To further investigate the mechanism of loss of CD46 from apoptotic cells, dual immunofluorescent microscopy images of normal and apoptotic unpermeabilised/unfixed Jurkat cells were analysed for CD46 and phosphatidylserine (Annexin V FITC) expression. Fluorescent images revealed CD46 was evenly distributed on normal cells with no staining for exposed phosphatidylserine. In contrast CD46 was shown clustered to the surface of apoptotic cells in bleb structures associated at site of strong phosphatidylserine exposure (Figure 5.10).

5.3.4.5 Western Blot Analysis of Nuclear and Cytoplasmic Cell Extracts for CD46

To shed further light on the mechanism of CD46 trafficking in apoptotic and necrotic cells, nuclear (Nuc) and cytoplasmic/membrane (Cyt) cell extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents. Western blotting was used to detect the presence of CD46 along with established markers of apoptosis (cleaved PARP), CD43 and lamin, a known nuclear component and substrate of activated caspase 3 (Figure 5.11). CD46 was first shown to reside in nuclear and cytoplasmic/membrane fractions of control cells. The validity of the extraction protocol was confirmed with the expected distribution of CD43 and lamin in the Cyt and Nuc fractions, respectively. A dramatic loss of CD46 signal was evident from necrotic cells although the cleavage of PARP was not evident.

In contrast, on apoptotic cells CD46 was shown lost from Nuc and Cyt fractions and the cleaved PARP signal was evident. Apoptotic Jurkat cells displayed weak CD43 and lamin signals.

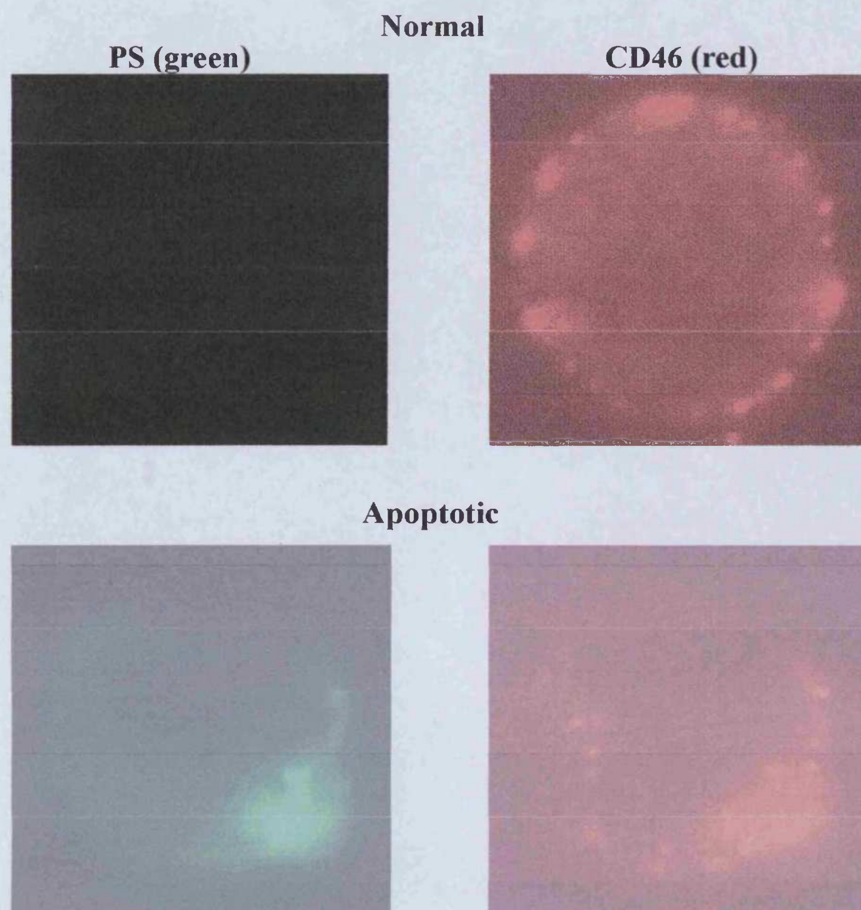


Figure 5.10 CD46 Clustered to Apoptotic Blebs where Phosphatidylserine Exposure is Prevalent. Double immunofluorescence staining of unpermeabilised Jurkat cells using the mouse anti-CD46 (11C5 clone) antibody (1 h 4°C) followed by Annexin V-FITC and the appropriate Alexa Red secondary antibody (1h 4°C) post camptothecin treatment (5μg/ml, 4 h) on untreated (normal) cells . Staining would detect only membrane components as cells are unpermeabilised and unfixed. CD46 staining (red) reveals clustering to apoptotic bleb where PS is prevalent (green).

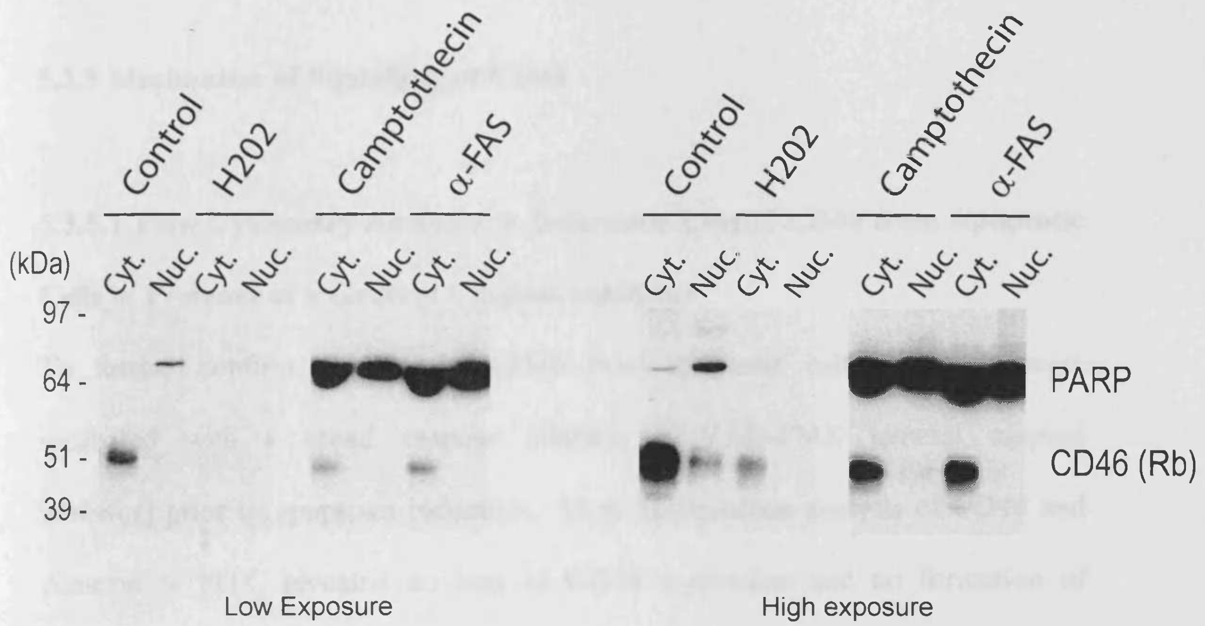


Figure 5.11 Western blot analysis of prepared cytoplasmic and nuclear fractions from control, apoptotic (camptothecin/Fas, 4 h) and necrotic (H2O2, 4 h) cells for CD46 (Rb polyclonal) expression along with a known cell marker of apoptosis (cleaved PARP). CD46 is lost from both apoptotic and necrotic cytoplasmic and nuclear cell fractions with an increase in cleaved PARP expression on apoptotic cells.

5.3.5 Mechanism of Signalling of CD46

5.3.5.1 Flow Cytometry Analysis to Determine Loss of CD46 from Apoptotic Cells in Presence of a General Caspase Inhibitor

To further confirm the loss of CD46 from apoptotic cells, the cells were incubated with a broad caspase inhibitor (Z-VAD-FMK general caspase inhibitor) prior to apoptosis induction. Dual fluorescence analysis of CD46 and Annexin V FITC revealed no loss of CD46 expression and no formation of Annexin V positive cells in the presence of the caspase inhibitor (Figure 5.12).

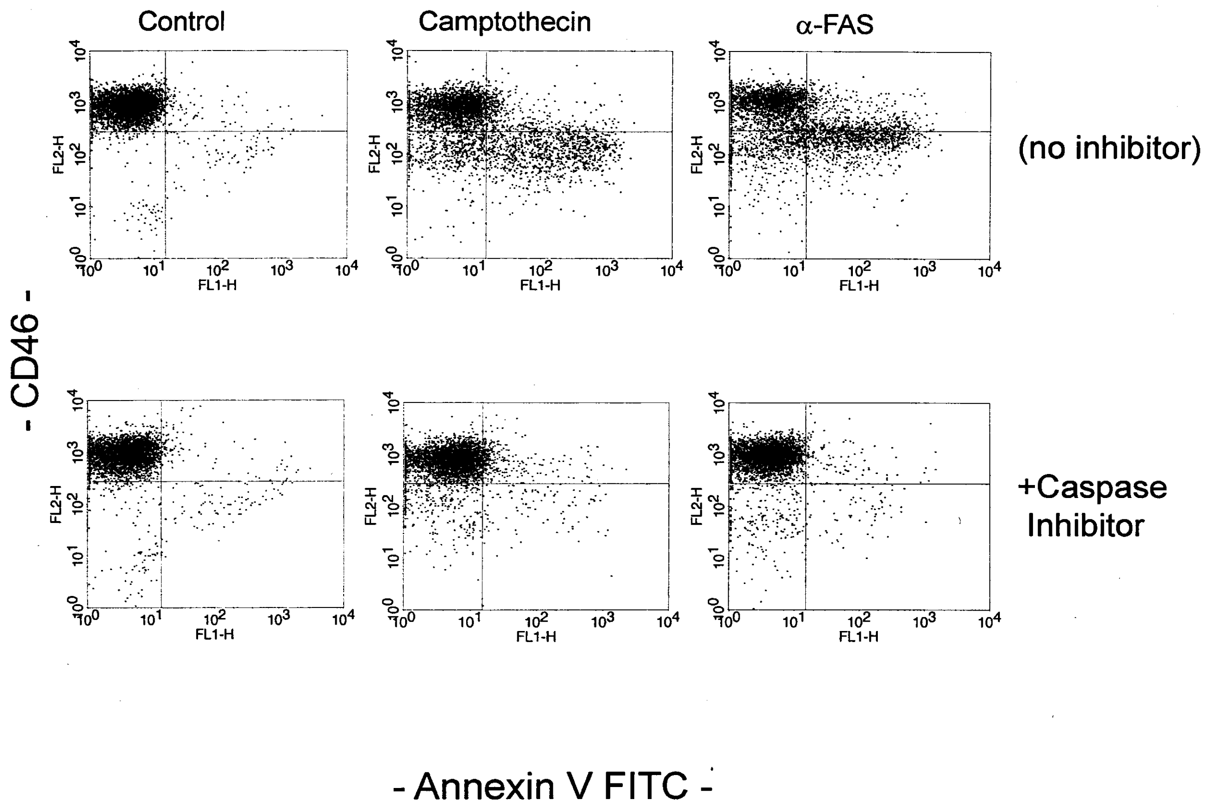


Figure 5.12. Dual fluorescence flow cytometry analysis of control, camptothecin, and anti-Fas treated Jurkat cells with or without a general caspase inhibitor (Z-VAD-FMK) and stained for CD46 and Annexin-V FITC. Cells were incubated with the inhibitor for 30 minutes at 37 degrees centigrade/5% CO2 prior to apoptosis induction (camptothecin/anti-Fas, 4 h). Loss of CD46 was totally abrogated when cells were incubated with the caspase inhibitor prior to apoptosis induction. Also there was no formation of Annexin V positive cells.

5.3.5.2 Flow Cytometry Analysis to Determine Whether the Release of CD46 Involves src Kinase Signalling

Cytoplasmic tails of CD46 (CYT-1 and CYT-2) encode putative signals for phosphorylation by kinases, which play pivotal roles in CD46 trafficking with intracellular processing and basolateral localisation. To investigate whether localisation of CD46 to apoptotic blebs involved signalling through CD46 tails, Jurkat cells were incubated with a selective inhibitor of the src family of protein tyrosine kinases (PP2 – 4-Amino-5- (4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine) prior to apoptosis induction. Dual fluorescence analysis of CD46 and Annexin V on apoptotic cells in the presence of the PP2 inhibitor showed CD46 was localising to apoptotic blebs for shedding and not controlled by src kinase signalling through CD46 tails (Figure 5.13).

5.3.5.3 Flow Cytometry Analysis to Determine Whether the Release of CD46 Involves Protein Kinase C Signalling

To determine further the signalling mechanisms involved in CD46 localisation to apoptotic blebs, Jurkat cells were incubated with a PKC inhibitor (N-Myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln) prior to apoptosis induction. Dual fluorescence analysis of CD46 and Annexin V on apoptotic cells in the presence of the PKC inhibitor showed CD46 was lost from apoptotic cells and was not controlled by protein kinase C signalling through CD46 tails (Figure 5.14).

5.3.5.4 Flow Cytometry Analysis to Determine the Release of CD46 Involves Casein Kinase Signalling

To investigate further whether the localisation of CD46 to apoptotic blebs is dependent on signalling through Casein Kinase 2 and CD46 cytoplasmic tails, Jurkat cells were incubated with a CK 2 inhibitor (TBBt) prior to apoptosis induction. Dual fluorescence analysis of CD46 and Annexin V on apoptotic cells in the presence of the CK 2 inhibitor showed CD46 was lost from apoptotic cells and was not controlled by Casein Kinase 2 signalling through CD46 tails (Figure 5.15).

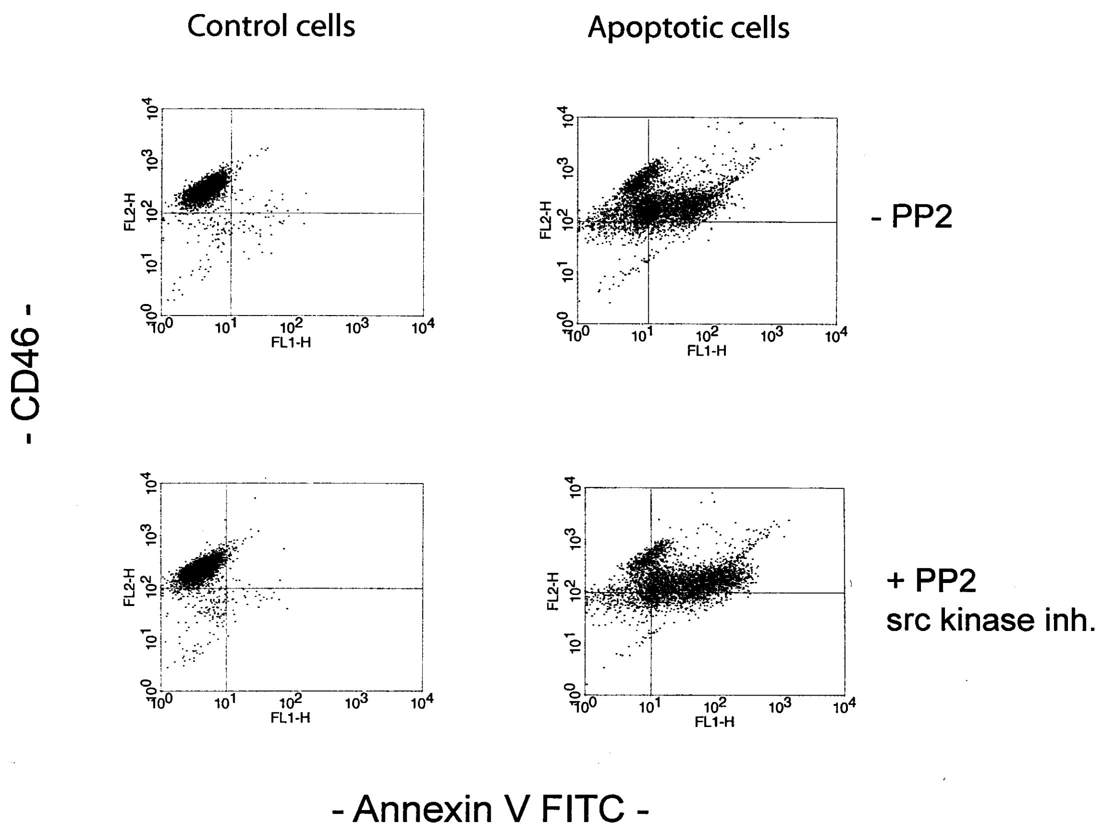


Figure 5.13 Dual fluorescence flow cytometry of control and apoptotic (camptothecin, 4 h) Jurkat cells incubated with or without the src kinase inhibitor (15 min, 37oC, 5% CO2) prior to apoptosis. Cells were stained for CD46 and Annexin V FITC. The presence of a src kinase inhibitor failed to control CD46 loss on apoptotic cells.

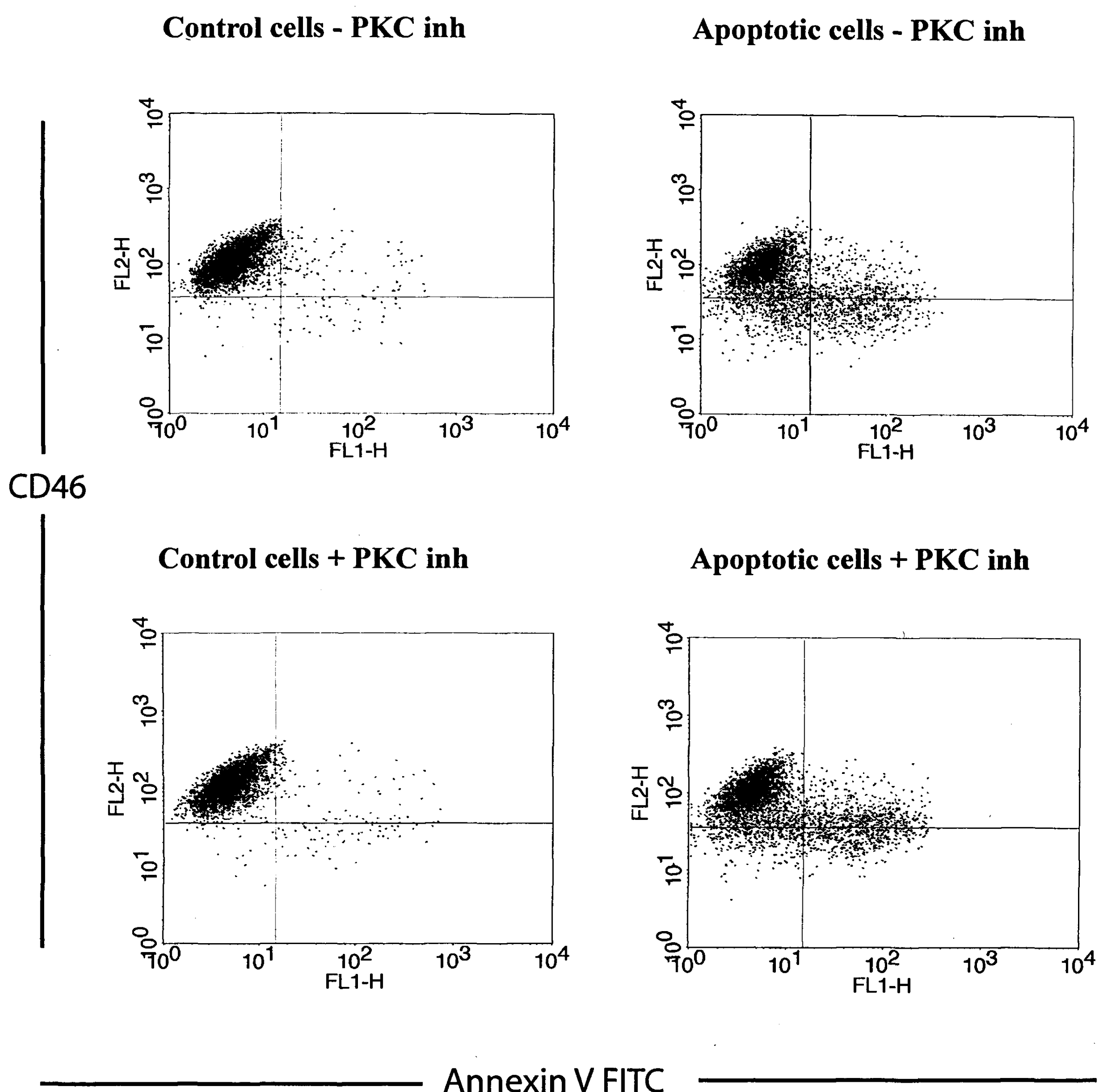


Figure 5.14 CD46 lost from apoptotic cells is not dependent on Protein Kinase C (PKC) signalling. Cells were incubated with a PKC inhibitor (N-Myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln) for 1 h prior to apoptosis induction (Camptothecin, 4 h) and CD46 and Annexin V expression was analysed by dual flow cytometry. The presence of a PKC inhibitor failed to control CD46 loss on apoptotic cells.

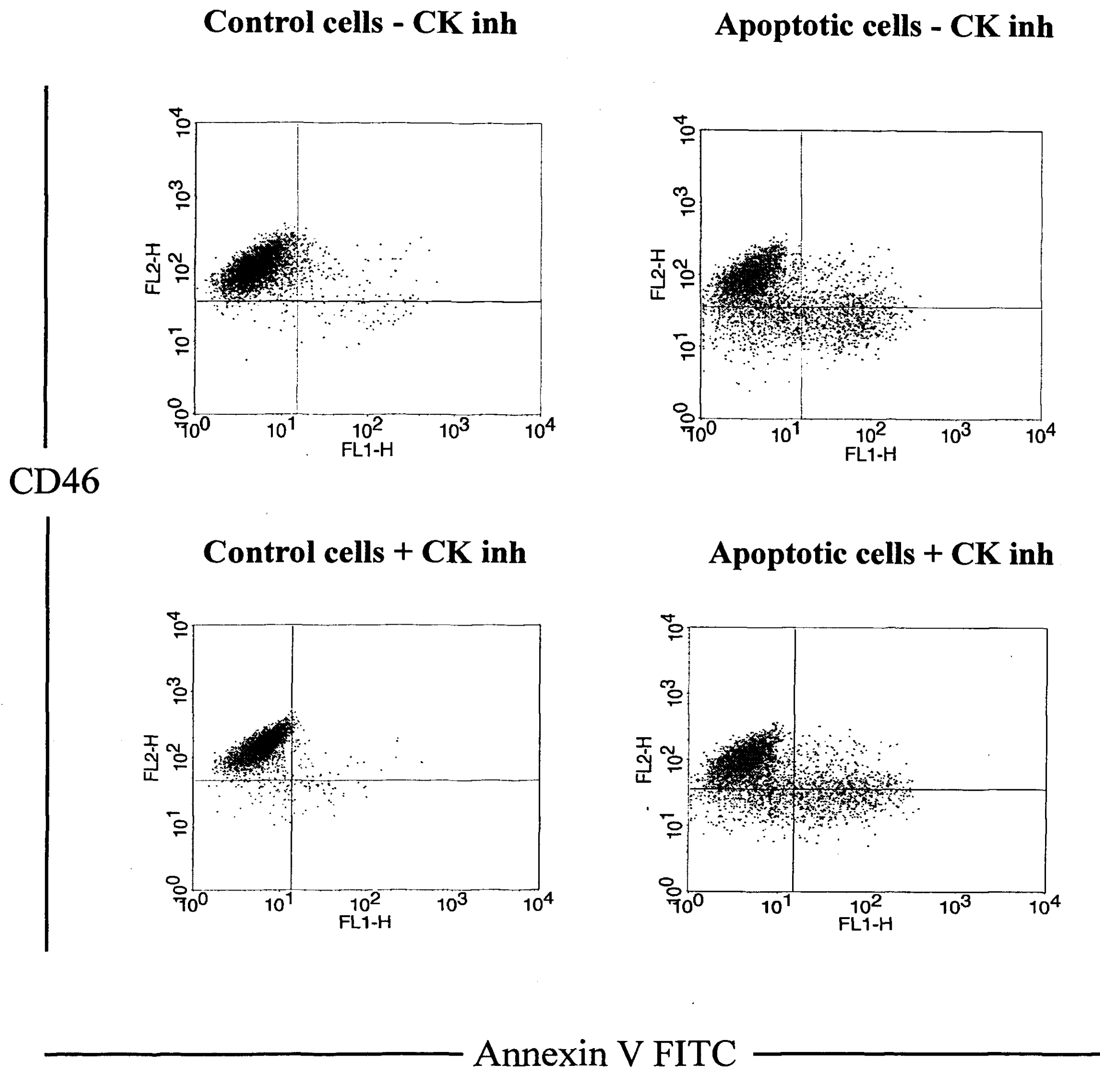


Figure 5.15 CD46 lost from apoptotic cells is not dependent on Casein Kinase II (CKII) signalling. Cells were incubated with a CK inhibitor (Tbbt) for 1 h prior to apoptosis induction (Camptothecin, 4 h) and CD46 and Annexin V expression was analysed by dual flow cytometry. The presence of a CK inhibitor failed to control CD46 loss on apoptotic cells.

5.4 Discussion

Although CD46 was found evenly distributed around the membrane, and was also present in cytoplasmic and nuclear stores of normal cells, the study observed clustering of CD46 to surface apoptotic blebs associated at sites of phosphatidylserine exposure, together with binding of C1q opsonin. During cell apoptosis (caspase-mediated) and necrosis further findings revealed CD46 expression was reduced from cytoplasmic/membrane (Cyt) and nuclear stores (Nuc), and therefore we proposed that CD46 from these stores along with membrane-bound CD46 was removed into apoptotic blebs which were then released as micro particles during the apoptotic process.

Consistent with the results from this study showing CD46 accumulating to blebs where C1q binding is also prevalent, previous results have shown C1q binding to surface blebs of apoptotic human keratinocytes (Korb and Ahearn 1997), HUVEC (Navratil, *et al.* 2001) and Jurkat cells (Nauta, *et al.* 2003, Nauta, *et al.* 2002) and therefore implies C1q has a vital role in clearance of apoptotic cells. It is well known that external PS serves as an apoptotic cell recognition signal for multiple phagocyte receptors (Fadok, *et al.* 2000, Ravichandran 2003, Savill 1997), and therefore C1q concentration to apoptotic blebs together with CD46 portrays the importance of these bleb structures in signalling to phagocytes to clear apoptotic cells before secondary necrosis ensues. The binding of C1q to apoptotic blebs by Western blot analysis also further reiterated the findings from the flow cytometry and fluorescent microscopy analysis in the previous chapter,

and that the observed binding of C1q to these bleb structures could be due to the proposed involvement of C1q detection of membrane bound nucleic acids on apoptotic cells and thus signalling to phagocytes for clearance of these apoptotic cells. Therefore, it would be important to ascertain the direct contribution of C1q as a receptor binding to NA as suggested by Palaniyar *et al* (Palaniyar, *et al.* 2004).

Thus far, the present experimental findings have hypothesised that during apoptosis CD46 is removed from cytoplasmic and nuclear stores and from the evenly distributed plasma membrane locations to surface bleb structures, which are released from the apoptotic cell surface. However, the exact mechanisms required for this phenomenon still need to be elucidated. CD43, a major sialoglycoprotein has recently been characterised as a SAMPs, which bind to siglecs on phagocytes. A lack of CD43 expression on micro organisms or on apoptotic cells can trigger phagocytosis (Crocker and Varki 2001). Studies by Eda *et al* revealed the susceptibility of Jurkat T cells to macrophage recognition at an early stage of apoptosis was due to CD43 on Jurkat cells forming a cap, which was involved in recognition. They showed CD43 capping was dependent on caspase activity and therefore proposed that caspase-dependent cytoplasmic events causing cytoskeletal changes were involved. One of the hypotheses considered was the involvement of moesin, a cytoskeletal protein of the ERM (ezlin/radixin/moesin) family that cross-links the intracellular domain of CD43 and actin filaments. During caspase activation this protein had previously been

shown to dephosphorylate and detach from the plasma membrane resulting in the disappearance of microvilli from the cell. Therefore, Eda *et al* suggested CD43 capping may be one of the consequences of this cytoplasmic disintegration, or if not, some cytoskeletal machinery could actively induce the movement of CD43 to form a cap. They also proposed that the loss of the CD43 cap from apoptotic cells could result from proteolytic cleavage of the protein, because CD43 is known to be proteolytically down-regulated upon stimulation or spontaneously, and the extracellular fragment of CD43 is released into plasma (Eda, *et al.* 2004). These findings, which highlighted a possible role for cytoskeletal proteins in transporting proteins for capping during apoptosis, convey a highly possible route for CD46 translocation.

Studies by Coleman *et al* showed the occurrence of apoptotic membrane blebbing was dependent on the function of ROCK (Rho-associated kinase). ROCK I is cleaved during apoptosis by activated caspases, generating a truncated kinase with increased intrinsic activity. This cleaved form is sufficient to drive cell contraction and membrane blebbing. The activity of ROCK proteins and consequent membrane blebbing are required for redistribution of fragmented DNA from the nuclear region into membrane blebs and apoptotic bodies (Coleman, *et al.* 2001). Therefore, it would be interesting to ascertain whether ROCK proteins could be involved in the translocation of CD46 from nuclear and cytoplasmic stores to apoptotic blebs.

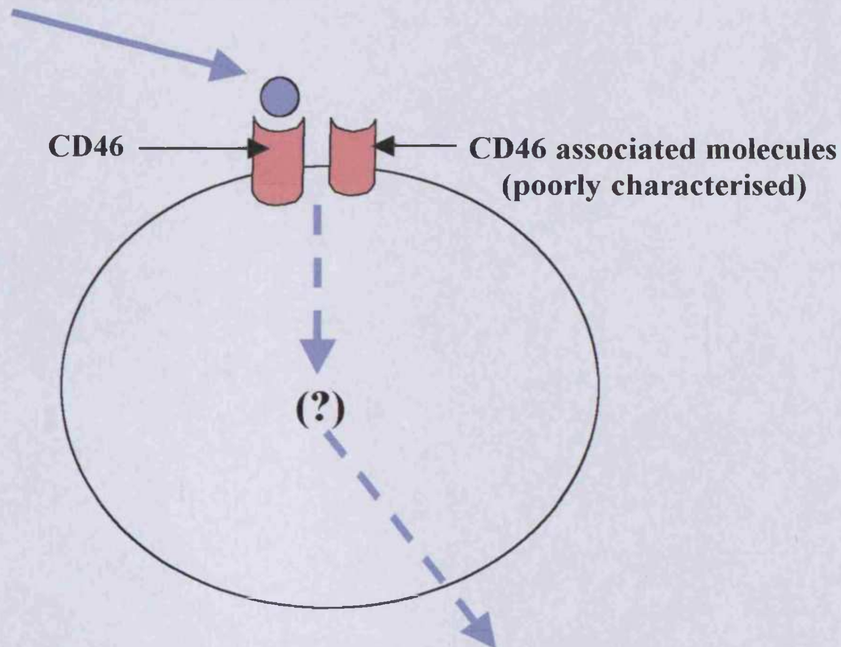
Recently, studies by Hakulinen *et al* on cancer cells, revealed CD46 to be constitutively shed from membranes in vesicles in an intact form, and as a

soluble form resulting from metalloproteinase (MMP) cleavage (Hakulinen, *et al.* 2004). The results in the present study also revealed necrotic T cell supernatants were highly enriched for sCD46 an indication that MMPs may be involved. However, the supernatant from apoptotic Jurkat T cells was not enriched with sCD46 indicating the majority of CD46 was released in blebs shed from the surface membrane and not as a cleaved soluble form. However the involvement of MMPs in the cleavage of the small amount of sCD46 observed in the supernatant of apoptotic cells could not be ruled out. Indeed the 35-kDa short form of CD46 was detached after 8 hours post apoptotic induction and may be the product of a cleaved form of CD46 by MMPs (please refer to section 5.3.4.2). The present observations have therefore suggested that the preferred mechanism of removal of CD46 to blebs for release from the cell is a phenomenon observed on apoptotic and not necrotic cells

CD46 is a widely expressed type 1 transmembrane regulatory glycoprotein that controls complement activation by serving as a membrane-bound cofactor for the plasma serine protease factor I to cleave C3b and C4b deposited on host cells. It is also known as a receptor for a wide variety of human pathogens including measles virus (MV), group A Streptococcus pyogenes, human herpes virus 6 (HHV6), Group B adenoviruses, and pathogenic Neisseria (gonorrhoea and meningitidis) (Dorig, *et al.* 1993, Gaggar, *et al.* 2003, Johansson, *et al.* 2003, Naniche, *et al.* 1993, Santoro, *et al.* 1999, Segerman, *et al.* 2003). Recently it has been reported to have a role in reproductive biology as expressed on the inner acrosomal membrane of spermatozoa (Riley-Vargas, *et al.* 2004).

It is still not clearly understood how and when CD46 is involved in intracellular signalling, or the key signalling pathways to which it interacts, but a number of reports have indicated the effects, which are believed to occur due to CD46 ligation of cellular responses (Figure 5.16).

CD46 ligands
CD46-specific antibodies
Complement-opsonised particles
Measles
Adenovirus (Group B)
Streptococcus (Group A)
HHV6
Neisseria



Cellular outcomes of CD46 signaling?
Cytokine production
Proliferation
Isotype switching
Nitric oxide production
Cell morphology (macropinocytosis)
Changes in MHC peptide presentation

Figure 5.16 A Schematic of the Effects of CD46 Ligation on Cellular Immune Responses (adapted from (Russell 2004)).

The role of CD46 as a complement activation inhibitor does not require signalling in the cell as it prevents the formation of C3/C5 convertases at the surface of the cell. However, binding of C3b to host cells during inflammation is potentially deleterious, and therefore CD46 may be involved in intracellular protective signalling (Wang, *et al.* 2000). Its role as a receptor for several human pathogens suggests CD46 is used to initiate a signalling event. Indeed, in studying measles virus interactions with CD46, it has been revealed that MV-induced signalling responses through CD46 result in decreased IL-12 secretion and increased release of IFNα/β synthesis by macrophages (Hirano, *et al.* 1999, Katayama, *et al.* 2000). It is also believed that CD46 is down regulated from the cell surface in persistent measles virus infection, potentially increasing susceptibility to complement-mediated lysis, and it is the cytoplasmic tails that have been shown to be involved. A cytoplasmic Tyr-X-X-Leu sequence has been identified as critical for CD46 down regulation (Yant, *et al.* 1997). This sequence resembles a sequence motif required for many membrane protein trafficking and receptor signalling events.

Only recently have questions started to be elucidated, and evidence collected to determine which isoforms of CD46 play a vital role in intracellular signalling (for review see (Russell 2004)). Molecular analysis of CD46 identified two cytoplasmic tails arising by alternative splicing (Post, *et al.* 1991). It is now believed that the generation of two cytoplasmic tails with different sequence characteristics contribute to signalling outcomes. The cytoplasmic tails of CD46

encode putative signals for Protein Kinase C, Casein Kinase 2, and src Kinases (see Fig 5.2) (Liszewski, *et al.* 1994). Cyt 1 possesses potential phosphorylation sites for Protein Kinases C and Casein Kinase 2, whereas Cyt 2 possesses sites for phosphorylation by src Kinases and Casein Kinase 2 (Liszewski, *et al.* 1994). It has been postulated that the differential phosphorylation of cytoplasmic tails and interaction with other signalling molecules dictates the differential intracellular trafficking, which determines the nature of the cellular response (Russell 2004). Studies by Wang *et al* revealed the predominant CD46 isoform in Jurkat T cells to be STP-BC, CYT-2 (BC2, 50%) while BC1 represented 15%, C2 20%, and C1 5%. Therefore CYT-2 was confirmed to be the major isoform (70-80%) in Jurkat cells. They also revealed that the CD46 CYT-2 in Jurkat cells was tyrosine phosphorylated by the src Kinase, Lck (Wang, *et al.* 2000).

Therefore, from previous CD46 cytoplasmic tail evidence, the present studies involved determining whether the phosphorylation of CYT-2 of CD46 in Jurkat cells contributed to the removal of CD46 to blebs and thus shedding during the apoptotic process. The results revealed that removal of CD46 to apoptotic blebs was not reliant upon phosphorylation of the cytoplasmic tail by either src kinases, casein kinases or protein kinase C, suggesting another signalling mechanism other than through the cytoplasmic tails must be involved to promote the removal of CD46 in blebs from apoptotic cells. The induction of apoptosis in this present study has also been shown to result in caspase-mediated apoptosis, and CD46 was lost from these cells. However, a direct link between the loss of

CD46 and caspase and PARP activity cannot be implied from the evidence thus far, and further experiments are warranted.

It is also believed that CD46 must undergo complex intracellular trafficking, and dependent on its function at the time, will relate to the cellular response (Russell 2004). A number of studies to date have determined how CD46 is internalised into the cell and trafficked back to the cell membrane. However, none of the studies have involved looking specifically at CD46 signalling during apoptosis, but the mechanisms could have a similar outcome. Irwin *et al* discovered that CD46 could be internalised from the cell surface *via* two alternative pathways depending upon the extent of cross-linking at the cell surface. CD46 is constitutively internalised *via* clathrin-coated pits, trafficked to multivesicular bodies, and recycled to the cell surface. However, CD46 that has been cross linked (e.g. antibody or measles virus) is known to induce extensions (pseudopodia) that internalise CD46 and its ligands in a process similar to macropinocytosis, which results in the degradation of cell surface CD46 (Crimeen-Irwin, *et al.* 2003; Russell 2004). Complement opsonisation of apoptotic cells is believed to initiate pathway-2 mediated internalisation through CD46 (Crimeen-Irwin, *et al.* 2003).

In summary, the present observations suggest CD46 is removed from cytoplasmic/membrane and nuclear stores and released in blebs from the surface of the apoptotic cell together with PS, C1q, and C3b, which are capable of

engaging well-known receptors on macrophages (PSR, cC1qR/CD91, CR3), and possibly to a CD46 receptor, which is yet to be characterised. It could be hypothesised that these structures act as appetisers to macrophages signalling and initiating the clearance of apoptotic cells (Figure 5.16). The role of CD46 in these microparticles remains unclear apart from ensuring that C3b is converted to iC3b. From these studies, and previous observations in Chapter 4, which revealed release of CD46 from apoptotic cells, it could be further argued that CD46 is a key and novel example of a 'self-associated molecular pattern, SAMP' behaving as a 'don't eat' signal on normal cells, and during apoptosis when this signal is lost it could be hypothesised that phagocytes are involved in the recognition and removal of these apoptotic cells by phagocytosis. Therefore, further experiments are warranted to determine whether the loss of CD46 from apoptotic cells primes the phagocytes into removing the apoptotic cells.

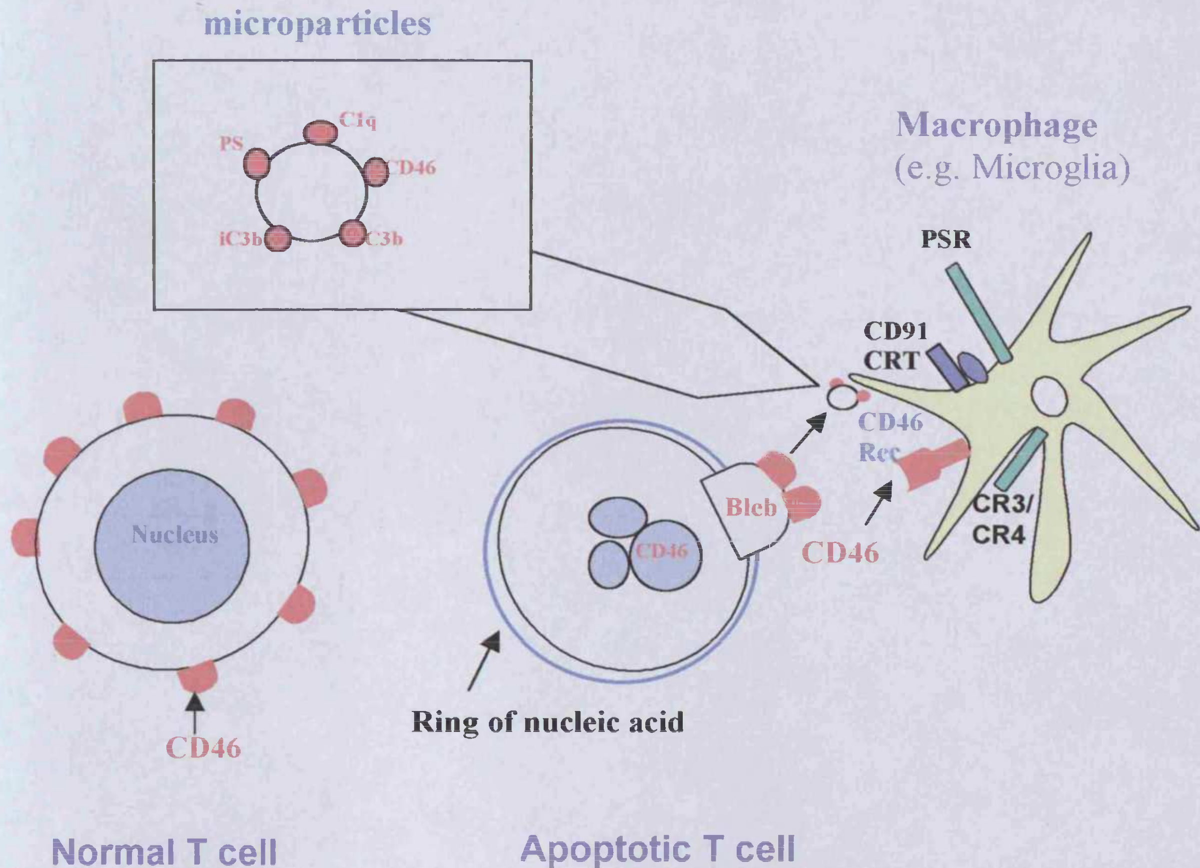


Figure 5.17 A Schematic of the Removal of CD46 to Membrane Blebs During Apoptosis, for example in the CNS, and the release of Blebs Resulting in their Proposed and Clearance by Microglia (Professional Macrophage).
Normal cells express CD46 evenly around the cell surface and during apoptosis CD46 is clustered to apoptotic blebs and shed from the cell surface in these blebs - enriched with CD46, PS, C1q, C3b and iC3b. Apoptotic cells also expose nucleic acid (NA) in the outer leaflet of the plasma membrane and promote activation of the complement system (C1q selective binding to NA). Phagocyte recognition of the apoptotic blebs could involve different receptors for PS, C1q (CD91/calreticulin, CRT), C3b/iC3b (complement receptor type 3, CR3) and other possible receptors for CD46, yet to be identified.

Chapter 6

Complement Biosynthesis and Expression of Phagocytic Receptors by Macrophage, Glial Cells and Neurones

6.1 Introduction

In tissues of the body, professional phagocytes usually remove apoptotic cells quickly and efficiently by either an innate or adaptive immune response. However, in the brain where an adaptive immune response is scarce, 'amateur' in addition to 'professional' phagocytes are believed to play key roles in recognising 'eat me' signals on apoptotic cells (ACAMPs) and pathogens (PAMPs), which lead to clearance of these different targets. The 'professional' phagocytes are believed to be microglia (Perry, *et al.* 1993), whereas astrocytes, neurones, and oligodendrocytes are important contributors to the population of 'amateur' phagocytes (Dong and Benveniste 2001, Flugel, *et al.* 2000, Martino, *et al.* 2001). Previous evidence by Parnaik *et al* revealed a difference in time between the clearance of apoptotic cells by professional (microglia) and amateur (astrocytes and epithelial cells) phagocytes. Microglia were shown to be extremely motile and capable of phagocytosing apoptotic cells on first contact, whereas amateur phagocytes had a delayed onset of ingestion generally a few hours after recognition (Parnaik, *et al.* 2000).

The aim of this study was (1) to ascertain by flow cytometry and RT-PCR analysis the capacity of professional and amateur phagocytes to express

complement and other phagocytic receptors involved in the clearance of apoptotic cells. This involved using the THP1 monocyte cell line (professional phagocyte) differentiated to macrophage by PMA treatment, and T98G astrocytoma cell line (amateur phagocyte), stimulated with IFNγ, (Aepfelbacher, *et al.* 1996, Farrar and Schreiber 1993, Gasque, *et al.* 1995, Gasque, *et al.* 1992, Knupfer, *et al.* 2000, Rehfeldt, *et al.* 1991). Preliminary studies by Dr. Philippe Gasque (unpublished observations) indicated that T98G was capable of phagocytosing apoptotic Jurkat T cells although the phagocytic index (percentage of cells capable of phagocytosing at least one apoptotic cell) was six-seven fold lower when compared to THP1. Therefore the present study aimed to determine to what extent the differential capacity to phagocytose apoptotic Jurkat was due to the expression of complement and phagocytic receptors (2) Analyse by RT-PCR the expression of phagocytic receptors by other brain cells (neurones and oligodendrocytes). To this aim several cell lines were used. For analysis of CNS neurones, Kelly, a human brain neuroblastoma cell line (ECACC, No. 92110411) was used. These cells possess a genomic amplification of the N-myc gene and express elevated levels of N-myc RNA or protein. For analysis of PNS neurones, Paju, a neural crest derived tumour cell line was used (kind donation from Leif C. Anderson, University of Helsinki). This cell line expressed neuronal cell markers ($CD56^{+}$ and $CD57^{+}$). For analysis of oligodendrocytes, HOG, a human oligodendrocyte cell line was used (kind donation from Dr G. Dawson, University of Chicago). This cell line was shown to express oligodendrocyte markers (MBP^{+} and $CNPase^{+}$)

6.1.1 Phorbol 12-Myristate 13-Acetate (PMA) Stimulation of THP1 Monocyte Cells

Previous evidence revealed PMA efficient in stimulating THP1 monocyte spreading (Aepfelbacher, *et al.* 1996, Rehfeldt, *et al.* 1991). Spreading of monocytes is essential for endothelial transmigration, tissue motility, and with relevance to this chapter macrophage differentiation characterised by filopodial and pseudopodial projections (Aepfelbacher, *et al.* 1996). This mechanism involves binding of integrins to either endothelial vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (i.e. $\alpha_4\beta_1$), or matrix fibronectin ($\alpha_5\beta_1$) (Aepfelbacher, *et al.* 1996).

6.1.2 Interferon-Gamma (IFNγ) stimulation of T98G

Astrocytes, which make up 50-60% of the brain cell population are believed to act as amateur phagocytes due to their strategic position at the blood brain barrier, a probable port of entry for most pathogens (Dong and Benveniste 2001). An astrocytoma cell line (T98G) was used in our analysis. Interferon gamma (IFNγ), known to be produced by T- and NK-cells, and important in host defence and immune regulation (Knupfer, *et al.* 2000), was used to boost phagocyte activity of T98G. IFNγ is also known to be the major cytokine capable of up regulating astrocyte biosynthesis of most complement components by a factor of 2-50 times (Gasque, *et al.* 1995, Gasque, *et al.* 1992).

6.1.3 Receptors on Amateur and Professional Phagocytes

An array of phagocytic receptors have been implicated in apoptotic cell clearance (for review refer to (Gregory and Devitt 2004)), and the aim of this chapter was to delineate further receptors which could be involved in the recognition and clearance of apoptotic cells by either amateur or professional phagocytes. This involved analysis at the mRNA (RT-PCR), and protein level (Flow cytometry).

6.1.4 Complement Biosynthesis by Amateur and Professional Phagocytes

Previous chapters have illustrated that complement activation on apoptotic cells, and at present there is also an increasing amount of evidence to suggest brain cells are capable of generating a full innate immune system to aid in the killing of pathogens/apoptotic cells (for review see (Gasque, *et al.* 2000, Morgan and Gasque 1996). Complement biosynthesis is known to occur within the CNS, and the four key cell types within the brain – astrocytes, oligodendrocytes, microglia, and neurones are all potential sites for complement biosynthesis (Morgan and Gasque 1996). Therefore, further work in this study involved characterising the expression/biosynthesis of complement components on phagocytes before and after phagocytosis at the mRNA (RT-PCR), and protein level (Flow cytometry) analysis.

6.2 Methods

6.2.1 Differentiation of THP1 Cells to Macrophage like cells with PMA

Cells (5 x 10^6) were isolated from culture and resuspended in 20ml of fresh F5 RPMI containing 10ng/ml PMA, and incubated at 37°C, 5% CO_2. On day 2 of stimulation the flask was shaken to detach semi-adherent cells, centrifuged at 800rpm for 3 minutes, and cells resuspended in 20ml of fresh F5 medium with 10ng/ml of PMA. On day 3, more than 50% of the cells were adherent to the flask. The medium was carefully discarded and fresh F5 medium with 10ng/ml of PMA was added. On day 4 of stimulation, the cells were fully differentiated. The flask was vigorously shaken and the cells resuspended at a density of 10^6 cells per ml for the phagocytosis assay.

6.2.2 T98G Stimulation with IFNγ to Induce a Phagocytic Phenotype

Cells (5 x 10^6) were isolated from culture and resuspended in 20ml of fresh F5 DMEM containing IFNγ (200 IU/ml), and incubated for 24 hours at 37°C, 5% CO_2.

6.2.3 Phagocytosis Assay

Jurkat cells (human T lymphocyte cell line) were harvested from culture and resuspended at a density of 10^6/ml in RPMI 1640 without FBS. Apoptosis was induced in 10ml of cells at 37°C, 5% CO_2 for 4 hours with Camptothecin (5μg/ml). Jurkat cells were extensively washed (3 times in RPMI) to remove

traces of camptothecin, and loaded with green calcein indicator (Sigma). Adherent THP1 differentiated with PMA and T98G differentiated with IFNγ were loaded with a red cell tracker (C2927, Molecular Probes) and calcein labelled apoptotic Jurkat added at a ratio of 1 apoptotic cell: 1 phagocyte. The flasks were incubated at 37°C for either 5 minutes, 30 minutes or 2 hours to allow THP1 and T98G to phagocytose the apoptotic Jurkat cells. Floating cells were removed by three washes in cold medium and the level of phagocytosis was analysed by flow cytometry and immunofluorescence microscopy.

6.2.4 Flow Cytometry to Determine the Expression of Phagocytic Receptors

Post PMA (THP1) and IFNγ (T98G) treatment (with or without addition of apoptotic Jurkat cells for 2 hours, followed by washing and incubation of the cells overnight in fresh 20 ml RPMI at 37°C, 5% CO_2) adherent macrophage cells were detached from the flask by incubation with 2ml of EDTA (10mM in NaCl) at 37°C, 5% CO_2 for 10 minutes. Cells were washed 2 times in 10ml PBS/BSA (1% w/v), resuspended at a final density of 5 x 10^5 cells, and 1 x 10^5 cells aliquoted into 5ml tubes. Immunofluorescence analysis of cells was performed, whereby cells were incubated with well-known macrophage markers – CR3 (Mo α CR3 (CD11b), 1/200), CR4 (Mo α CR4 (CD11c), 1/200), Phosphatidylserine receptor (Rb α PSR, 1/500), CD91 (Mo α CD91, 1/200), and calreticulin (Mo α CRT, 1/200), followed by a specific phycoerythrin (PE) conjugated $F(ab)_2$ goat antibody to mouse or rabbit IgG (1/200), and analysed by

flow cytometry. For all experiments undifferentiated cells were used as a comparison.

6.2.5 Flow Cytometry to Determine Complement Expression/Biosynthesis before and after Phagocytosis.

PMA treated (THP1), and IFNγ treated (T98G) cells, prior to and post exposure to apoptotic Jurkat cells for 2 hours, followed by washing and incubation of the cells overnight in fresh 20 ml RPMI at 37°C, 5% CO_2, were detached from the flask by incubation with 2ml of EDTA (10mM in NaCl) at 37°C, 5% CO_2 for 10 minutes. Cells were washed 2 times in 10ml PBS/BSA (1% w/v), and fixed with 1ml formaldehyde (3% in PBS) for 30 minutes at room temperature. The cells were then washed three times in 1ml PBS/BSA, and reactive groups were blocked by incubating the cells with 1ml glycine (2% in PBS) for 5 minutes at room temperature. Cells were washed 3 times in PBS/BSA, and permeabilised with 0.1 % triton X100 for 5 minutes at room temperature. Cells were finally washed 3 times in PBS/BSA, resuspended at a final density of 5 x 10^5 cells, and 1 x 10^5 cells/well were aliquoted into round-bottomed 96 well plates. For the detection of complement components, immunofluorescence analysis of cells was performed, whereby cells were incubated with antibodies recognising C1q (Rb α C1q, 1/200), C3 (Rb α hu C3 (L440), 1/200 and Mo α hu C3 (C3/30), 1/20), C4 (Rb α C4, 1/200), MAC (Mo α hu C5b9 (B7) 1/50), followed by phycoerythrin (PE conjugated $F(ab)_2$ goat antibody to mouse or rabbit IgG (1/200). For all

experiments untreated THP1/T98G were used as a comparison. Cells were analysed by flow cytometry.

6.2.6 RNA Extraction from THP1 & T98G Cells Followed by RT-PCR Analysis for Complement Expression

RNA was extracted from untreated, treated THP1 (PMA) and T98G (IFNγ) cells prior to and post phagocytosis of apoptotic Jurkat cells by the method described previously (2.8.1). RT PCR was performed to determine the level of expression of complement components on THP1 derived macrophages and T98G before and after phagocytosis. The primers for the various complement components were selected from Invitrogen Custom primers website:-
(http://www.invitrogen.com/content.cfm?pageID=9716&fuseaction=cpd.selectPrimers) (Table 6.1).

The optimum annealing temperature for the primers was within the range of 50-60°C. The annealing temperature adopted for the PCR reaction was 58°C.

6.2.7 RNA Extraction and RT-PCR to Screen for Phagocytic Receptors Involved in the Clearance of Apoptotic Cells by Amateur and Professional Phagocytes

RNA was extracted from the professional macrophage, THP1 (PMA) and the amateur macrophages - T98G (IFNγ) (astrocyte), Paju and Kelly (neurones) and HOG (oligodendrocytes) by the method described previously (2.8.1). RT-PCR was performed using selected primers to determine semi quantitatively the

expression of specific macrophage receptors (Table 6.2). The mean optical density for each PCR product was measured using the Image J Software program, and graph produced of the ratio compared to Actin:

$$\text{(i.e. } \frac{\text{Mean optical density of phagocyte receptor.)}}{\text{Mean optical density of Actin}}$$

Table 6.1 Primers Used in the RT-PCR Screen for Complement Components

Primer	Access Number: NCBI Genebank	Sequence	Expected Band size (bp)
Actin			
Upper	BC009848	5'CCTTCCAGCAGATGTGGATT3'	467
Lower		5'CAGAACACTCAGCCCTGACA3'	
Human C1q (1)	BC066295		
Upper		5'GCAGCCCAGAAAGAGACAAC3'	449
Lower		5'TCCCTGGAAGAGCTGAAGAA3'	
Human C1q (2)	BC066295		
Upper		5'ACAAATCCAGCAGCCACCATACT3'	447
Lower		5'TTGATAAAGAAGATAGCCTGAAAA3'	
Human C1r	BC035220		
Upper		5'GATCTATGCCAACGGGAAGA3'	407
Lower		5'TGGTGGTGTAACGGAAGTCA3'	
Human C1s	BC056903		
Upper		5'TGTGGTGCATTGTCCTGTTT3'	368
Lower		5'AAACCCCGTAAAACGCTCTT3'	
Human C3	K02765		
Upper		5'GGAAAAGGAGGATGGAAAGC3'	460
Lower		5'GTCCTGGCATTGTTTCTGGT3'	
Human C4	K02403		
Upper		5'ACGGCTTCCAGGTTAAGGTT3'	494
Lower		5'TGACACTTTGCTGCCAAAAG3'	
Human C5	M57729		
Upper		5'AGTGTGTGGAAGGGTGGAAG3'	392
Lower		5'AGGGAAAGAGCATACGCAGA3'	
Human C5	M57729		
Upper		5'GATTTGGCAGTGGCTTGGCTACAG3'	522
Lower		5'ACGCGGCTCCTTCACAGACTTTC3'	
Human C9			
Upper	NM001737	5'ATCGGGATGGAAACACTCTG3'	836
Lower		5'TTATGGAAGAGGCCCAGTTG3'	

Table 6.2 Primers Used in the RT-PCR Screen for Phagocytic Receptors

Primer		Access Number: NCBI Genebank	Sequence	Expected Band size (bp)
Scavenger receptors:				
CD68	**Upper**	BT009923	5'AAGAGCCACAAAACCACCAC3'	575
	Lower		5'TTGTACTCCACCGCCATGTA3'	
SRA1	**Upper**	NM138715	5'GGAACACATGAGCAACATGG3'	517
	Lower		5'AGTGGGACCTCGATCTCCTT3'	
SRB1	**Upper**	NM005505	5'CTGTGGGTGAGATCATGTGG3'	216
	Lower		5'GCCAGAAGTCAACCTTGCTC3'	
CD36	**Upper**	NM001001548	5'ATGTAACCCAGGACGCTGAG3'	592
	Lower		5'GCCTTGGATGGAAGAACAAA3'	
Lox1	**Upper**	AF035776	5'CTGGAGGGACAGATCTCAGC3'	441
	Lower		5'TAAGTGGGGCATCAAAGGAG3'	
Receptors for complement and associated molecules:				
CRT	**Upper**	NM004343	5'GTTTCGAGCCTTTCAGCAAC3'	469
	Lower		5'CAGTCCTCAGGCTTGGAGTC3'	
CD91	**Upper**	BC052593	5'TGCTACTGCAACAGCAGCTT3'	485
	Lower		5'TGTGTCACCTCGTTCTCCTG3'	
CR3	**Upper**	BC096346	5'GGATGACCTCAGCATCACCT3'	507
	Lower		5'TTCTCTGAGGCCGTGAAGTT3'	
CR4	**Upper**	BC038237	5'TGCCTGTCAGCATCAACTTC3'	541
	Lower		5'TTTGCCTCCTCCATCATTTC3'	

Receptors for microbial components:			
CD14 **Upper**	NM000591	5'GGTTCCTGCTCAGCTACTGG3'	595
Lower		5'CTTGGCTGGCAGTCCTTTAG3'	
Receptors for sugars/PL's:			
PSR **Upper**	BC066654	5'TCCCAGGGAACTCATCAAAG3'	268
Lower		5'TACCGTCTTGTGCCATACCA3'	
MER **Upper**	NM006343	5'AGCTTGGGAGTCAGTGAGGA3'	430
Lower		5'AGCTTGGGAGTCAGTGAGGA3'	
FcγR **Upper**	NM000566	5'GTTCCAGTTGATGGGCAAGT3'	567
Lower		5'TGTCACAGATGCATTCAGCA3'	
CD93 **Upper**	AJ295142	5'CACACAAGGGTCCTTCCACT3'	549
Lower		5'TAACTGTCTGCCGCATTCTG3'	
CD31 **Upper**	BC022512	5'GAGTCCTGCTGACCCTTCTG3'	350
Lower		5'CACTCCTTCCACCAACACCT3'	
ABCA1 **Upper**	AF285167	5'CTGCTAAGGAGGGAGCCTTT3'	502
Lower		5'AGTTCCAGGCTGGGGTACTT3'	
α5β3 **Upper**	NM002210	5'AACTCAAGCAAAAGGGAGCA3'	227
Lower		5'GGGTTGCAAGCCTGTTGTAT3'	
SHPS **Upper**	D86043	5'AGTGGTTCAGAGGAGCTGGA3'	395
Lower		5'GTCCACGTTGGTCTGGAAGT3'	

6.3 Results

6.3.1 Expression of Phagocytic Receptors by THP1 Monocyte and T98G Astrocyte Prior to and after Cell Activation

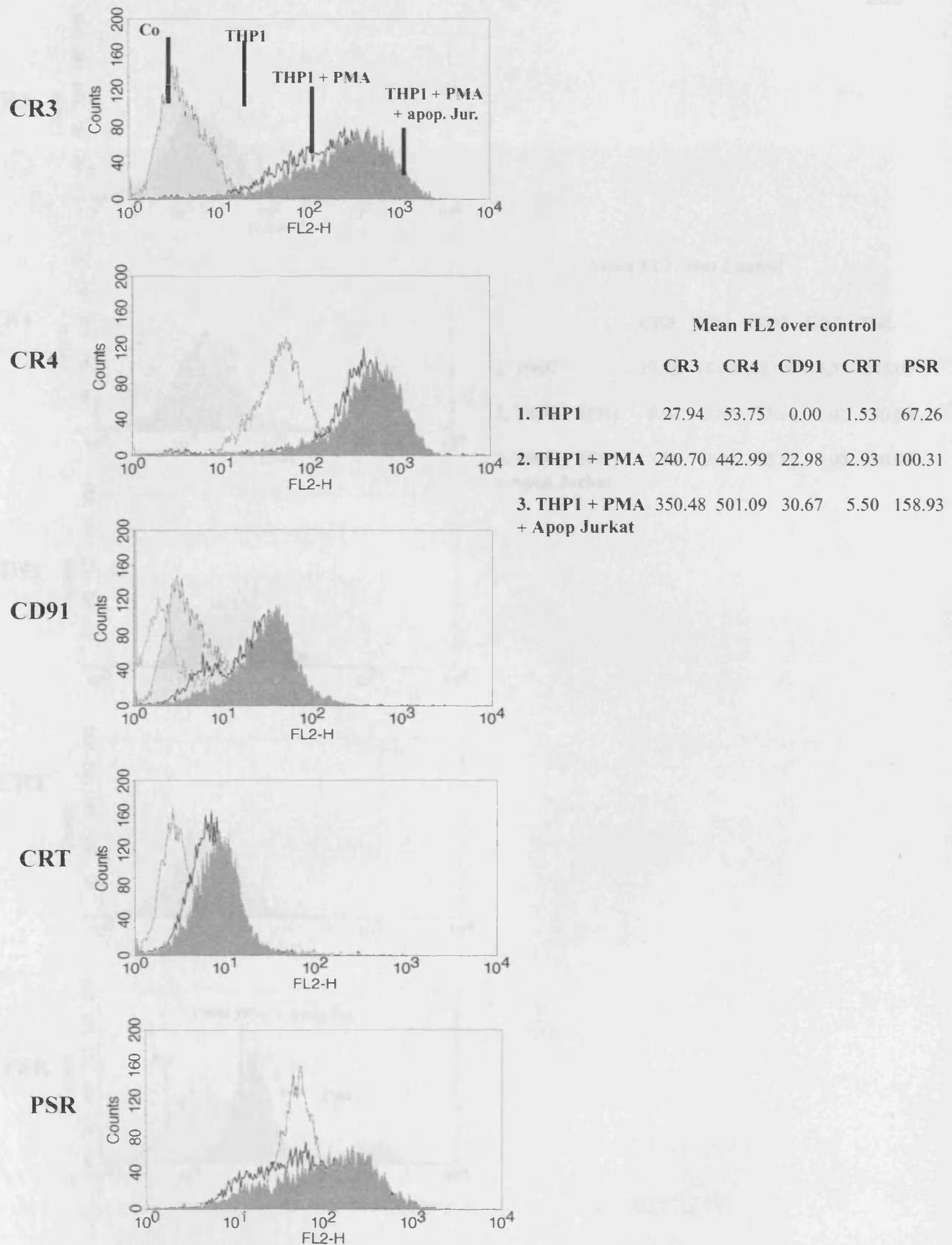
Previous work by Philippe Gasque had shown both THP1 and T98G were capable of phagocytosing but at different levels of efficacy (i.e. THP1 >> T98G) (Gasque, et al. 1992) Therefore, to ascertain whether THP1 and T98G were expressing phagocytic receptors involved in the clearance of apoptotic cells, fluorescence analysis for specific surface macrophage receptors was performed. Unstimulated THP1 cells readily stained for CR3, CR4 and PSR, with no membrane CD91 or CRT staining observed. Post PMA treatment a significant increase in CR3, CR4 and PSR staining was observed and weak staining for CD91 was also evident. CRT staining remained low post PMA treatment (Figure 6.1). Unstimulated T98G cells stained weakly for CR3, CR4, CD91 and CRT but stained strongly for PSR (Figure 6.2). Post IFNγ treatment T98G cell staining of CR3, CR4 and CRT was lost, CD91 staining remained weak and PSR staining was slightly weaker compared to unstimulated cells. For T98G there was no observed difference in expression of receptors prior to or post addition of apoptotic Jurkat cells to differentiated cells. However, for THP1 an increase in staining for CR3, CR4 and PSR (1.2-1.5 fold) was observed post addition of apoptotic Jurkat cells to differentiated THP1 cells (2 hours incubation).

Dr Philippe Gasque also confirmed the phagocytic activity of fluorescent red loaded phagocytes (THP1 and T98G loaded with red cell tracker) using

fluorescent calcein green loaded apoptotic Jurkat and analysis by Flow cytometry and immunofluorescent microscopy. Almost 60-70% of the red PMA-treated THP1 cells were found loaded with green apoptotic Jurkat cells within 2 hours. In contrast, the phagocytic index for T98G before or after treatment with IFNγ was only 6-8% after 2 hours (Figure 6.3, Dr Philippe Gasque's unpublished observations).

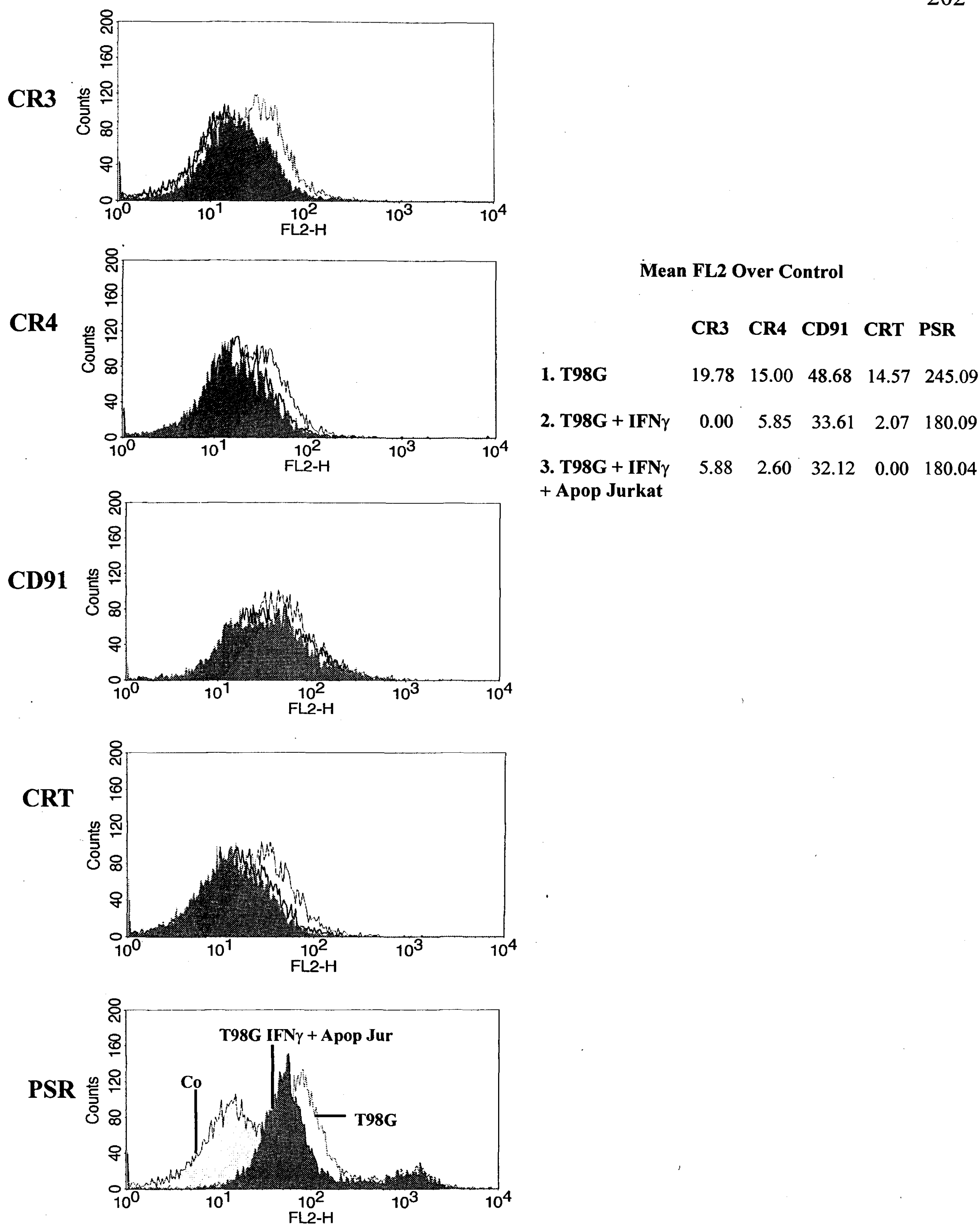
6.3.2 Flow Cytometry Analysis for Expression of Complement on Permeabilised THP1 and T98G Cells

In order to define complement expression/biosynthesis by THP1 and T98G prior to and after phagocytosis of Jurkat cells, the profile of complement component expression (C1q, C3, C4, C9) was assessed by flow cytometry on permeabilised cells (Figure 6.4 and 6.5). Unstimulated THP1 and T98G cells were strongly stained for C1q and C4 and this significantly decreased post stimulation. Both cells also stained for C3 but to a lesser extent to that observed for C1q and C4. Interestingly, in response to the different regimes (PMA, IFNγ) C3 staining increased approximately 2 fold on THP1 but remained relatively the same on T98G. Both THP1 and T98G showed no significant C9 staining. No significant difference was noticed when the cells had phagocytosed apoptotic Jurkat.



Mean FL2 over control	CR3	CR4	CD91	CRT	PSR
1. THP1	27.94	53.75	0.00	1.53	67.26
2. THP1 + PMA	240.70	442.99	22.98	2.93	100.31
3. THP1 + PMA + Apop Jurkat	350.48	501.09	30.67	5.50	158.93

Figure 6.1 Single flow cytometry analysis to determine THP1 phagocytic receptor expression after PMA treatment (10 ng/ml, 3 days) and after addition of apoptotic Jurkat (2 h). Cells were stained for well known macrophage surface receptors - CR3, CR4, CD91, Calreticulin (CRT) and PSR followed by a specific rPE conjugated secondary antibody. Light grey filled profiles represent secondary antibody only, grey lines represent THP1 (unstimulated), black lines represent THP1 stimulated with PMA and dark grey filled profiles represent THP1 (stimulated with PMA) + addition of apoptotic Jurkat cells.

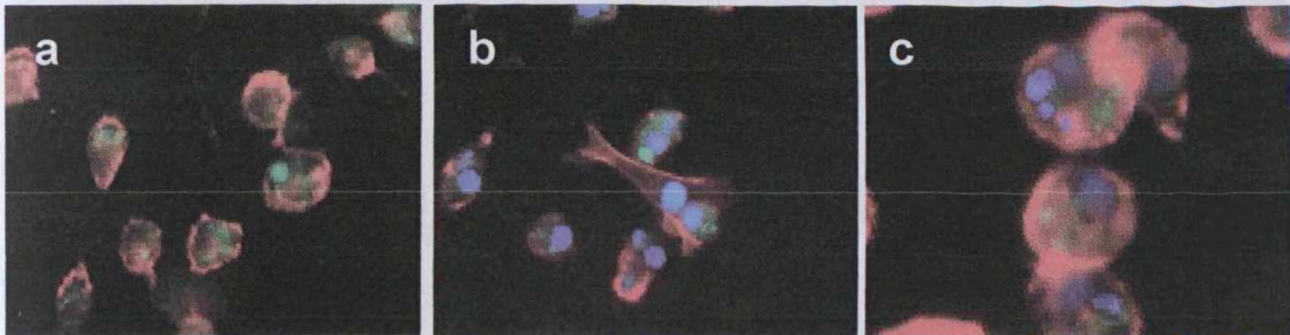


Mean FL2 Over Control

	CR3	CR4	CD91	CRT	PSR
1. T98G	19.78	15.00	48.68	14.57	245.09
2. T98G + IFNγ	0.00	5.85	33.61	2.07	180.09
3. T98G + IFNγ + Apop Jurkat	5.88	2.60	32.12	0.00	180.04

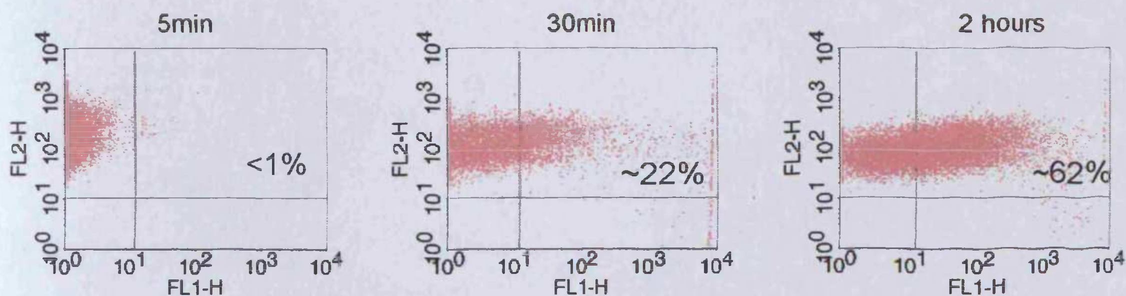
Figure 6.2 Single flow cytometry analysis to determine T98G phagocyte receptor expression after IFNγ treatment (200u/ml, 24 h) and after addition of apoptotic Jurkat (2 h). Cells were stained for well known macrophage surface receptors - CR3,CR4, CD91, Calreticulin (CRT) and PSR followed by a specific rPE conjugated secondary antibody. Light grey filled profiles represent secondary antibody only, grey lines represent T98G (unstimulated), black lines represent T98G stimulated with IFNγ and dark grey filled profiles represent T98G (stimulated with IFNγ) + addition of apoptotic Jurkat cells.

A.



THP1 (macrophage)
Jurkat (apoptotic cell)
Dapi (nuclear staining)

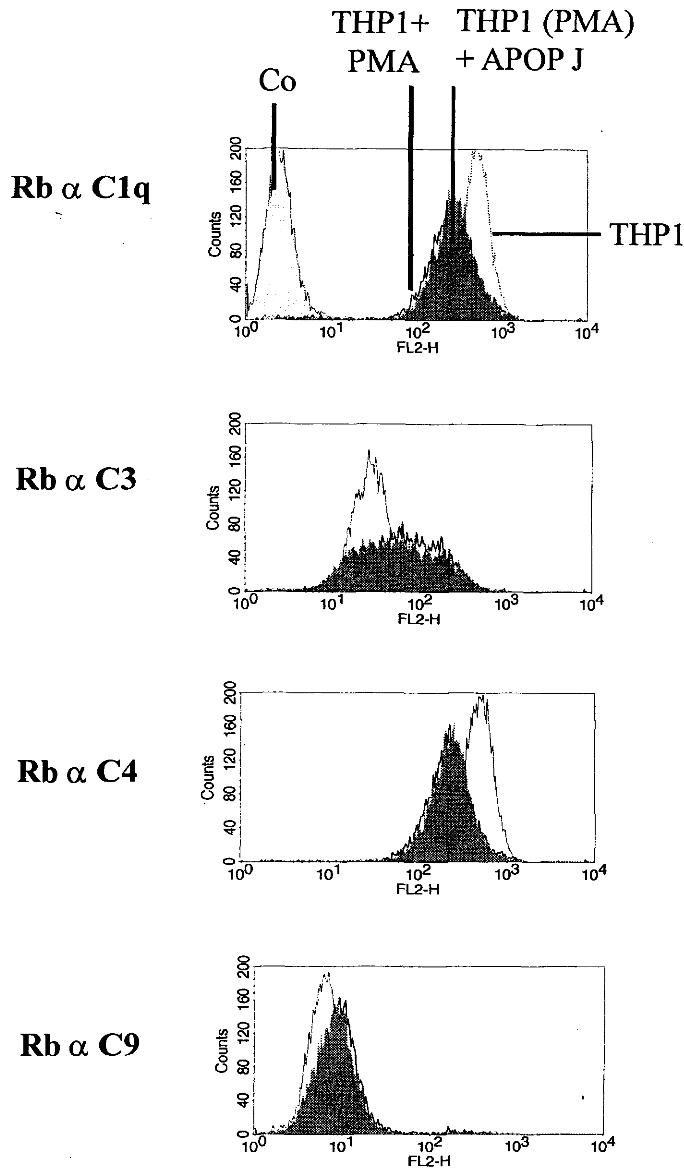
B.



FACS analysis: Red THP1 cells phagocytosing green calcein loaded apoptotic jurkat cells.

Fig 6.3: A. Immunofluorescence analysis: Adherent THP1 cells differentiated with PMA and loaded with the red cell tracker (C2927, Molecular probes) were phagocytosing apoptotic Jurkat T cells loaded with green calcein indicator. Nuclei were counterstained with DAPI (blue). Magnification (a,b, x400) (c, x1000).
B. FACS analysis: PMA-treated THP1 (red, adherent) and apoptotic Jurkat cell (green, in suspension), as above, were incubated at a ratio 1:1 and flasks were placed at 37°C for either 5min, 30min or 2hours. Floating cells were removed by three washes in cold medium and the level of phagocytosis was analysed by FACS. Almost 60-70% of THP1 cells were shown to phagocytose apoptotic cells in 2h. Of note, only 6-8% of T98G were capable of phagocytosing apoptotic jurkat cells after 2hours (data not shown).

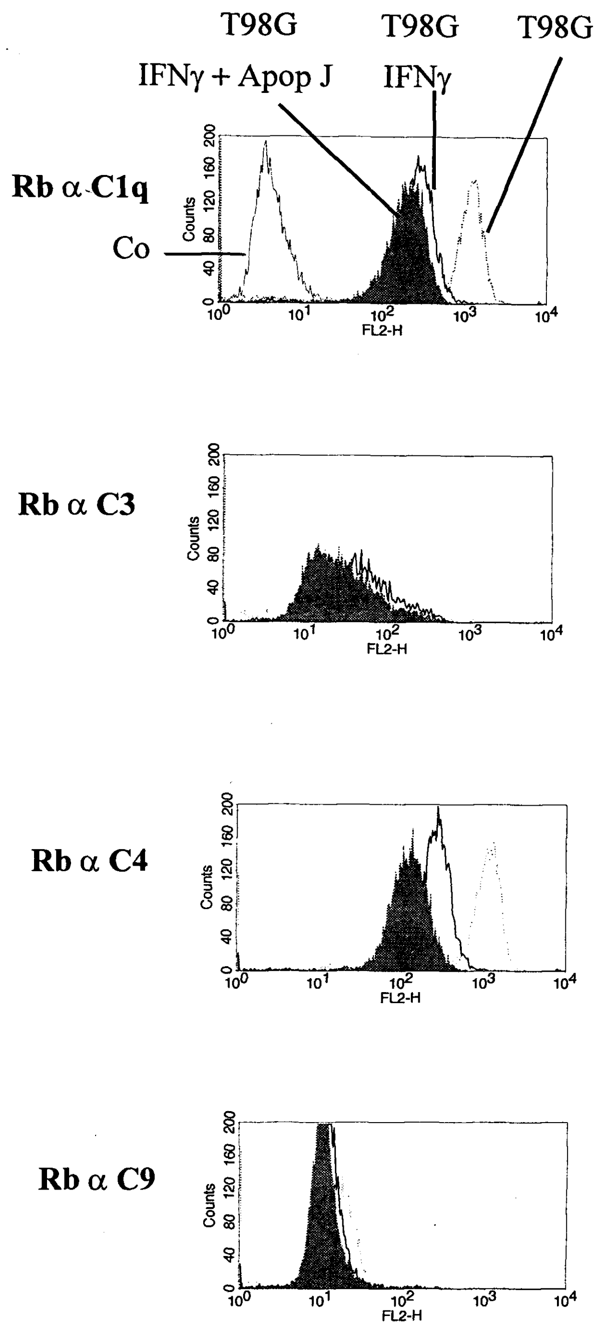
These data were obtained by Dr P. Gasque (unpublished observations).



Mean FL2 over control

	C1q	C3	C4	C9
1. THP1	492.35	37.83	488.45	6.00
3. THP1 + PMA	288.49	89.82	248.02	10.02
4. THP1 + PMA + Apop Jurkat	309.07	89.63	254.21	9.31

Figure 6.4 Single flow cytometry analysis of permeabilised THP1 cells for complement expression. Cells were stained for C1q, C3, C4 and C9 followed by a specific rPE conjugated secondary antibody, Light grey filled profiles represent secondary antibody only, grey lines represent THP1 (undifferentiated), black lines represent THP1 differentiated with PMA and grey filled profiles represent THP1 (differentiated with PMA) + addition of apoptotic Jurkat cells.



Mean FL2 over control

	C1q	C3	C4	C9
1. T98G	1127.21	59.01	1079.41	31.28
2. T98G + IFNγ	266.79	52.18	245.98	7.88
3. T98G + IFNγ + Apop Jurkat	202.93	35.63	130.95	8.56

Figure 6.5 Single flow cytometry analysis for permeabilised T98G cells for complement expression. Cells were stained for C1q, C3, C4 and C9 followed by a specific rPE conjugated secondary antibody. Light grey profiles represent secondary antibody only, grey lines represent T98G (unstimulated), black lines represent T98G stimulated with IFNγ and grey filled profiles represent T98G (stimulated with IFNγ) + addition of apoptotic Jurkat cells.

6.3.3 RT-PCR Analysis for Complement Expression by THP1 and T98G Cells

In order to further clarify complement expression/biosynthesis by THP1 and T98G prior to and after phagocytosis of Jurkat cells, the profile of complement component expression (C1q, C1r, C1s, C3, C4, C5, C9) at the mRNA level was assessed by RT-PCR (Figure 6.6 and 6.7). Differentiated THP1 cells (prior to and after phagocytosis) showed a loss of C1q expression compared to undifferentiated cells. However, unstimulated T98G cells showed only a weak C1q expression and this was lost post IFNγ treatment. Of note, to different sets of primers were used for C1q with expected product sizes of 449 bp (C1q 1) and 447 bp (C1q 2). Only the C1q 1 primers were successful in amplifying the cDNA of correct size, and therefore both sets of primers need further evaluation on their ability to amplify. THP1 cells revealed extremely weak expression for C1s and C1r, which was lost post treatment with PMA or post addition of Jurkat to PMA treated THP1. T98G unstimulated and stimulated cells expressed C1s and C1r. The expression of C1r was shown lost post addition of apoptotic Jurkat to T98G cells. Unstimulated THP1 cells expressed C3, and a decrease in expression was revealed post PMA treatment, which conflicted with the observed increased C3 expression by flow cytometry analysis. A further decrease in expression was observed post addition of apoptotic Jurkat cells and where one cell preparation revealed no C3 expression. Unstimulated T98G cells expressed C3, and a small increase in expression was observed post stimulation, which differed from the flow cytometry results where no change in expression was

shown. However, post addition of apoptotic Jurkat a decreased expression of C3 was observed.

Undifferentiated THP1 cells expressed C5 but a loss of expression was observed on stimulated cells. The observed C5 product size is different in two cell preparations due to the use of two different sets of primers with expected product sizes of 392 and 522 bp (refer to Table 6.1). There was no C4 expression observed which conflicted the high expression revealed by the flow cytometry analysis. No C9 expression was observed on either cell type. The primers were shown to successfully amplify the correct size of cDNA for C1q, Cls, C1r, C3 and C5 on both T98G and THP1 cells. However, no expression of C4 or C9 expression was observed on either cells, and therefore further experiments will need to be performed to address the capacity of the primers to amplify the cDNA of correct size. As described, set 2 primers for C1q failed to amplify a product in contrast to set 1 (Figure 6.7).

6.3.4 RT-PCR Analysis for Phagocytic Receptor Expression on Amateur and Professional Phagocytes.

In order to compare the expression of specific phagocytic receptors on amateur and professional phagocytes a number of cell lines were used (THP1, T98G, Paju, Kelly, HOG), screened by RT-PCR for receptor expression (Figure 6.8, 6.9, and 6.10), and the mean optical density for each PCR product measured (Figure 6.11, 6.12 and 6.13). THP1, the professional phagocyte was shown to express a number of receptors ranging from scavenger receptors (CD68, SRB1, CD36), to

receptors for complement (CR3, CR4, CRT), to receptors for sugars and phospholipids (PSR, FcγR) to individually classed receptors CD93, CD31, $\alpha_5\beta_3$ integrin, ATP-binding cassette transporter A1 (ABCA1) and Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1 (SHPS) (Figure 6.8A). Although THP1 are known to express CD91 protein, as shown by the flow cytometry data, the CD91 primers failed to amplify a specific band. In comparison to THP1, the amateur phagocyte, T98G astrocyte model was shown to express a smaller diversity of receptors, with only CRT expression detected (Figure 6.8B). Interestingly, the neurones (Paju and Kelly) were shown to express a wider range of phagocytic markers than T98G cells including some of the scavenger receptors, CRT, $\alpha_5\beta_3$ integrin, SHPS, CD93, CD14 and PSR (Figure 6.9A,B), and HOG, the oligodendrocyte, was shown to also express some of the scavenger receptors, CRT, $\alpha_5\beta_3$ integrin, SHPS, PSR, and in addition expression of MER was observed (Figure 6.10).

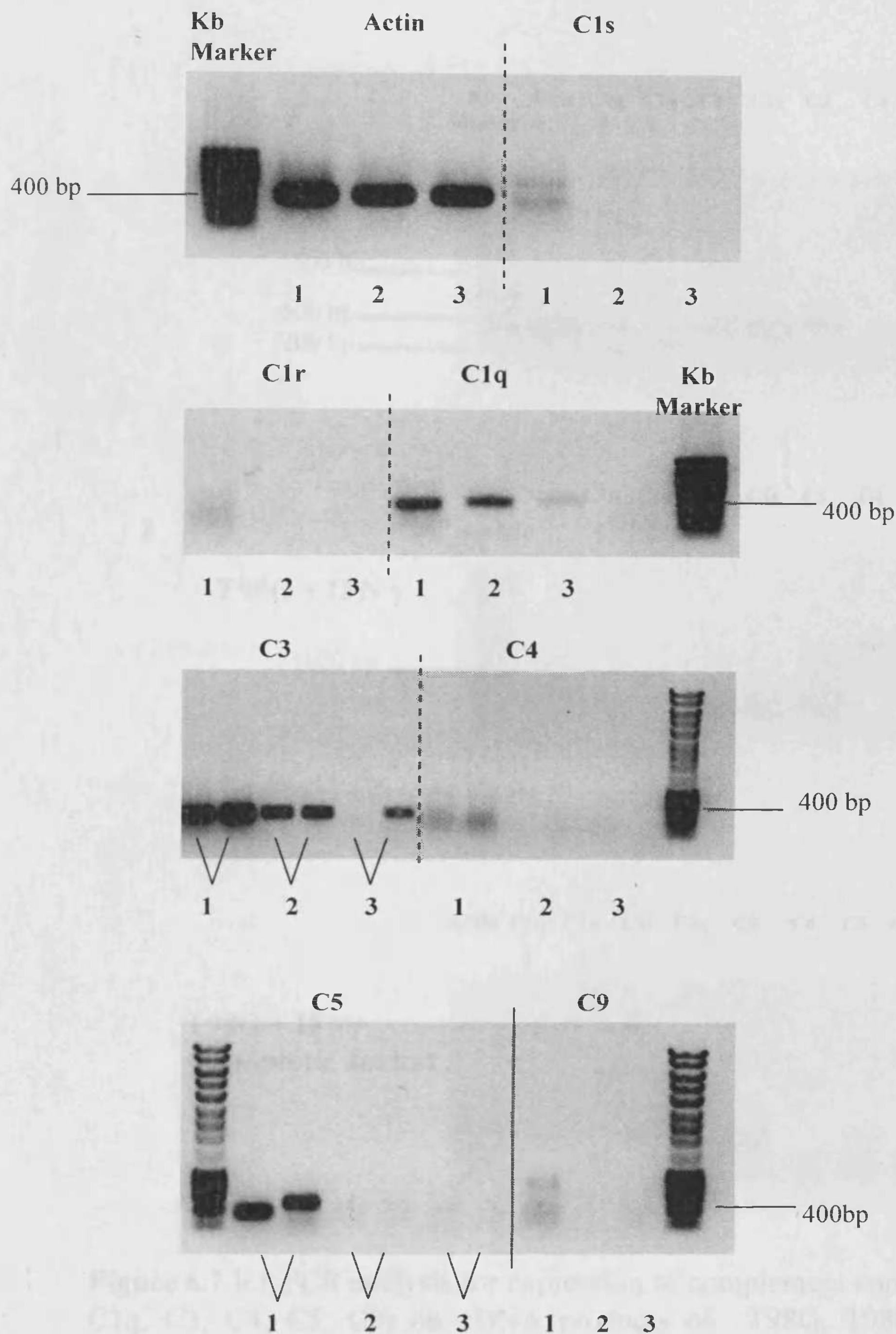
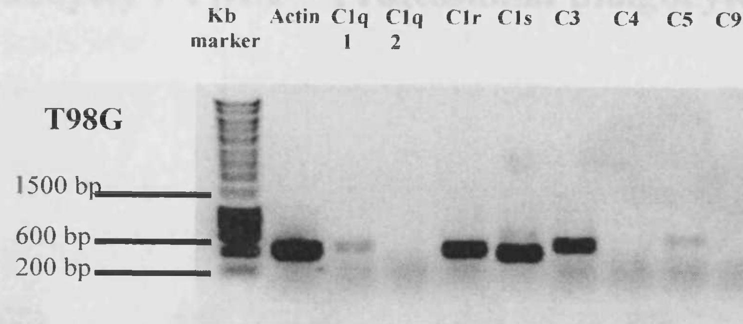
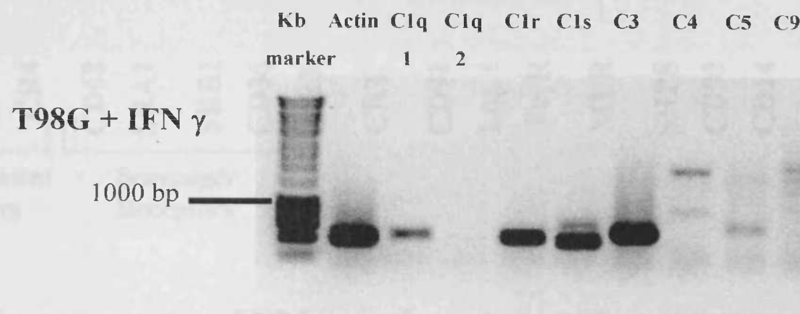


Figure 6.6 RT PCR analysis for expression of complement components (C1r, C1q, C3, C4, C5, C9) on cDNA products of THP1 undifferentiated (1), THP1 differentiated with PMA (2) THP1(differentiated with PMA) + addition of apoptotic Jurkat (3) obtained after RT-PCR (30 cycles) of 4μg of total RNA from these cells. Ethidium bromide staining of agarose gel (0.7% w/v). A 1 Kb DNA ladder was used as a marker of DNA size. Samples were run at 100 V until complete separation of DNA fragments, and the gel analysed in a UV box





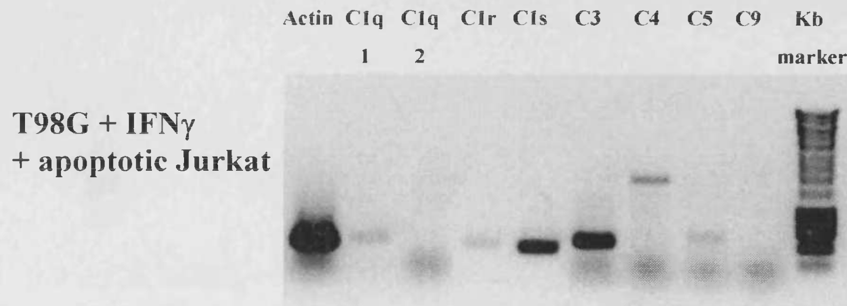
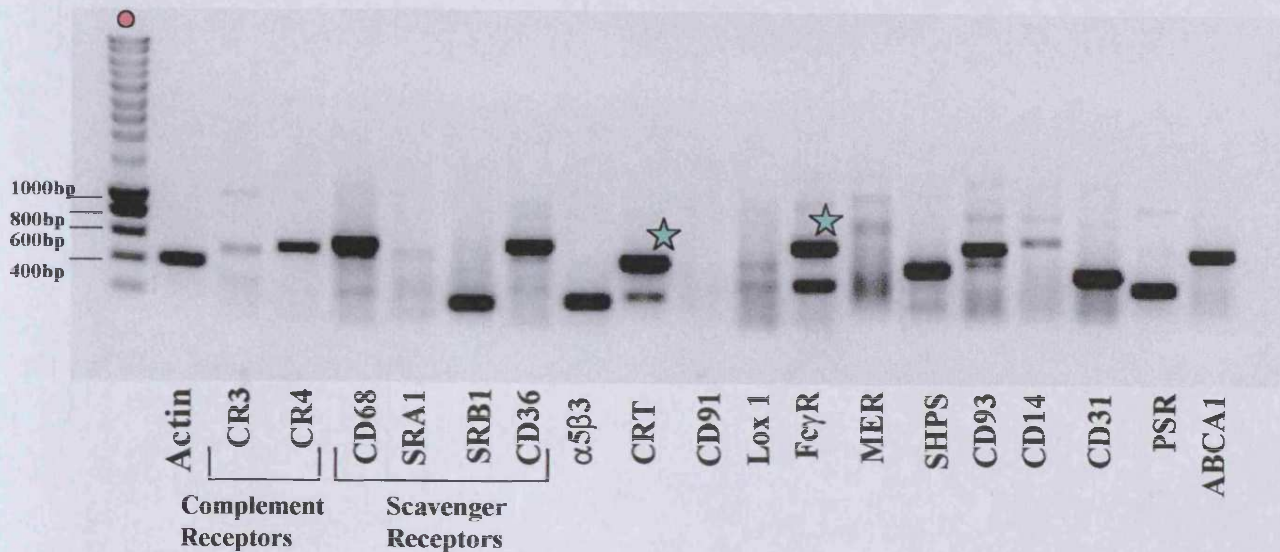


Figure 6.7 RT PCR analysis for expression of complement components (C1r, C1q, C3, C4, C5, C9) on cDNA products of T98G, T98G post IFNγ treatment and T98G (IFNγ) + addition of apoptotic Jurkat obtained after RT-PCR (30 cycles) of 4μg of total RNA from these cells. Ethidium bromide staining of agarose gel (0.7% w/v). A 1 Kb DNA ladder was used as a marker of DNA size. Samples were run at 100 V until complete separation of DNA fragments, and the gel analysed in a UV box

A. THP1 (monocyte) + PMA Professional phagocyte



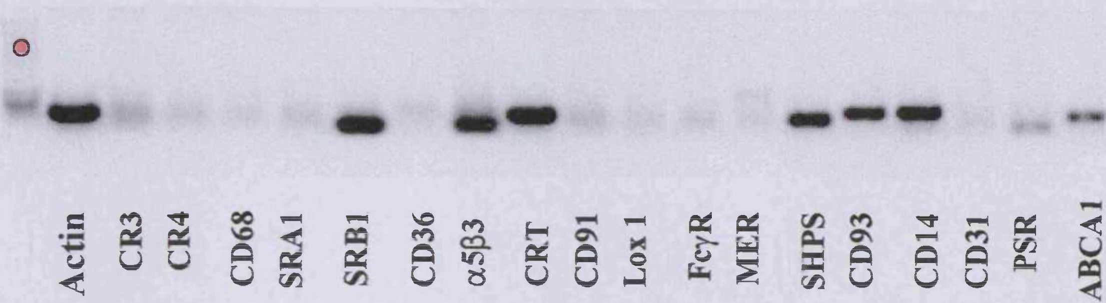
B. T98G (astrocyte) + IFNγ Amateur phagocyte

Figure 6.8 RT PCR analysis for expression of macrophage receptors on cDNA products of (A). Professional (THP1) and (B). Amateur (T98G) phagocytes obtained after RT-PCR (30 cycles) of 4μg of total RNA from these cells. Ethidium bromide staining of agarose gel (0.7% w/v).

○ A 1 Kb DNA ladder was used as a marker of DNA size. Samples were run at 100 V until complete separation of DNA fragments, and the gel analysed in a UV box

☆ - band of expected size. The amplification of the second band is likely to be due to either non specific coupling of the primers or to an alternative spliced form of the cDNA.

A. Paju Unstimulated (peripheral nerve neurones) **Amateur phagocyte**



B. Kelly Unstimulated (CNS neurones) **Amateur phagocyte**

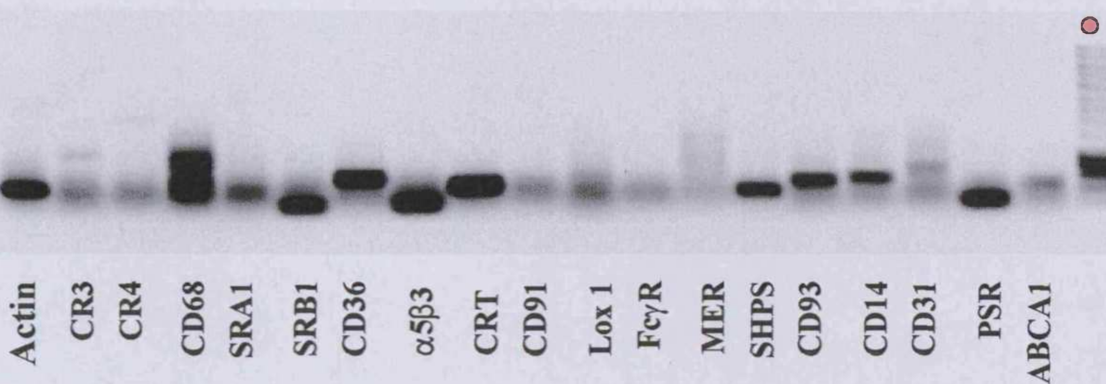


Figure 6.9 RT PCR analysis for expression of phagocytic receptors on cDNA products of neurones (A). Paju and (B). Kelly, the amateur phagocytes obtained after RT-PCR (30 cycles) of 4μg of total RNA from these cells. Ethidium bromide staining of agarose gel (0.7% w/v).

● A 1 Kb DNA ladder was used as a marker of DNA size.

Samples were run at 100 V until complete separation of DNA fragments, and the gel analysed in a UV box

HOG (Oligodendrocyte) Amateur phagocyte

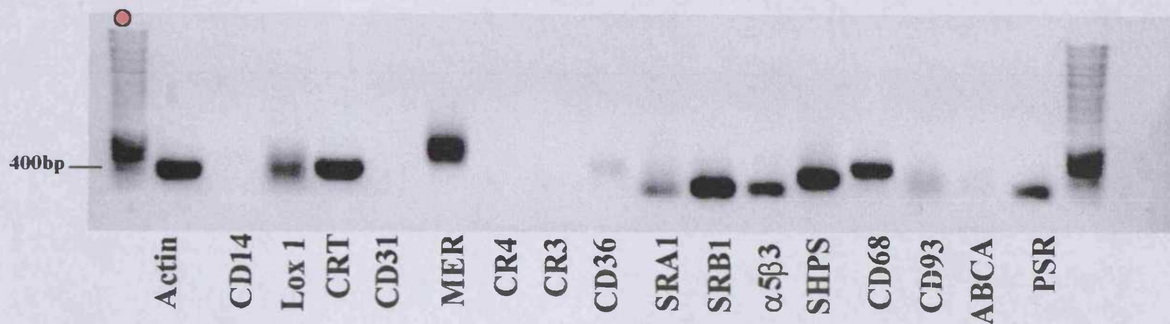


Figure 6.10 RT PCR analysis for expression of phagocytic receptors on cDNA products of HOG oligodendrocytes (amateur phagocytes) obtained after RT-PCR (30 cycles) of 4μg of total RNA from these cells. Ethidium bromide staining of agarose gel (0.7% w/v).

● A 1 Kb DNA ladder was used as a marker of DNA size. Samples were run at 100 V until complete separation of DNA fragments, and the gel analysed in a UV box

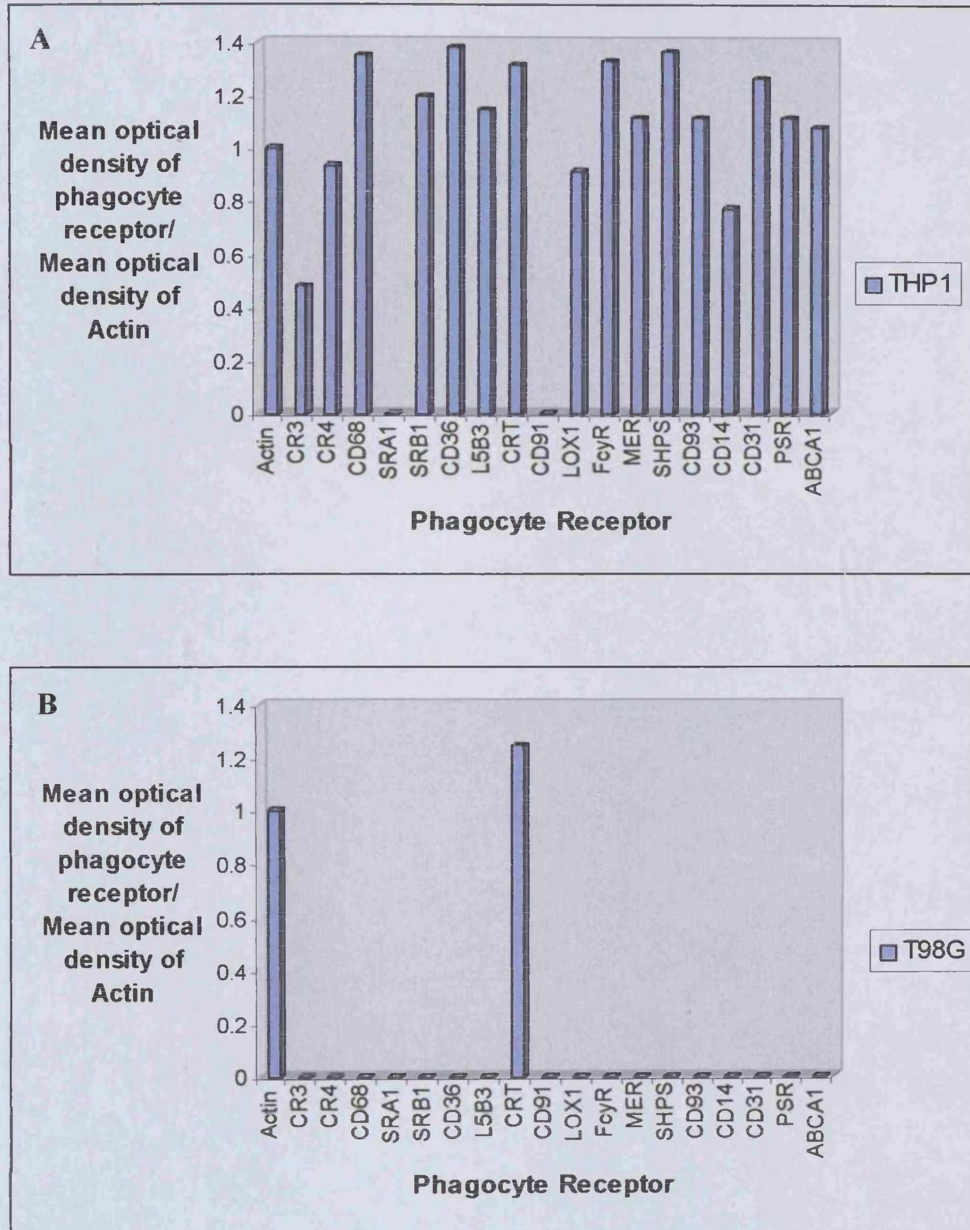
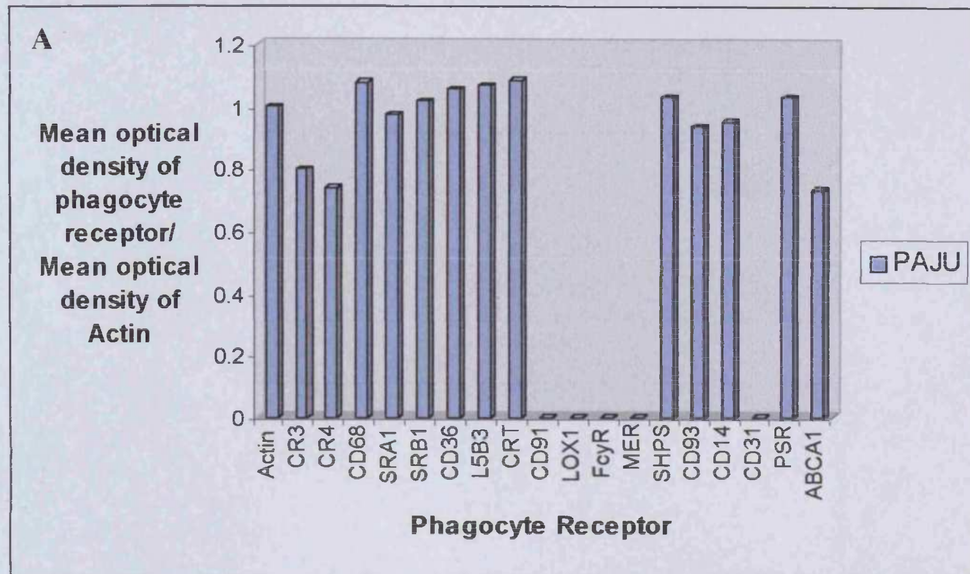


Figure 6.11 Histogram to reveal semi-quantitative PCR analysis of Phagocyte receptors by (A) THP1 (PMA) and (B) T98G (IFNγ). The mean optical density of each band of PCR product was measured and the ratio of product in relation to quantity of Actin was determined (Mean optical density of phagocyte Receptor) / Mean optical density of Actin



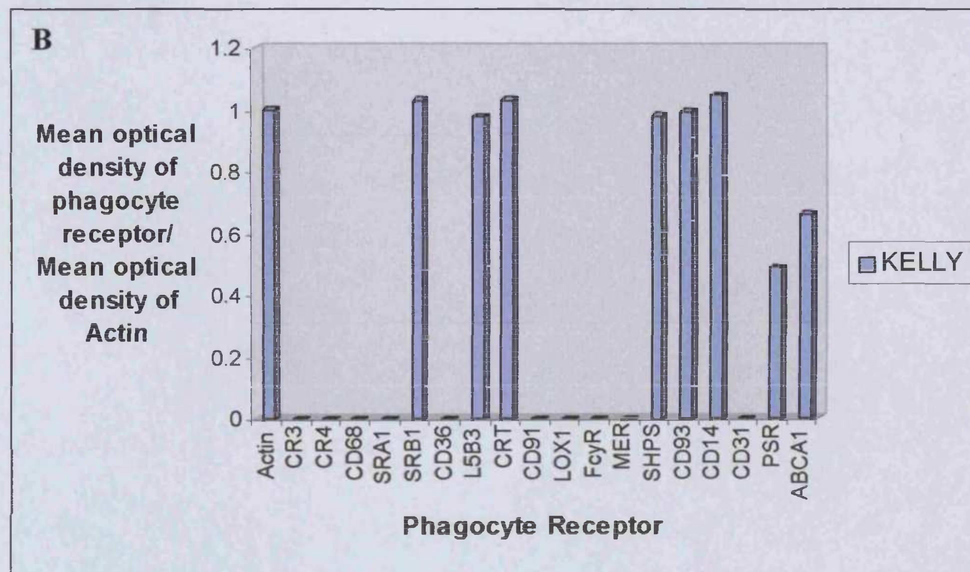


Figure 6.12 Histogram to reveal semi-quantitative PCR analysis of phagocyte receptors by (A) Paju and (B) Kelly. The mean optical density of each band of PCR product was measured and the ratio of product in relation to quantity of Actin was determined $\left(\frac{\text{Mean optical density of phagocyte receptor}}{\text{Mean optical density of Actin}}\right)$

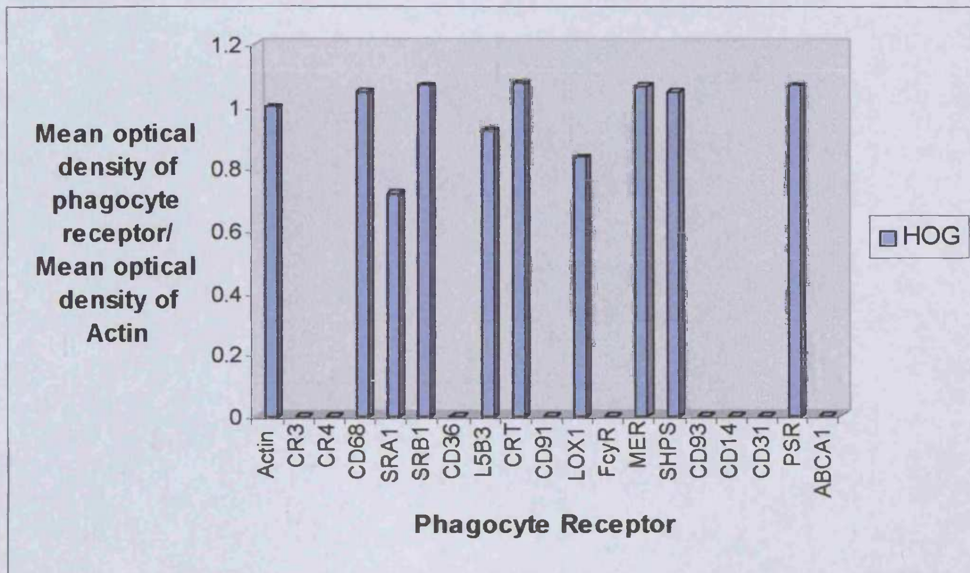


Figure 6.13 Histogram to reveal semi-quantitative PCR analysis of phagocyte receptors by HOG. The mean optical density of each band of PCR product was measured and the ratio of product in relation to quantity of Actin was determined

$$\frac{\text{(Mean optical density of phagocyte receptor)}}{\text{Mean optical density of Actin}}$$

6.4 Discussion

Analysis of the macrophage phenotype of both professional (monocytes) and amateur (astrocytes, neurones, oligodendrocytes) phagocytes has confirmed the original concept that 'professional' and 'amateur' phagocytes are believed to both possess membrane-bound pattern recognition receptors (PRRs) capable of recognising PAMPs on pathogens, and ACAMPs on apoptotic cells, leading to their clearance (Gregory 2000, Parnaik, et al. 2000). The majority of reported PRRs have also been shown to be expressed by brain cells (De Simone, et al. 2004, Gasque, et al. 2000), and this was evident from the present study whereby an array of surface receptors was shown expressed by neurones (Kelly and Paju cell lines) and oligodendrocytes (HOG cell line), however, interestingly there was a limitation to the amount of receptors expressed by one astrocyte cell line, T98G, with only CRT (RT-PCR), CD91 and PSR (showing significant staining by flow cytometry data) being expressed on stimulated cells. Therefore, further work would require extending the analysis to other astrocyte cell lines and of astrocyte primary cultures.

The analysis for receptor expression by THP1 (monocyte) and T98G (astrocyte) cells using flow cytometry (detecting membrane expression) and RT-PCR (detecting gene expression) revealed conflicting findings. Stimulated THP1 (PMA) cells were shown to express CR3, CR4, and PSR by both flow cytometry and RT-PCR, however weak CD91 staining at the cell surface was shown by flow cytometry but no expression was evident by RT-PCR. A plausible

explanation for the difference could include the set of CD91 primers that may not have been optimal for amplification. THP1/PMA were found to express CRT mRNA but this failed to materialise in the expression of CRT at the cell membrane (flow cytometry data). This could indicate that CRT may be expressed intracellularly or secreted. No amplification by RT-PCR for CD91 was observed for T98G, whereas weak surface staining by flow cytometry was also evident as seen for THP1. Flow cytometry analysis on T98G revealed only very weak surface staining of CR3, CR4 and CRT and strong staining of PSR, however post IFNγ treatment the cells showed no CR3, CR4 and CRT staining and PSR staining remained strong. RT-PCR analysis was performed on T98G cells post IFNγ treatment and therefore would explain why no CR3 or CR4 expression was evident. However, RT-PCR revealed CRT expression but no PSR expression by T98G. The results could indicate that CRT is not expressed at the cell surface of differentiated T98G cells (as depicted for THP1) or is only expressed at low levels, which cannot be detected by the antibody. The expression of PSR by flow cytometry and not by RT-PCR analysis could be due to an artefact of the PSR antibody or the set of primers may not have been optimal for amplification.

For complementary studies different primers need to be used and checked for specificity and functional activity, and in addition the localisation of the protein or detection of intracellular stores requires further analyses on permeabilised cells, and the specificity of the antibody will need to be validated by Western blotting with the identification of the protein with the expected molecular mass.

In addition to THP1 (professional phagocyte) and T98G (proposed amateur phagocyte) the RT-PCR analysis also aimed to detect the expression of a wide range of different phagocytic receptors by other brain cells (neurones – Paju and Kelly and oligodendrocytes -HOG cell lines) believed to be important contributors to the population of amateur phagocytes in the brain. The first class of receptors, membrane bound PRRs involved in direct binding to apoptotic ligands include some of the scavenger receptors (SR-AI, SR-BI, LOX1, CD68) binding to OxLDL sites (Erdosova, et al. 2002, Fukasawa, et al. 1996, Oka, et al. 1998, Platt, et al. 1996), CD14 binding to ICAM3 (Devitt, et al. 1998), CD31 involved in either detachment or adhesion to CD31 ligand (Brown, et al. 2002), CD93 binding to as yet unidentified ligand (Norsworthy, et al. 2004), SHPS binding to CD47 (Tada, et al. 2003) and PSR binding to PS (Fadok, et al. 2000). Interestingly in the present study, scavenger receptors, SHPS, and PSR were found expressed on both professional (monocytes) and amateur phagocytes (neurones, oligodendrocytes), whereas CD14 and CD93 expression was restricted to monocytes and neurones, and CD31 expressed only on monocytes. No expression of PRRs involved in direct interactions with ligands on the apoptotic surface was observed on astrocytes, and we did not have a cell line to model human CNS microglia. Therefore, the present study, using THP1, the professional phagocyte, correlated with previous evidence, whereby in the CNS, CD93 expression was found abundant on the professional microglia, but also on endothelial cells and a subset of neurones particularly in disease conditions

(Gasque *et al*, unpublished observations), and CD14 was expressed on quiescent and reactive microglia (Moffatt, et al. 1999). Previous evidence has also shown that engulfment of apoptotic neurones by microglia is dependent on carbohydrate lectin interactions, and an interaction between PS and an unidentified receptor for PS on microglia (Witting, et al. 2000). However, conflicting evidence for a role of PS mediated clearance has been reported by Chan *et al*, who revealed neither the structural PS-derivative phospho-L-serine nor the PS-binding protein Annexin V caused an inhibitory effect on phagocytosis of apoptotic thymocytes by rat microglia (Chan, et al. 2001). These contrasting findings could suggest that microglia are capable of phagocytosing apoptotic thymocytes and apoptotic neurones by different receptor signalling mechanisms, or an identical receptor could have different affinities for each cell type.

A second group of receptors consist of membrane bound PRRs recognising ACAMPs through bridging molecules. These include calreticulin (CRT) in association with CD91 binding to the C1q bridging molecule (Ogden, et al. 2001), $\alpha_5\beta_3$ integrin cooperating with CD36 in binding to the glycoprotein thrombospondin (TSP) molecular bridge (Savill 1992), MER receptor tyrosine kinase interacting with the bridging molecule Gas 6 (Ishimoto, et al. 2000, Scott, et al. 2001), ABCA1 binding to Annexin I (Luciani and Chimini 1996), and FcγR binding to C-reactive protein (CRP) or serum amyloid P (SAP) (Mold, et al. 2002). In the present study, calreticulin (CRT) but not its associated molecule CD91 (due to primers not being optimal for amplification), was found expressed

on both professional and amateur phagocytes including astrocytes, which only revealed expression for this specific receptor. The $\alpha_5\beta_3$ integrin and its associated molecule CD36 were shown expressed on professional (monocytes) and amateur phagocytes (neurones and oligodendrocytes), which was in agreement with previous evidence which revealed a Vitronectin ($\alpha_5\beta_3$) receptor-mediated mechanism in the clearance of apoptotic neurones by microglia (Witting, et al. 2000). In addition, THP1 monocytes were shown to express ABCA1 and FcγR. Fc Receptors have previously been found on microglia mediating phagocytosis of specific targets through binding of immunoglobulins (Smith 2001).

The complement receptors CR3 and CR4 are also known to bind bridging molecules C3b and iC3b, and promoting phagocytosis of apoptotic cells (Mevorach, et al. 1998, Takizawa, et al. 1996). Microglia, the 'professional' macrophages of the brain, located within the parenchyma and Kolmer cells of the choroid plexus were shown to abundantly express CR3 and CR4 (Gasque, et al. 2000). The present study confirmed expression of these receptors only by professional phagocytes (differentiated THP1).

Therefore, the present observations are in agreement with the different status of the CNS phagocyte, either having a role as a resident phagocyte (*i.e.* professional) with a plethora of receptors, or as cells, which are not otherwise readily phagocytic, but have evolved to adapt to this new role (*i.e.* amateur). Treatment of THP1 with PMA revealed a good phagocytic activity and

correlated with an increased expression of several phagocytic receptors. In contrast, T98G was shown to have a low level of phagocytic activity and IFNγ did not improve this activity. Furthermore, in agreement with their lower phagocytic activity we found that they were expressing low levels of CRT and failed to co express CR3 and CR4 post stimulation. PSR was the only receptor expressed at high levels.

The phagocytes could undergo different signalling events mediated through the engagement of one or more of their specific receptors, which dictates the final immune response. In addition amateur phagocytes may only be activated to clear apoptotic cells when the professional phagocyte is overwhelmed. In particular, the neuronal cell lines were shown to express an array of receptors and therefore could be considered as the most favourable amateur CNS phagocyte to aid the professionals in clearance of apoptotic cells. There has been only one published report to date conveying neurones may be involved in phagocytosis of apoptotic T cells (Flugel, et al. 2000), therefore further functional experiments are required to confirm their ability to successfully phagocytose when the professional phagocytes of the CNS are overwhelmed. Redundancy conveys the presence of multiple receptors, which ensure phagocytosis when one or more of the receptors are switched on. Efficiency suggests different receptors engage with different stages along the apoptotic pathway, resulting in a series of 'first-line' and 'back-up' mechanisms that are required for rapid clearance when apoptosis of the target cell occurs.

The present studies flow cytometry and RT-PCR analysis for complement expression by professional (THP1) and amateur (T98G) phagocytes confirmed the hypothesis presented by Medzhitov and Janeway (1997), that a plethora of innate immune molecules are expressed by activated professional and amateur brain cells to recognise PAMPs and ACAMPs (Medzhitov and Janeway 1997). A number of secreted PRRs have previously been characterised and these include the defence collagen, C1q, and the collectins, MBL and SPA (Tenner 1999). C1q is known to bind to apoptotic surface blebs (Korb and Ahearn 1997), and there is increased evidence that brain cells can generate a full innate immune system to mount a response against apoptotic cells (See review, (Gasque, et al. 2000)). Levi-Strauss and Mallat were the first to confirm that brain cells were able to produce complement. They revealed cultured rodent astrocyte cell lines and primary murine astrocytes produced C3 and factor B (Levi-Strauss and Mallat 1987). In the present study, professional (THP1) and amateur (T98G) phagocytes were shown to express C1q, in agreement with previous evidence, which had portrayed expression on astrocytes (Barnum 2002, Gasque, et al. 1993), and microglia (Walker, et al. 1995). However, an increase in C1q expression post stimulation was not observed for either THP1 (monocyte) or T98G (astrocyte) phagocytes, indicating C1q levels could be a limiting factor to clear apoptotic cells. Of note, the finding that C1q expression was not increased after IFNγ stimulation of T98G and in fact was decreased contrasted previous

evidence, whereby synthesis of all complement components was enhanced (Barnum 2002, Gasque, et al. 1992).

THP1 and T98G were shown to express C3, which confirmed previous evidence of expression of C3 on astrocytes, microglia, neurones and oligodendrocytes (Gasque, et al. 1993, Gasque, et al. 1992, Gasque and Morgan 1996, Walker, et al. 1995, Walker and McGeer 1993). In contrast to the findings for C1q an increase in C3 expression was observed on stimulated THP1 professional phagocytes by flow cytometry but not on stimulated T98G amateur phagocytes. However, RT-PCR analysis revealed a decreased expression of C3 on stimulated THP1 cells compared to unstimulated cells and an increase in expression on stimulated T98G cells. The explanations for the conflicting results could be that the amount of cDNA used per cell preparation might have been slightly different, or secondly the primers were not successful in amplifying the cDNA in each cell preparation or finally the increase in C3 staining in the flow cytometry analysis might have been due to an artefact of the C3 antibody.

C3 is known to be opsonised on the apoptotic cell surface to aid in the recognition of the cell by phagocyte receptors CR3 and CR4 (Mevorach, et al. 1998). The binding of professional phagocytes by way of CR3/CR4 recognition of C3b is suggested in the present flow cytometry study, with increased CR3/CR4 expression observed on stimulated THP1 but not T98G. Evidence to date has also revealed astrocytes to express receptors for complement components and fragments (CR1, CR2, C3aR and C5aR) with the exception of

CR3 and CR4 (Gasque, et al. 1996, Gasque, et al. 1995). Therefore the present findings for T98G uphold this evidence.

Flow cytometry analysis of THP1 and T98G cells revealed C4 expression on unstimulated cells, which supported previous evidence of expression on astrocytes (Gasque, et al. 1993, Walker, et al. 1998) and the level of expression was decreased post stimulation. In contrast, RT-PCR analysis revealed no C4 expression on THP1 or T98G cells, which conflicted the high expression revealed by the flow cytometry analysis. The set of primers may not have been optimal for amplification in these experiments and therefore their ability to amplify needs confirmation. There was also no C9 expression observed, and therefore if the primers were checked for optimal amplification ability this would indicate a prevention of cell lysis by complement and safe clearance of apoptotic cells in an anti-inflammatory response.

Therefore, in summary, THP1 (professional) and T98G (amateur) were shown to express complement components, but counter intuitively a significant decrease in complement expression following phagocytosis of apoptotic cells was observed (i.e. observed decrease in C1q expression).

Chapter 7

General Discussion

7.1 Introduction

Safe and efficient removal of apoptotic cells is of paramount importance across a range of multicellular organisms in maintaining homeostasis and preventing injury. With focus on the CNS, it is vital that the resident professional, and amateur phagocytes work together to efficiently remove apoptotic cells and other toxic cell debris, thus preventing associated pathologies that arise from insufficient clearance (e.g. Huntington's disease, Parkinson's disease, and Alzheimer's disease) (Yuan and Yankner 2000). It has been proposed that a synergy exists between exposed "Eat me" and low levels of "Don't eat me" signals to instruct amateur or professional phagocytes, *via* their surface PRRs, to rapidly dispose of apoptotic cells before secondary necrosis ensues, thus limiting the release of toxic and pro-inflammatory cell debris (Elward and Gasque 2003, Grimsley and Ravichandran 2003, Savill and Fadok 2000).

7.2 C1q and other complement opsonins on apoptotic cells: proposed, "Eat me signals"

Cells undergoing apoptosis display a number of "Eat me signals". Some are well characterised, such as exposure of PS normally restricted to the inner membrane leaflet or poorly characterised such as changes in surface sugars detected by

phagocyte lectins. Other "Eat me" signals have also been defined for their capacity to bind adhesive bridging molecules such as innate immune defence collagen molecules MBL, surfactant proteins SP-A, SP-D and complement C1q (Tenner 1999). The key role of C1q has been confirmed by comprehensive studies demonstrating that C1q deficient animals were severely impaired in the clearance of apoptotic cells (Botto, et al. 1998). C1q has also been shown to act as a bridging molecule between, as yet, unidentified C1q binding sites on apoptotic cells and the phagocyte calreticulin (CRT)/CD91 complex (Vandivier, et al. 2002). The present Clq findings confirmed previous evidence whereby C1q was shown to bind directly to apoptotic cells and apoptotic blebs and released in C1q-enriched microparticles found in plasma (Nauta, et al. 2002). It also further argued for a central role for C1q in the early detection of membrane bound nucleic acid (NA) on apoptotic cells long before they expose the canonical PS "eat me" signal. DNA has long been known as an activator of the complement pathway through its interaction with both the collagen-like region and the globular region of C1q (Jiang, et al. 1992). A recent report by Palaniyar *et al* portrayed major defence collagens (SP-D, MBL, C1q but not SP-A) were involved in binding to carbohydrate (pentose sugar-based anionic phosphate) polymers including free DNA, RNA and nucleic acids present on apoptotic cells (Palaniyar, et al. 2004). Direct binding and competition studies indicated the collagen and globular domains were involved in interactions with DNA (Palaniyar, et al. 2004). Work in the present study has revealed DNAse and RNAse treated apoptotic cells failed to activate the classical pathway on apoptotic

cells with reduced C1q and C3 levels at the cell surface. This contrasted with the finding observed when necrotic cells were treated with DNAse/RNAse, which suggested that other mechanisms (e.g. cardiolipin exposed on mitochondria) may be driving C1q binding and complement activation on necrotic cells (Peitsch, et al. 1988).

C1q could therefore contribute a dual role in phagocytosis of apoptotic cells, firstly as an activator of the classical complement pathway to promote opsonisation by C3 fragments, and secondly via a role as a bridging molecule between complement receptors and as yet unidentified ligand on apoptotic cells. Macrophages have been previously shown to synthesise and also display C1q as a type II cell surface molecule (Kaul and Loos 1995), and the present study has shown C1q to be expressed on THP1 and T98G phagocytes. Therefore, further studies to ascertain the direct contribution of C1q as a receptor binding to NA, a proposed "Eat me" signal, to mediate safe clearance of apoptotic cells is required.

7.3 Expression of CD46 on Apoptotic Cells: is CD46 a SAMP?

Host cells use the membrane complement regulatory proteins (CD46, CD55, CD59) to prevent assembly of the MAC and resultant pro-inflammatory cell lysis. CD55 binds to and breaks up the classical and alternative C3/C5 convertase enzyme, CD46 acts as a cofactor for factor I allowing it to cleave C4b and C3b opsonins deposited on self tissues, and CD59 binds to C8 in the C5b-8 complex and blocks further incorporation of C9 to form the MAC (Morgan 1999, van Beek, et al. 2003). During apoptosis, down-regulation of these complement

regulatory proteins has been reported (Hara, et al. 1996, Jones and Morgan 1995, Tsuji, et al. 1994), however, their role, if any, in promoting phagocytosis remains to be elucidated.

The present study has revealed that during apoptosis CD46 is removed from cytoplasmic/membrane and nuclear stores to surface bleb structures, which are released from the apoptotic cell. However, the exact mechanisms involved remain to be elucidated. The population of apoptotic cells that showed decreased CD46 expression also had robust complement activation (C3b) on their cell surface, which ultimately would make them more appetising to macrophages expressing C3b receptors. In this context and given that CD46 is ubiquitously expressed on all nucleated cells it could be argued that CD46 is a key and novel example of a 'self-associated molecular pattern, SAMP' behaving as a "Don't eat me" signal to control the outcome of the apoptotic cell by the innate immune system. Dr P. Gasque has shown that fifteen different human cell lines and primary human cell cultures induced to undergo apoptosis were rapidly losing CD46 at the cell surface and furthermore, Crry, the CD46-like protein in rodents, was also found to be dramatically reduced in several apoptotic cell models and possibly capping into blebs. Therefore, the question that remains to be answered is whether CD46/Crry is a universal SAMP to control the innate immune recognition by professional and amateur phagocytes in analogy to MHC-class I inhibiting NK cell killing. *In vivo* experiments along these lines are highly warranted. CD46 genetic defects in humans or Crry experimental deficiency have been shown associated with severe pathologies including haemolytic

uremic syndrome (HUS) and embryonic lethality (Richards, et al. 2003, Xu, et al. 2000). It is still unclear whether the immunoregulatory properties of CD46/Crry are exclusively dependent on their capacity to control complement opsonisation. Crry is capable of protecting from innate immune recognition in a complement-independent manner stressing the plausible role of a CD46/Crry counter receptor (CD46R), which still needs to be identified (Caragine, et al. 2002). It will therefore be important to ascertain whether canonical innate immune cells (macrophages, neutrophils, mast cells, dendritic cells) express the putative immunoregulatory CD46R. A CD46-human IgG4 Fc fusion protein was produced and shown to bind to macrophage (THP and HL60) (K. Elward, unpublished observation). Purification of the CD46R interacting with CD46 and subsequent analysis of the protein sequence by mass spectrometry is required.

In contrast to CD46, the majority of GPI-anchored CD55 and CD59 proteins remained attached to the apoptotic cells, which support the hypothesis that apoptotic cells are capable of controlling the cytotoxic and cytolytic activities of the MAC. This also reiterates the importance of the complement regulators in controlling the route to cell death, whether *via* a favourable anti-inflammatory apoptotic process or *via* an adverse pro-inflammatory necrotic process. The results also revealed a novel observation that necrotic cells rapidly lose soluble forms of CD55, CD46 and CD59, and therefore became incapable of controlling C3 opsonisation and MAC formation. This finding confirmed recent evidence

whereby soluble CD46 was shown to be shed from necrotic tumour cells in response to matrix metallo-proteases (Hakulinen, et al. 2004).

In addition to CD46 concentration to apoptotic blebs, complement C1q and PS, along with C3b/iC3b, were shown opsonised at the surface of apoptotic cells and clustered to bleb structures. The position of PS, a well characterised "Eat me" signal (Fadok, et al. 2000) may be involved in promoting phagocytosis.

7.4 The Decreased Expression of CD46 from apoptotic cells does not Require CD46-Mediated Protein Phosphorylation

Only recently has evidence started to accumulate to determine which isoforms of CD46 are involved in intracellular signalling (for review see (Russell 2004)). The present findings revealed removal of CD46 to apoptotic blebs was not dependant on phosphorylation of the cytoplasmic tail by src kinases, casein kinases or protein kinase C, and another signalling mechanism must therefore be identified. The induction of apoptosis was shown to result in caspase-mediated apoptosis, and CD46 was lost from these cells. However, a direct link between the loss of CD46 and caspase and PARP activity cannot be implied. It is therefore paramount to elucidate the exact signalling events involved in translocation of CD46 into apoptotic blebs. Of note, interestingly apoptotic MCF7 (breast cancer cell line) did not lose CD46 although they do not express caspase 3 (P. Gasque observations (Elward, et al. 2005))

Studies by Eda *et al* revealed the susceptibility of Jurkat T cells to macrophage recognition at an early stage of apoptosis was due to the loss of CD43 (an anti-adhesive molecule) on Jurkat cells. They showed CD43 capping was dependent on caspase activity and therefore proposed that caspase-dependent cytoplasmic events causing cytoskeletal changes were involved. One of the hypotheses considered was the involvement of moesin, a cytoskeletal protein of the ERM (ezlin/radixin/moesin) family that cross-links the intracellular domain of CD43 and actin filaments. During caspase activation moesin had previously been shown to dephosphorylate and detach from the plasma membrane resulting in the disappearance of microvilli from the cell. Therefore, Eda *et al* suggested CD43 capping may be one of the consequences of this cytoplasmic disintegration, or if not, some cytoskeletal machinery could actively induce the movement of CD43 to form a cap. They also proposed that the loss of the CD43 cap from apoptotic cells could result from proteolytic cleavage of the protein, because CD43 is known to be proteolytically down-regulated upon stimulation or spontaneously, and the extra cellular fragment of CD43 is released into plasma (Eda, et al. 2004). These findings, which highlighted a possible role for cytoskeletal proteins in transporting proteins for capping during apoptosis, convey a highly possible route for CD46 translocation.

Studies by Coleman *et al* showed the occurrence of apoptotic membrane blebbing was dependent on the function of ROCK (Rho-associated kinase). ROCK I is cleaved during apoptosis by activated caspases, generating a truncated kinase with increased intrinsic activity. This cleaved form is sufficient to drive

cell contraction and membrane blebbing. The activity of ROCK proteins and consequent membrane blebbing are required for redistribution of fragmented DNA from the nuclear region into membrane blebs and apoptotic bodies (Coleman, et al. 2001). Therefore, it would be interesting to ascertain whether ROCK proteins could be involved in the translocation of CD46 from nuclear and cytoplasmic stores to apoptotic blebs.

Recently, studies by Hakulinen *et al* on cancer cells, revealed CD46 to be constitutively shed from membranes in vesicles in an intact form, and as a soluble form resulting from metalloproteinase (MMP) cleavage (Hakulinen, et al. 2004). The results in the present study also revealed necrotic Jurkat T cell supernatants were highly enriched for sCD46 an indication that MMPs may be involved. However, the supernatant from apoptotic Jurkat T cells was not enriched with sCD46 indicating the majority of CD46 was released in blebs shed from the surface membrane and not as a cleaved soluble form. However the involvement of MMPs in the cleavage of the small amount of sCD46 observed in the supernatant of apoptotic cells could not be ruled out. The present observations have therefore suggested that the preferred mechanism of signalling and clustering to blebs for CD46 release is a phenomenon observed on apoptotic and not necrotic cells.

7.5 Expression of PRRs on Monocytes, Glial Cells and Neurones

Aside from the analysis for "Eat me" signals and "Don't Eat" me signals on apoptotic cells, the associated PRRs expressed on professional and amateur

phagocytes were analysed, and complement expression/biosynthesis by phagocytes prior to and post phagocytosis was determined. The present results confirmed the already portrayed different status of the CNS phagocyte, either having a role as a professional phagocyte (i.e. macrophages/microglia) (Perry, et al. 1993) or as cells, which are not otherwise readily phagocytic, but have evolved to adapt to this new role (*i.e.* amateur) (Dong and Benveniste 2001, Flugel, et al. 2000, Martino, et al. 2001).

Treatment of THP1 (monocyte cell line) with PMA revealed a good phagocytic activity and correlated with an increased expression of several phagocytic receptors. In contrast, T98G (astrocyte cell line) was shown to have a low level of phagocytic activity and IFNγ did not improve this activity. Furthermore, in agreement with their lower phagocytic activity we found that they were expressing low levels of CRT and failed to express high levels of CR3 and CR4 post stimulation. PSR was the only receptor expressed at high levels, although this data will need further investigation. Further work would require extending the analysis to other astrocyte cell lines and primary cultures.

The majority of reported PRRs have also been shown to be expressed by brain cells (De Simone, et al. 2004, Gasque, et al. 2000), and this was evident from the present study whereby an array of surface receptors was shown expressed by neurones (Kelly and Paju cell lines) and oligodendrocytes (HOG cell line). In particular, the neuronal cell lines expressed the highest number of receptors out of all the proposed amateur phagocytes analysed, and therefore could be considered as the most favourable amateur CNS phagocyte to aid the

professionals in clearance of apoptotic cells. There has been only one published report to date conveying neurones may be involved in phagocytosis of apoptotic T cells (Flugel, et al. 2000), and further functional experiments are required to confirm their ability to successfully phagocytose when the professional phagocytes of the CNS are overwhelmed.

7.6 Expression of Complement by THP1 and T98G

Professional (THP1) and amateur (T98G) phagocytes were shown to express complement components (C1q, C3 and C4) but a significant increase in complement biosynthesis was not required to aid in phagocytosis, instead a decrease in complement expression following phagocytosis of apoptotic cells was observed (i.e. observed decrease in C1q expression).

In conclusion, the present findings convey an emerging role for the complement regulatory protein CD46 as a "Don't eat me" signal. CD46 has been shown to concentrate to apoptotic blebs from cytoplasmic/membrane and nuclear stores and is released from the membrane in the form of micro-particles, which are opsonised with PS, C1q, and C3b, NA as well as CD46, which engage well known receptors on phagocytes (PSR, cC1qR/CD91, CR3), and to a CD46 receptor, which is yet to be characterised. It could be proposed that these micro-particles provide 'appetisers' to the surrounding phagocytes to prepare for a bigger meal, the apoptotic corpses. The role of CD46 in micro-particles, apart from ensuring C3b is converted to iC3b, remains to be fully elucidated. The

clearance of these micro-particles by CNS professional or amateur phagocytes, without the need for further complement biosynthesis, is of paramount importance before injury ensues. However, the exact signalling mechanisms for clearance still need further characterisation.

7.7 Future Objectives

1. The present findings stress the emerging role of CD46 as a "Don't eat me" SAMP signal and NA as an "Eat me signal" interacting with C1q. Further experiments are required to delineate the exact signalling mechanisms involved between NA and the proposed receptor, C1q and between CD46 and as yet unidentified CD46 receptor on phagocytes. The current understanding of "Don't eat me" signals (CD46, CD200, CD47) in contrast to "Eat me" signals is still primitive, and therefore more research is needed to identify and characterise the signalling mechanisms of these SAMP's to phagocytes across a range of species.
2. CD46 is known to be ubiquitously expressed on all nucleated cells, and therefore could be characterised as a vital 'self-associated molecular pattern, SAMP' behaving as a "don't eat" me signal to control the demise of the dying cell by the innate immune system. In addition to the apoptotic Jurkat model studied in the present work, characterisation of CD46 as a "Don't eat me" signal across other cells is warranted, and work at present in the laboratory has confirmed fifteen different human cell lines and primary human cell cultures induced to undergo apoptosis

rapidly lose CD46 at the cell surface. Crry, the CD46-like protein in rodents, was also shown dramatically reduced in several apoptotic cell models and possibly through capping into blebs. Therefore experiments are required to determine whether CD46/Crry is a universal SAMP to control the innate immune recognition by professional and amateur phagocytes.

3. The exact signalling mechanisms involved in the removal of CD46 from cytoplasmic/membrane and nuclear stores to apoptotic blebs for release from the apoptotic cell needs to be elucidated.
4. Characterisation of specific receptors on professional and amateur CNS phagocytes has been addressed with the interesting observation that neurones could play an important role in phagocytosis of apoptotic cells. Further studies are required to delineate the possible cellular functions of receptor/ligand interactions, and possible cross talks between receptors involved in recognition of "eat me" and "don't eat me" signals to instruct a non-phlogistic response.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 43, pp. 36342–36354, October 28, 2005
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CD46 Plays a Key Role in Tailoring Innate Immune Recognition of Apoptotic and Necrotic Cells*

Received for publication, June 16, 2005, and in revised form, August 3, 2005 Published, JBC Papers in Press, August 8, 2005, DOI 10.1074/jbc.M506579200

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Complement is the canonical innate immune system involved in host defense and tissue repair with the clearance of cell debris. In contrast to the robust armory mounted against microbial *nonself*-pathogens, complement is selectively activated on *altered self* (*i.e.* apoptotic and necrotic cells) to instruct the safe demise by poorly characterized mechanisms. Our data shed new light on the role of complement C1q in sensing nucleic acids (NA) rapidly exposed on apoptotic Jurkat T cell membranes and in driving C3 opsonization but without the lytic membrane attack complex. DNA/RNase-treated apoptotic cells failed to activate complement. We found that several other apoptotic cell models, including senescent keratinocytes, ionophore-treated sperm cells, and CMK-derived platelets, stained for cleaved caspase 3 were rapidly losing the key complement regulator CD46. CD46 from nuclear and membrane stores was found to cluster into blebs and shed into microparticles together with NA, phosphatidylserine, C1q, and factor H. Classical and alternative pathways of complement were involved in the recognition of H_2O_2-treated necrotic cells. Membrane attack complex was detected on necrotic cells possibly as a result of CD46 and CD59 shedding into soluble forms. Our data highlight a novel and universal paradigm whereby the complement innate immune system is using two synergistic strategies with the recognition of altered self-NA and missing self-CD46 signals to instruct and tailor the efficient removal of apoptotic and necrotic cells in immunoprivileged sites.

In analogy to the specificity of the adaptive immune response, innate immunity is extremely selective and has divided the universe into innocuous "*self*" and potentially noxious (danger) substances ranging from toxic cell debris ("*altered self*," *e.g.* apoptotic cells, necrotic cells, amyloid fibrils, and prion infectious agent) to nonself-microorganisms.

Hence, selective innate immune recognition is established essentially according to particular molecular patterns (*i.e.* apoptotic cell-associated molecular patterns (ACAMPs),[2] pathogenic protein-associated molecular patterns (PPAMPs), and pathogen-associated molecular patterns (PAMPs)) (1–4). Ultimately, "professional" and "amateur" phagocytes recognize ACAMP/PPAMP/PAMPs "eat me" signals through specific soluble and membrane-bound pattern-recognition receptors (*e.g.* complement collectins and phagocytic receptors), which lead to clearance of the different target cells (4–7).

The complement system is the canonical innate immune system capable of recognizing a plethora of PAMPs, PPAMPs, and ACAMPs essentially through mannan-binding lectin (MBL) and C1q to initiate the activation of the complement cascade (7–9). Historically, C1q was first shown to bind to antigen-antibody complexes, but it is increasingly evident that C1q multimeric structure is key to the selective recognition of apoptotic cells, toxic amyloid fibrils, and the pathogenic prion agent by mechanisms that remain poorly understood (10–18). These events will lead to the opsonization of the target with C1q, C4, and C3 opsonins recognized by macrophages bearing complement pattern-recognition receptors such as CR1, CR3, and CR4 (where CR indicates complement receptor) (7, 19, 20).

Furthermore, innate immune recognition is based also on recognition of molecular markers specific for self to prevent uncontrolled phagocytosis (3). These markers are gene products expressed only on the surface of normal uninfected cells of the host but not on microbial cells. They are lost from the membrane of infected cells or abnormal cells such as senescent, apoptotic, necrotic, and transformed cells. Recognition of these "don't eat me" signals (also known as self-associated molecular patterns (SAMPs)) (4, 7) by different soluble and membrane-bound pattern-recognition receptors is coupled to the inhibition of the innate immune response (3, 21). The best characterized example of the marker of self is the recognition of the major histocompatibility complex class I molecules by various inhibitory receptors expressed on natural killer (NK) cells (22). As major histocompatibility complex I is constitutively expressed on all cells (apart from erythrocytes) and is often down-regulated as a result of viral infection or cellular transformation, recognition by NK cells of a missing self-ligand allows them to selectively eliminate infected and transformed cells and spare normal, healthy self-cells.

The missing self-strategy is not unique to NK cell function and is widely used by the innate immune system (for review see Refs. 3, 4, 7, and 21). CD47, CD200, and sialic acids have been recognized recently as classical SAMPs involved in the control of phagocytosis by macrophages (23–28). Another well known example is the regulation of the complement system by membrane-bound complement regulatory proteins (CRegs, *e.g.* CD46, CD55, and CD59) (for comprehensive review see Refs. 29–31). As cells of "nonself" origin, including pathogens, lack these host gene products, activation of the complement cascade can proceed uninhibited resulting in lysis or phagocytosis of the target cells.

Unequivocally, complement plays a critical role in the scavenging of toxic cell debris for safe and efficient removal by phagocytes before

* This work was supported by the Medical Research Council (to P. G. and K. E.), the UK Multiple Sclerosis Society (to P. G. and M. G.), and the Wellcome Trust (to C. L. H., M. M., and B. P. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: ACAMPs, apoptotic cell-associated molecular patterns; NA, nucleic acids; MAC, membrane attack complex; DAPI, 4,6-diamidino-2-phenylindole; PPAMPs, pathogenic protein-associated molecular patterns; PAMPs, PAMPS for pathogen-associated molecular patterns; NK, natural killer; FACS, fluorescence-activated cell sorter; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase; Z, benzyloxycarbonyl; FMK, fluoromethyl ketone; FITC, fluorescein isothiocyanate; SAMPs, self-associated molecular patterns; NHS, normal human serum; CR, complement receptor; s, soluble; PS, phosphatidylserine; CRegs, complement regulatory proteins; MBL, mannan-binding lectin.

engaging the pro-inflammatory response (mediated by C5a) and secondary necrosis (because of the membrane attack complex (MAC)). Neighboring cells can express a full complement system on demand (32), and C1q will flag target cells through its capacity to bind to apoptotic blebs and necrotic cells. The mechanisms for selective C1q binding to abnormal cells remain poorly characterized, although natural IgM antibodies have been shown to be required for C1q-dependent complement opsonization of late apoptotic/necrotic cells (33–35). Of note, in immunoprivileged sites such as the testis and brain, which lack an adaptive immune response (hence IgM), it is plausible that other signals are involved to initiate the activation of the complement classical pathway (36).

We recently argued that the expression of CRegs could be modulated when the cells undergo programmed cell death (4). It has already been reported that CRegs are reduced on dying cells (37–39); however, it is unknown how CRegs are lost from apoptotic/necrotic cells and to what extent the loss of one or more CRegs provide key signals to promote the recognition and clearance of these dying cells and to ensure the anti-inflammatory/pro-inflammatory responses, respectively.

This study describes for the first time the molecular mechanisms and routes involved in the recognition and decoding of apoptotic and necrotic cells by the canonical innate immune complement system. Unique knowledge of these pathways will be discussed in the context of physiological and pathological settings.

MATERIALS AND METHODS

Cells and Reagents—Human Jurkat E6 T lymphocyte cell line obtained from the European Collection of Cell Cultures (ECACC) (Porton Down, Salisbury, UK) was cultured at 37 °C, 5% CO_2 in RPMI 1640 containing 5% heat-inactivated fetal bovine serum, penicillin/streptomycin (50 μg/ml), L-glutamine (2 mg/ml), sodium pyruvate (1 mg/ml), and fungizone (2.5 μg/ml) (Invitrogen) at a density of 1×10^6/ml in 75-cm^2 tissue culture flasks (Greiner Bio-One Ltd., Stonehouse, Gloucestershire, UK). Human cell lines originally sourced from the ECACC were cultured in the laboratory as described previously (40) (epithelial cells: HeLa, MCF7, ECV304, SW13, HepG2, and A549; glial cells: T98G; myeloid cells: THP1, HL60, and CMK). The human oligodendrocyte cell line (HOG) was from Dr. G. Dawson (Department of Biochemistry, University of Chicago). HMC1 mast cell line and the EAHy929 endothelial cell line were kindly provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN) and Dr. C. J. S. Edgell (University of North Carolina), respectively. Primary cultures of human keratinocytes and serum-free media were obtained from Invitrogen. Primary cultures of human fibroblasts (8399) were from the ATCC (Manassas, VA). Spermatozoa were isolated from healthy volunteers as described and treated with 20 μM calcium ionophore to induce the acrosome reaction (41).

Induction and Detection of Apoptosis and Necrosis—Jurkat cells were harvested from culture, washed once, and resuspended at a density of 10^6/ml in RPMI 1640 with L-glutamine, sodium pyruvate, and antibiotics but without fetal bovine serum. Apoptosis was induced for 4 h using 10^7 cells treated with either 5 μg/ml camptothecin (Sigma), 80 ng/ml anti-Fas monoclonal antibody (clone CH11, Upstate Signaling Solutions, Milton Keynes, UK), UV light treatment (400 mJ/6-well plate, UV Stratalinker 2400, Stratagene) (42), or after γ-irradiation (4000 centigrays, in-house facility) followed by 8 h in culture. Necrosis was induced by incubation of 10^7 cells in 10 ml of culture medium for 4 h with oxidant H_2O_2 (0.01 M) (Sigma) (43). In some experiments, cells were incubated for 30 min with either a broad spectrum caspase inhibitor (Z-VAD-FMK, 5 μM, Sigma) or a specific caspase 3 inhibitor (*N*-acetyl-Glu-Ser-Met-Asp-al, 5 μM, Sigma) prior to the induction of apoptosis. UV light treatment (400 mJ/6-well plate) was carried out to induce reliable apoptosis of all the other cell lines used in this study. Monitoring of apoptosis and necrosis was performed as follows: 1) FITC-labeled annexin V (1:50) (Pharmingen) and propidium iodide (PI) (5 μg/ml) (Sigma); 2) analysis by fluorescent microscopy (unpermeabilized cells) and flow cytometry (FACScan flow cytometer, BD Biosciences); 3) immunocytochemistry staining with anti-cleaved caspase 2) (Asp-175) polyclonal antibody (1:100) (Cell Signaling Technology Inc.) and anti-poly(ADP-ribose) polymerase (PARP) p85 fragment polyclonal antibody (1:100) (Promega, Southampton, UK); 3) trypan blue staining (Sigma, diluted 1:1 with cells) and PI uptake on unpermeabilized cells and analysis by fluorescent microscopy; 4) Western blot analysis of cell lysates using either the anti-PARP p85 fragment protein, the anti-cleaved caspase 3, or the anti-lamin antibody (Pharmingen). In routine conditions, 30–60% of the cells responded to the apoptotic treatment, whereas H_2O_2 oxidative treatment produced over 90% of necrotic cells. To isolate apoptotic cells (PI^{dim} and AnV^{dim}) from viable cells (negative for both), camptothecin-treated Jurkat cells were sorted using the Moflow cytomics equipment (Cytometry Facility, Central Biotechnology Services, Cardiff University, UK).

Complement Activation and Complement Regulation—Apoptotic and necrotic Jurkat cells were incubated with normal human serum (NHS) (25%) as a source of complement for either 15 min (to assess early C1q binding) or for 1 h at room temperature. Heat inactivation of NHS was carried out at 56–60 °C for 30 min. In some experiments, apoptotic or necrotic cells were treated with a mixture of DNase I/RNase (Sigma, 10 μg/ml in RPMI, 30 min at 37 °C) before incubation with NHS. Anti-complement antibodies were either from commercial sources (see TABLE ONE) or were kindly provided by Dr. M. Fontaine (INSERM U519, Rouen, France), Dr. P. W Taylor (CIBA Geigy Pharmaceuticals, Sussex, UK), and Professor O. Gotze (University of Gottingen, Germany). Cells were analyzed by dual fluorescence flow cytometry and by microscopy analysis essentially as described (40).

Expression Profile of Complement Regulators and Other Surface Markers on Apoptotic/Necrotic Cells—In order to define complement regulators (CD46, CD55, and CD59) and other surface markers (i.e. annexin I and CD43) with a modulated expression during the course of apoptosis/necrosis, the profile of surface molecule expression was assessed by flow cytometry, essentially as described (40). In some experiments, Jurkat cells were incubated with a selective inhibitor of the Src family of protein-tyrosine kinases (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d] pyrimidine, 1 μM), for 15 min prior to apoptosis induction. Casein kinase II inhibitor (TBBT) and protein kinase C inhibitor (Myr-N-FARKGALRQ) were from Calbiochem and were used at 10 μM for 1 h. Mouse antibodies against human CD59 (clone BRIC 229^{IgG2a}) and human DAF (CD55, clone BRIC 216^{IgG1}) were from the International Blood Group Reference Laboratory (Bristol, UK). We used different mouse monoclonal antibodies against human CD46 as follows: clone J4.48^{IgG1} against the SCR1 domain from Serotec (Oxford, UK); clone Tra 2.10^{IgG1} against SCR1; clone GB24^{IgG1} against SCR3/4 (from Prof. J. Atkinson, Washington University, St. Louis, MO), clone MEM258^{IgG1} (from Dr. V. Horejsi, Institute of Molecular Genetics, Prague, Czech Republic); or clone 11C5^{IgG1} (from Dr. E. Rubinstein, INSERM U268, Paris, France) (44). The rabbit polyclonal anti-human CD46 and anti-human CD55 were produced in-house. The mouse anti-CD47 (clone BRIC 126^{IgG2b}) was from International Blood Group Reference Laboratory. Mouse monoclonal anti-CD43 and anti-annexin I antibodies were from Pharmingen.

TABLE ONE

Routes of complement activation and opsonization on apoptotic and necrotic Jurkat cells; tailoring innate immune recognition on altered self-cells by complement

Part a, for microscopy analyses, Jurkat cells were induced to undergo apoptosis (camptothecin-treated cells, 4 h) and necrosis (H_2O_2, 4 h), and the hallmarks of programmed cell death and necrosis were confirmed by staining with either trypan blue, propidium iodide, or annexin V-FITC on unfixed cells followed by microscopy analysis. Annexin V-positive apoptotic cells excluded trypan blue, confirming membrane integrity, and were weakly stained for PI, indicating the exposure of nucleic acids at the cell membrane (memb). Robust nucleic acid staining with PI was localized to nuclei (nucl) in necrotic cells, and annexin V strongly stained damaged membranes. Part b, for FACS analyses, the route and level of complement activation on apoptotic cells *versus* necrotic cells were ascertained by incubation of cells 4 h post-treatment with NHS (1:4 in RPMI for 15 min for C1q staining and 30 min otherwise) followed by immunostaining using (*R*)-PE-conjugated secondary antibodies and FACS analysis. Control cells failed to activate the complement system, and we observed only background stainings (data not shown; see Fig. 1). Data indicate the mean fluorescence (FL2) above background using either isotype control anti-mouse antibodies (Pharmingen) and non-immune control rabbit antiserum. The antibodies used are as follows: C1q, mouse (Mo) anti-C1q, clone 12A5B7 (ascites 1:1000; ATCC); rabbit (Rb) anti-human C1q (1:200; Dako, Ely, UK); goat (Gt) anti-human C1q (1:1000; Calbiochem and Merck). For C4, goat anti-C4 (1:200; from ATAB and from ICL, Dr. Marc Fontaine). For C2, rabbit anti-human C2 (1:1000; Nordic, Tilburg, The Netherlands) and goat anti-human C2 (1:1000; Calbiochem). For C3, mouse anti-human C3 (clone H1C3^{IgG1}, 1 μg/ml) and affinity-purified rabbit anti-C3 (L440, 5 μg/ml) (Dr. M. Fontaine); goat anti-human C3 (1:200; Calbiochem); mouse anti-human C3b neoepitope (clone C3/30) tissue culture supernatant, 1:20; Dr. P. W. Taylor); mouse anti-human iC3b neoepitope, (1:1000; Quidel, Technoclone, Dorking, UK); mouse anti-human C3d (clone BGRL11^{IgM}, 2.4 μg/ml, International Blood Group Reference Laboratory (IBGRL)); rabbit anti-human C3d (1:200; Dako). For MAC, rabbit anti-human C5 (1:200; Dr. Fontaine); sheep (Sh) anti-human C9 (Binding Site, Birmingham, UK); mouse anti-human C5b9 neoepitope (clone B7^{IgG1}; in-house, 10 μg/ml). For factor B, affinity-purified rabbit anti-human Bb (Prof. O. Gotze, University of Gottingen, Germany). For factor H, affinity-purified rabbit anti-human factor H (L740, 0.5 μg/ml; Dr. M. Fontaine). Isotype-matched control mouse antibodies were from Pharmingen. Primary antibodies were followed by (*R*)-phycoerythrin (R-PE)-conjugated goat anti-mouse (1:200; Dako) or goat anti-rabbit Ig (1:200; Sigma). The level of staining was assessed by FACS analysis (data are expressed as mean FL2 over negative controls; $n = 3$). Of note, untreated cells failed to activate the classical and alternative pathways of complement (see Fig. 1). The specificity of all polyclonal antibodies was tested and confirmed as reported previously (68). The abbreviations used are as follows: Mo, mouse; Rb, rabbit; Gt, goat; Sh, sheep.

***a*, Hallmarks of necrosis and apoptosis (microscopy analysis, $n = 3$)**

Treatments	Camptothecin-treated cells		H_2O_2-treated cells	
	Staining	**%**	**Staining**	**%**
Trypan blue	Negative	~90–95	Positive	>95
PI	Weak (memb)	~40–50	Strong (nucl)	>95
Annexin V-FITC	Strong (memb)	~30–35	Strong (memb)	~50–60%

***b*, Routes and level of complement activation (FACS analyses, $n = 3$)**

Immunostainings	Camptothecin (mean FL2)	H_2O_2 (mean FL2)
Mo α-C1q (12A5B7, ATCC)	4.3	19.7
Rb α-C1q (Dako)	37	183.5
Gt α-C1q (Calbiochem)	47.3	605.9
Gt α-C4 (ATAB, Dr. M. Fontaine)	104.3	1031.8
Gt α-C4 (ICL, Dr. M. Fontaine)	71.2	1187.8
Rb α-C2 (Nordic)	25.6	251.3
Gt α-C2 (Calbiochem)	73.4	371.2
Mo α-C3 H1C3 (Dr. M. Fontaine)	26.6	183.8
Rb α-C3c (L440, Dr. M. Fontaine)	17.13	230.7
Gt α-C3 (Calbiochem)	11.7	1297.9
Mo α-C3b neo (C3/30, Dr. Taylor)	62.8	1345
Mo α-iC3b neo (Quidel)	72.4	1030
Mo α-C3d (BGRL11, IBGRL)	4.30	56.9
Rb α-C3d (Dako)	25.50	712.6
Rb α-C5 (Dr. M. Fontaine)	2.31	56.6
Sh α-C9 (binding site)	3.6	158.7
Mo α-C9 neo (B7, in-house)	12.1	42.5
Rb α-Bb (Prof. O. Gotze)	5.5	149.1
Rb α-fH (L740, Dr. M. Fontaine)	26.5	81

Preparation of Lysates from Total Cells and Nuclear, Cytoplasmic, and Bleb-enriched Fractions Using Normal, Apoptotic, and Necrotic Jurkat—Cell lysates of normal, apoptotic, and necrotic Jurkat cells were prepared from 1×10^7 cells using lysis buffer (500 μl of 1% Triton X-100, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin in phosphate-buffered saline). Nuclear and cytoplasmic fractions were prepared according to the manufacturer's instructions (NE-PER, 78833 kit from Pierce). The fetal calf serum-free medium bathing treated cells (10 ml) was dialyzed, freeze-dried, and resolubilized in Laemmli buffer (500 μl in total) to detect the soluble and vesicle forms of proteins shed from the cells. To isolate specifically microparticles released by apoptotic/necrotic cells, 10 ml of culture supernatants were collected from the initial spin at 2000 rpm for 10 min and ultracentrifuged (100,000 × *g*, 1 h, room temperature) in a Beckman TL-100 ultracentifuge with a TLA 100.3 rotor. Bleb/vesicle-enriched pellets were finally resolubilized in Laemmli loading buffer (500 μl in total) (45). The remaining 10 ml of supernatant containing soluble proteins was dialyzed overnight in phosphate-buffered saline, freeze-dried, and resuspended in 500 μl of Laemmli loading buffer. Samples were separated on SDS-PAGE, and Western blotting was performed using rabbit polyclonal antibodies against CD55, CD46, cleaved caspase 3, and cleaved PARP. Precast gels (4–20% acrylamide) and prestained protein markers were from Invitrogen. Double immunoblots were also performed using mouse anti-CD46 (MEM258, V. Horejsi), mouse anti-CD43 (MEM59 V. Horejsi), and mouse anti-lamin (Pharmingen). The mouse anti-β-tubulin was used to test for equal protein loading (Sigma).

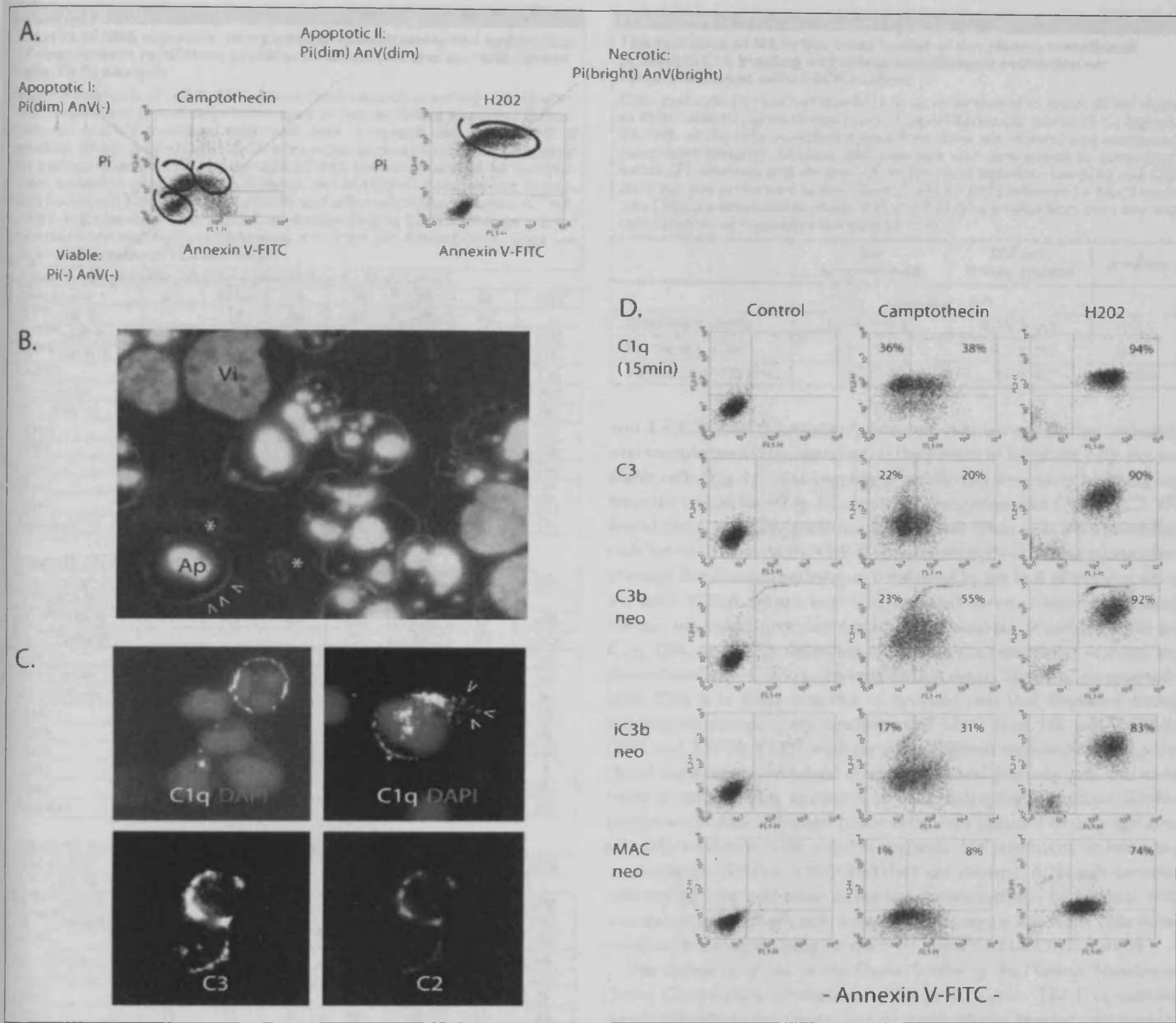

FIGURE 1. **Tailoring complement activation on apoptotic and necrotic cells.** *A*, P_i and annexin V-FITC cell scatter histograms of camptothecin-treated cells clearly depict the presence of the following: 1) viable cells; 2) a subset of very early apoptotic cells (apoptotic I) expressing nucleic acid at the cell surface (PI dim); and 3) a subset of apoptotic cells (apoptotic II) with nucleic acid and phosphatidylserine exposure at the cell surface (P_i^{dim} and AnV ^{dim}). Necrotic cells were strongly stained with PI (nuclear staining) and annexin V-FITC. *B*, camptothecin-treated cells (4 h) were seeded onto glass slides, fixed with acetone, and stained with DAPI. Apoptotic cells (*Ap*) with condensed and fragmented nuclei were also displaying nucleic acid exposure at the cell membrane (ring structure as indicated by *white arrowheads*). 30–40% of the cells remained viable (*Vi*). Small vesicle-like membranes were also stained with DAPI (*). Magnification ×1000. *C*, camptothecin-treated Jurkat cells incubated with NHS (1:4, 15 min) were stained for C1q (rabbit) or double-stained for C3bneo (mouse) and C2 (rabbit) before being spotted onto glass slides. Cells were fixed with acetone, and nuclei were counterstained with DAPI. C1q staining was either evenly distributed around the cell surface of apoptotic cells or displaying a granular pattern with vesicles detaching from the cells (*arrowhead*). Viable cells with intact nuclei failed to stained for C1q, C3b, and C2 (data not shown). *D*, Jurkat cells were either untreated (control) or induced to undergo apoptosis (4 h, 5 μg/ml camptothecin) and necrosis (4 h, H_2O_2), then incubated with 25% NHS, and analyzed by two-color flow cytometry for complement opsonization and annexin V FITC. Data are from one experiment and with reproducible findings ($n = 4$).

Statistical Analysis—Statistical analyses were performed using Sigmaplot and Sigmastat software package. Differences between parameters were analyzed using unpaired Student's t tests. The *p* values were considered statistically significant at *p* less than 0.05.

RESULTS

NA Exposure Precedes Phosphatidylserine (PS) "Flip-flop" on Apoptotic Cells—First, Jurkat T lymphocytes were induced to undergo apoptosis or necrosis by chemical treatments using either campt[illegible], an inhibitor of topoisomerase I, or the oxidant H_2O_2, respectively. Second, three more apoptotic triggers were tested by exposure of Jurkat to anti-Fas IgM antibody, ionizing γ-irradiation or UV light treatment. The 4-h time point was selected to give ~30% of apoptotic cells with intact plasma membranes. These cells were weakly positive for PI (membrane staining) and excluded trypan blue (TABLE ONE), whereas the necrotic cells were membrane-permeable and strongly stained with trypan blue and PI^{bright} (TABLE ONE). When the camptothecin-treated cells were analyzed by FACS, we clearly identified three distinct cell populations as follows: viable cells, cells stained with PI but not with annexin V (called apoptotic I), and cells stained with PI and annexin V (apoptotic II cells)

TABLE TWO

Kinetics of DNA exposure, complement opsonization, and expression of complement regulatory proteins on apoptotic and necrotic Jurkat cells; FACS analysis

The key hallmarks of cell death and the overall capacity to activate and control the complement system were ascertained in two models of apoptosis (camptothecin and UV-irradiated cells) and were compared with one model of necrosis (H_2O_2-treated cells). Cells were either untreated (control) or treated for various times (30 min to overnight (O/N)). Immunostainings for complement activation products (C1q, C3bneo, and MACneo), complement regulatory proteins (CD46, CD55, and CD59), and other cell surface markers (CD43, CD47, and annexin I) were carried out as described in TABLE ONE (n = 3 for camptothecin and H_2O_2 experiments; n = 2 for UV-treated cells). Data are expressed as mean of FL2 intensity.

(Model I: Camptothecin, inhibitor topoisomerase I, - treated cells)

Time course	*Co*	*30min*	*1h*	*2h*	*4h*	*8h*	*O/N*
PI ^{dim} cells %	-	12	20	24	45	42	-
PI ^{bright} cells %	-	<1	<1	<1	<2	15	>95
PS ^{bright} cells %	-	-	-	5	35	60	>95
Gt αC1q	-	26	48	57	62	123	125
Mo αC3b neo	<10	15	34	42	185	314	760
Mo α MAC neo	-	-	-	-	-	18	167
CD46	110	110	95	87	42	35	32
CD55	68	61	58	58	59	55	46
CD59	364	354	345	360	325	329	330
CD43	90	84	54	16	7	4	3
CD47	439	411	459	489	511	554	527
Annexin I	<5	<5	8	10	37	56	111

(Model II: UV-treated cells)

Time course	*Co*	*30min*	*1h*	*2h*	*4h*	*8h*	*O/N*
PI ^{dim} cells %	-	5	23	34	50	70	-
PI ^{bright} cells %	-	<1	<1	<1	<5	15	>95
PS ^{bright} cells %	-	-	-	5	63	90	>95
Gt αC1q	-	13	25	37	47	nd	nd
Mo α C3b neo	<10	20	37	40	221	nd	nd
Mo α MAC neo	-	-	-	-	6	nd	nd
CD46	127	114	106	99	27	23	12
CD55	72	67	65	68	60	56	48
CD59	360	325	314	310	304	355	340
CD43	139	132	82	41	35	9	5
CD47	369	419	442	465	478	480	475
Annexin I	<5	<5	12	23	36	44	67

(Model III: Oxidative Necrosis: H_2O_2 treated cells)

Time course	*Co*	*30min*	*1h*	*2h*	*4h*	*8h*	*O/N*
PI ^{dim} cells %	-	-	<10	-	-	-	-
PI ^{bright} cells %	-	21	62	76	82	>95	>95
PS ^{bright} cells %	-	19	64	65	76	>95	>95
Gt αC1q	-	45	243	560	754	nd	nd
Mo αC3b neo		200	347	871	1560	nd	nd
Mo α MAC neo	-	34	30	45	87	121	232
CD46	101	86	47	28	22	25	21
CD55	71	72	70	60	48	45	34
CD59	362	321	305	287	240	210	167
CD43	84	54	16	7	4	4	4
CD47	355	368	417	492	554	569	420
Annexin I	9	16	25	44	120	280	9

(Fig. 1*A*). Most interestingly, time course FACS analyses revealed that NA exposure, assessed by PI staining, was already prominent at 30 min long before exposure of PS (peak at 4 h) in several apoptotic models (TABLE TWO). DAPI staining of camptothecin-treated cells confirmed NA exposure at the cell membrane of apoptotic but not viable cells (Fig. 1*B*, *Ap*, *white arrowheads*). Small NA-positive vesicles were also observed, probably released from apoptotic Jurkat (Fig. 1*B*, *).

Innate Immune Recognition and Tailoring of Complement Activation on Altered Self (Apoptotic Cells Versus Necrotic Cells) While Preserving Normal Self-cells—Complement activation was tested by incubation of treated cells with NHS, and the level of complement proteins on target cells was analyzed by double microscopy and FACS analysis using several anti-complement antibodies and annexin V FITC (Fig. 1, C and *D*, and TABLES ONE and TWO). Control cells failed to activate the complement system when exposed to NHS for 30–60 min (Fig. 1, *C* and *D*, and TABLE TWO). Immunofluorescent microscopy further indicated that complement C1q opsonized at the surface of apoptotic cells but not viable cells (Fig. 1*C*) was capping in vesicle-like structures blebbing out from the cell surface (Fig. 1*C*, *arrowhead*) together with C3b and C2. We found that C1q staining was most prominent when cells were incubated only for 10–15 min with NHS. Complement activation was taking place through the classical pathway as confirmed by the lack of staining when we used EDTA serum and C1q-depleted serum (data not shown). Hence, we found prominent staining of annexin V-positive cells for C1q, C3b, and iC3b following camptothecin treatment, whereas the monoclonal anti-C5b9 neoepitope did not detect the MAC on apoptotic cells. This is in sharp contrast to necrotic cells that displayed strong staining for complement opsonins and MAC (Fig. 1*D*, and TABLES ONE and TWO). FACS analysis using different monoclonal and polyclonal antibodies confirmed that the classical pathway was the main route of complement activation on cells undergoing apoptosis. Similar results were obtained when Jurkat cells were induced to undergo apoptosis by treatment with anti-Fas antibody, UV treatment, or following γ-irradiation (TABLE TWO and data not shown). Although necrotic cells bound C1q, activation of the alternative pathway by necrotic cells was also observed, with only a weak Bb staining on apoptotic cells compared with strong staining on necrotic cells (TABLE ONE, Part b).

TABLE THREE

The exposure of NA in the outer leaflet of the plasma membrane promotes C1q binding and drives complement activation on apoptotic Jurkat cells; FACS analysis

Camptothecin-treated Jurkat cells (4 h) were incubated in either RPMI alone or RPMI with 10 μg/ml DNase I and 10 μg/ml RNase (30 min at 37 °C; Sigma). 90–95% of the cells excluded trypan blue (data not shown) and confirmed membrane integrity. Nucleic acid exposure was determined by propidium iodide (PI) staining, and the level of complement opsonization (C1q and C3b staining) was performed as described (TABLE ONE) followed by FACS analysis. Data are expressed as mean of FL2 ± S.D. The p value from the t test was calculated using Sigmaplot software (n = 4).

	No pretreatment	DNase-, RNase-treated	*p* values
	Mean FL2 ± S.D.		
Propidium iodide	41.25 ± 8.4	8.75 ± 3.4	0.002
Rabbit anti-C1q	52.2 ± 8.9	4.7 ± 0.9	0.001
Mouse anti-C3b neo	157 ± 35.5	41 ± 6.7	0.005

The Exposure of NA in the Outer Leaflet of the Plasma Membrane Drives Complement Activation on Apoptotic Cells—The C1q staining clearly identified two populations of camptothecin-treated cells double-stained or not for PS exposure (Fig. 1D). Most interestingly, this pattern was reminiscent of the PI/annexin V staining pattern (Fig. 1*A*). We hypothesized that NA exposure at the cell membrane may contribute to the initiation of the classical pathway given that DNA has long been known as a C1q-binding molecule (46). To test this, we compared the level of C1q binding and C3 opsonization prior to and after DNase/RNase treatment of camptothecin-treated Jurkat cells. Treated cells remained viable and displayed markedly reduced levels of PI staining at the cell membrane (TABLE THREE). The removal of NA dramatically affected the activation of the classical pathway on apoptotic cells with reduced C1q binding and C3b levels at the cell surface (p < 0.005). In contrast, only a 15–20% reduction of C1q binding was observed when necrotic cells were treated with DNase/RNase (data not shown), suggesting that other mechanisms (*e.g.* cardiolipin exposed on mitochondria) are driving C1q binding and complement activation on necrotic cells (47).

Apoptotic and Necrotic Cells Control Differentially the Complement System—Previous studies have reported a reduction in the expression of CRegs in response to apoptosis (37–39). To confirm these findings and

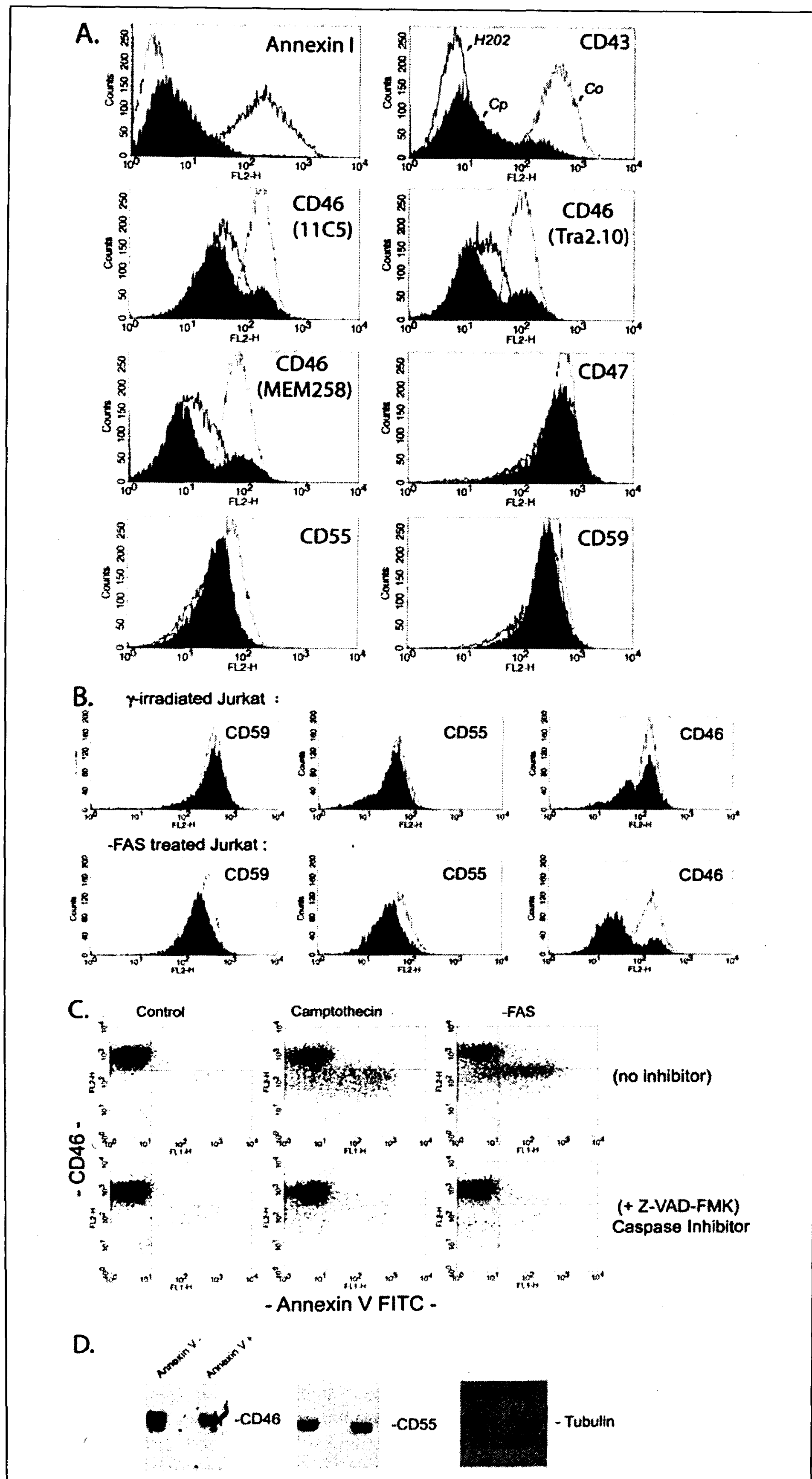

FIGURE 2. **Expression of complement regulatory proteins on apoptotic *versus* necrotic Jurkat T cells; dramatic loss of CD46 on apoptotic cells is caspase-dependent but Src kinase-independent.** *A*, control cells (*thin line*) or cells treated for 4 h with camptothecin (*filled histogram*) and H_2O_2 (*solid line*) were stained for the membrane complement regulators (CD46, CD55, and CD59) and other cytoplasmic/cell surface proteins (annexin I, CD43, and CD47) and analyzed by single flow cytometry using mouse anti-CD antigens and (*R*)-phycoerythrin-conjugated goat anti-mouse antibodies (FL2 channel). Of note, the cytoplasmic annexin I protein has been reported to translocate to the cell surface on apoptotic cells and was used as an internal control in our experiments (48). CD43 is a highly sialylated membrane protein that is known to be rapidly shed from apoptotic cells (49). *B*, the expression of CD46, CD55, and CD59 was also analyzed when Jurkat cells were exposed to different apoptotic paradigms using the anti-Fas antibody (4 h) or following γ-irradiation and 8 h in culture. Both treatments induced more than 40% annexin V-positive cells with intact cell membrane as confirmed by trypan blue exclusion (data not shown). *C*, Jurkat cells were incubated with either a general caspase (Z-VAD-FMK) or an Src kinase (PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d] pyrimidine)) inhibitor (69) for 1 h prior to apoptosis induction (camptothecin or anti-Fas for 4 h). *D*, Western blot analysis of camptothecin-treated cell lysates following cell sorting in annexin V-negative and annexin V-positive cell fractions. Equivalent cell numbers were used, and we confirmed equal protein gel loading using the anti-tubulin antibody.

TABLE FOUR

CD46 is rapidly and universally lost from apoptotic cells; FACS analysis

Several different human cell lines from different tissue sources as well as primary cultures were induced to apoptosis (UV treatment, 4 h) and tested for CD46 expression and other cell markers. Over 50–60% of the cells were strongly stained using annexin V-FITC and excluded trypan blue, confirming the canonical apoptotic phenotype ($n = 2$).

(MEAN of FL2) for:	CD46		CD55		CD59		CD47	
(n=2)	No UV	4h post UV	No UV	4h post UV	No UV	4h post UV	No UV	4h post UV
(Epithelial cells):								
Hela cervix	643	161	1458	965	824	620	222	157
MCF7 breast	429	183	68	58	757	750	749	774
ECV304 bladder	88	34	68	67	384	412	91	89
SW13 adrenal gland	88	23	4.5	7	253	216	23	23
HepG2 liver	147	59	64	51	635	567	nd	nd
A549 lung	228	94	300	279	1010	1103	185	149
(Fibroblasts):								
Primary (ATCC)	82.4	12	79	67	944	1002	226	235
(Endothelial cells):								
EAhy929 pass. 52	1868	675	1441	1290	8149	7990	2035	2156
(Myeloid cells):								
HL60 neutrophils	203	54	109	94	217	234	nd	nd
HMC1 mast cells	328	156	244	210	849	1011	nd	nd
CMK megakaryoblast	304	89	145	122	118	155	31	39
(Brain cells):								
T98G astrocyte	291	116	545	511	3823	3910	nd	nd
HOG oligodendrocyte	232	89	189	123	1375	1527	410	408

ascertain the contribution of CRegs to the selective regulation of complement on apoptotic cells and necrotic cells, flow cytometry analyses were performed using monoclonal antibodies to the key membrane CRegs, CD46, CD55, and CD59, alongside other antibodies against cytosolic and membrane proteins (annexin I, CD43, and CD47). Jurkat control cells (Fig. 2A) expressed high levels of all CRegs together with CD43 and CD47. The annexin I staining was used as an internal control and was detected at the cell surface only after apoptosis and necrosis as reported previously (48). We confirmed that CD43 was rapidly lost from apoptotic and necrotic cells with a distinct bimodal distribution observed on apoptotic cells (49). A high loss of CD46 expression was observed on camptothecin- and H_2O_2-treated cells, with a distinct bimodal distribution of expression apparent on apoptotic induced cells, where one population retained a level of expression similar to that of normal cells (untreated) (mean FL2 = 245 ± 38), whereas the second population expressed a reduced level (at least a log reduction) (21 ± 6). CD55 and CD59 expression were slightly affected when cells were induced into apoptosis and necrosis (Fig. 2*A*). From the expression of CD59, it can be concluded that complement activation is under safe control and limiting the formation of cytolytic and cytotoxic MAC formation, yet allowing opsonization possibly to facilitate phagocytosis. CD47 expression was not affected on apoptotic or necrotic cells. We used an even larger panel of anti-CD antibodies (CD2, CD3, CD4, CD5, CD9, CD11a, CD29, CD31, CD45, CD53, CD54, CD81, CD90, CD94, CD100, CD208, and HAL-I, all kindly provided by Prof. V. Horejsi), and we failed to find a modulated cell surface expression of these markers on apoptotic and necrotic cells (data not shown).

In addition to the camptothecin apoptotic model described earlier, a γ-irradiated model (optimal level of apoptosis, 8 h post-treatment), an α-Fas model (optimal level of apoptosis, 4 h), and a UV-induced apoptosis model (4 h) were established to examine the loss of CD46. We observed the distinct bimodal distribution of CD46 expression as assessed by FACS analysis with a population of decreased expression compared with normal in these three new models (Fig. 2*B* and TABLE TWO). Kinetics analyses indicated that CD46 was rapidly lost from the apoptotic cell surface as early as 2 h post-treatment (TABLE TWO). Moreover, we found that the rate of CD46 loss was identical whether the apoptotic cells were incubated in the presence or absence of complement, suggesting that C3b, the natural CD46 ligand, is not implicated in CD46 fate on apoptotic cells (data not shown).

The Decreased Expression of CD46 Correlates with Caspase-dependent Apoptotic Cell Death but Does Not Require Caspase 3 or CD46-mediated Protein Phosphorylation—To ascertain whether the loss of CD46 membrane antigen staining was restricted to apoptotic cells, dual fluorescence analysis was performed, whereby monoclonal antibody staining was followed by annexin V FITC and (R)-phycoerythrin-conjugated goat anti-mouse immunoglobulin (Fig. 2*B*). Camptothecin and αFas-treated Jurkat cells showed a clear population of annexin +ve cells with a remarkable 10-fold decreased expression of CD46. Most interestingly, the loss of CD46 from Jurkat cells was totally abrogated when cells were incubated with a broad caspase inhibitor (Z-VAD-FMK) prior to apoptosis induction (Fig. 2*B*). However, a caspase 3 inhibitor (*N*-acetyl-Glu-Ser-Met-Asp-al) failed to reduce significantly the loss of CD46 from the apoptotic Jurkat cells (data not shown), and this is in agreement with our findings that CD46 loss is still taking place in a caspase 3-deficient cell line, the MCF7 breast cancer cell line treated with UV light (TABLE FOUR) (42). Cytoplasmic tails of CD46 (CYT-1 and CYT-2) encode putative signals for phosphorylation by kinases, which play pivotal roles in CD46 trafficking with intracellular processing and basolateral localization. Inhibitors of kinases controlling the phosphorylation status of CD46 tails, *i.e.* 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d] pyrimidine for Src kinases, TBBT for casein kinase II, and Myr-N-FARKGALRQ for protein kinase C, failed to control CD46 loss on apoptotic cells (data not shown).

To obtain pure cell populations of normal and apoptotic Jurkat, cells were sorted on the basis of expression of PI/annexin V staining using the

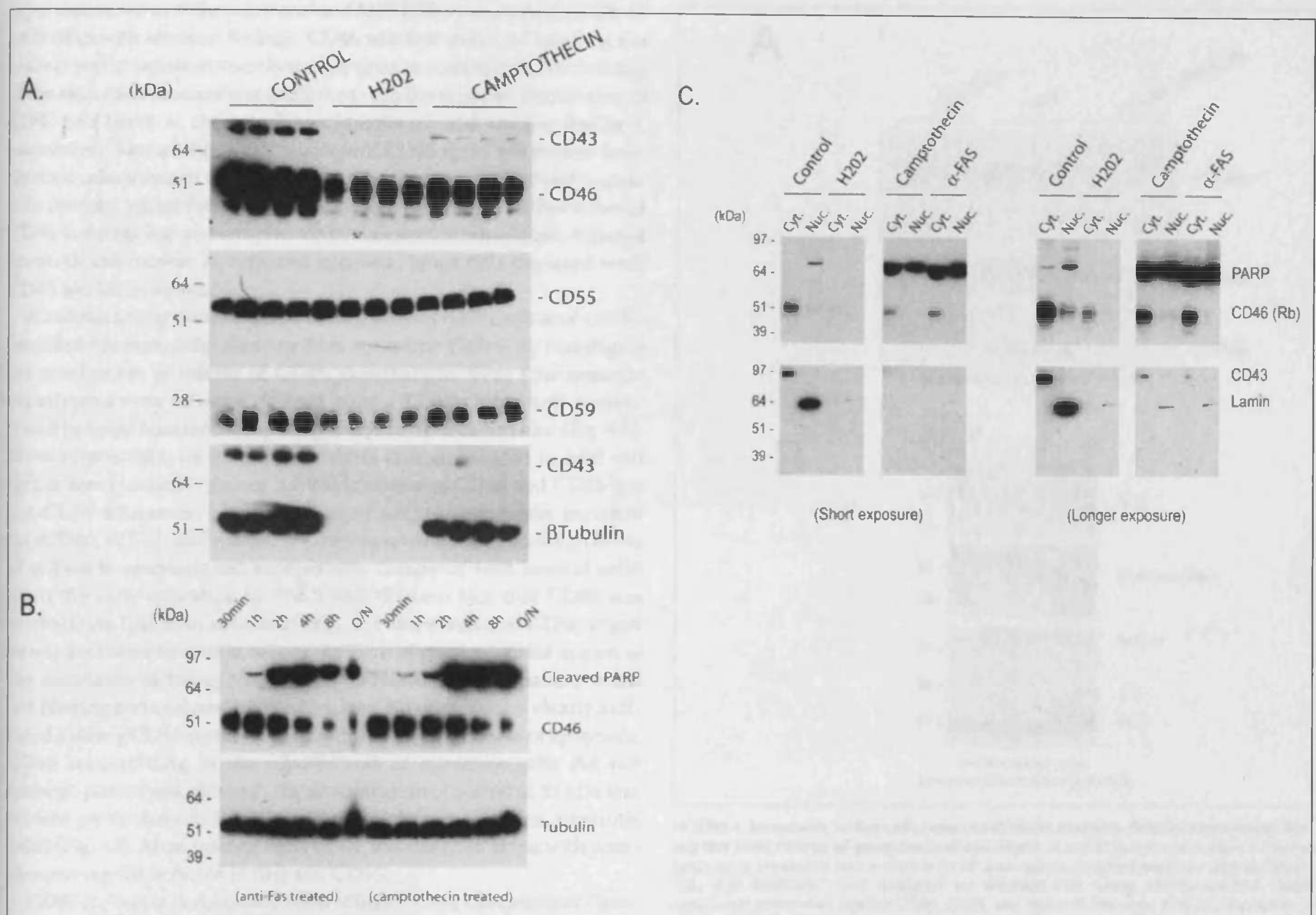

FIGURE 3. **The loss of CD46 and other complement regulators is a rapid event and correlates with the fingerprints of cell death.** The expression of the complement regulators was analyzed by Western blotting of total cell lysates using control cells, necrotic cells (H_2O_2), and apoptotic cells (camptothecin or anti-Fas-treated Jurkat cells, 4-h time point). *A*, data from four separate experiments. β-Tubulin was used as an internal control of equal protein loading, although the signal was absent from H_2O_2-treated cells because of the rapid degradation of the cytoskeletal pool of proteins. *B*, kinetics of CD46 loss using two different apoptotic paradigms and correlation with the overall fingerprint of apoptosis (cleaved PARP signal). *C*, membrane/cytoplasmic (*Cyt.*) and nuclear (*Nuc.*) fractions were prepared according to the manufacturer's instructions (Pierce) and analyzed by Western blotting for CD46 and CD43 expression, along with the known cell markers of apoptosis (cleaved PARP and lamin). Note that two sets are presented of short or longer exposures to facilitate the analysis of the complex set of data. First, we confirmed that CD43 was detected only in the (*Cyt.*) fraction, whereas lamin and PARP were found highly enriched in the (*Nuc.*) fraction of control cells. Second, CD46 signal was distributed in both fractions, and the intensity of the signal was dramatically reduced when cells were induced to necrosis (H_2O_2) or apoptosis (camptothecin and anti-Fas, 4 h post-treatment). Third, we confirmed by double Western blot analyses that the loss of the CD46 signal in apoptotic cells correlated with strong signals for cleaved caspase 3 (data not shown) and cleaved PARP, whereas the level of lamin, a caspase 3 substrate, was dramatically reduced.

cytomation MoFlo high speed cell sorter. Western blot analysis of the cell lysates indicated that total CD46 expression was reduced on apoptotic (annexin +ve) by a factor of 3–4-fold compared with normal (annexin −ve) cells, whereas CD55 levels remained the same (Fig. 2*D*). Given that cell surface expression of CD46 on apoptotic cells is reduced by at least 10-fold (FACS), our Western blot data suggest that shedding and protein internalization may together be responsible for the rapid and dramatic loss of CD46 at the cell surface of apoptotic Jurkat.

Different Mechanisms Control the Loss of CD46 from Apoptotic Cells and Necrotic Cells—Several Western blot experiments were performed to clarify further the mechanism involved in the loss of CReg expression by apoptotic versus necrotic cells. Treated Jurkat cells were prepared and analyzed by double Western blot immunostaining (Fig. 3, $n = 4$). Analysis of total cell lysate preparations confirmed CD55 expression to be unaffected after induction of apoptosis and necrosis. CD59 expression was reduced only when cells were treated with H_2O_2, whereas CD46 expression showed a profound difference between control and treated cells, with a greater than 50% loss of expression from apoptotic and necrotic cell lysates (Fig. 3*A*). CD43 expression was confirmed to be dramatically reduced from apoptotic cells, whereas necrotic cells were largely CD43-free. Of note, the loss of this anti-adhesive molecule at the cell surface is thought to be involved as a signal for macrophage recognition and removal of the dying cell. β-Tubulin was used as an internal control of equal protein loading, although the signal was totally absent from necrotic cells, indicative of the loss of the cytoskeletal architecture and integrity after H_2O_2 treatment. Most interestingly, kinetics studies indicated that the rapid loss of CD46 expression coincided with the overall hallmarks of programmed cell death in two different models (anti-Fas and camptothecin-treated Jurkat cells) (Fig. 3*B*). For example, at 2 h post-treatment, we found a strong cleaved PARP signal that was associated with a reduction in CD46 total expression. At 8 h, almost 90% of CD46 was lost from the apoptotic cell lysates.

To shed further light on the mechanism of CD46 trafficking in apoptotic and necrotic cells, nuclear and cytoplasmic/membrane cell extracts were prepared using the NE-PER kit from Pierce. Western blotting was used to detect the presence of CD46 along with established markers of apoptosis (cleaved caspase 3neo and cleaved PARPneo), CD43, and lamin, a known nuclear component and substrate of activated caspase 3 (Fig. 3*C*). In these

experiments, we used the polyclonal and MEM258 monoclonal anti-CD46 antibodies with identical findings. CD46 was first shown to reside in the nuclear and cytoplasmic/membrane fractions in control cells. The validity of the extraction protocol was confirmed with the expected distribution of CD43 and lamin in the cytoplasmic/membrane and nuclear fractions, respectively. Remarkably, a dramatic loss of CD46 signal was evident from necrotic cells, although without involving the cleavage of PARP and caspase 3. In contrast, robust PARP and caspase 3 signals coincided with the loss of CD46 in the nuclear and cytoplasmic/membrane fractions of two different apoptotic cell models. As expected, apoptotic Jurkat cells displayed weak CD43 and lamin signals.

Identification of Soluble CD46 Shed from Necrotic Cells and CD46-enriched Microparticles Budding from Apoptotic Cells—To investigate the mechanism of release of CD46, supernatants from four separate experiments were collected, filtered using a 0.2-μm filter, and concentrated by lyophilization before being analyzed by Western blot (Fig. 4A). Most surprisingly, we found that control cells (incubated in fetal calf serum-free medium) released soluble (s) forms of CD46 and CD55 but not CD59. Moreover, necrotic cell supernatants were highly enriched for sCD46, sCD55, and sCD59. We were surprised not to find high levels of sCD46 in apoptotic cell supernatants compared with control cells, given the early indication by FACS and Western blot that CD46 was dramatically lost from apoptotic cells. We suspected that CD46 might be released from apoptotic cells in the form of microparticles as part of the membrane blebbing. Hence, we lyophilized the supernatants without filtering and analyzed by Western blot. Kinetics studies clearly indicated a strong CD46 signal accumulating during the course of apoptosis. CD46 accumulating in the supernatants of apoptotic cells did not undergo proteolysis, although the accumulation of a band at 35 kDa was evident particularly in 8 h late and overnight samples (late apoptotic cells) (Fig. 4*B*). Most interestingly, CD46 was detected along with complement regulator factor H (fH) and CD55.

CD46 Is Present in Apoptotic Blebs Where PS and Complement Opsonins Are Prevalent before Being Released from Surface Membranes—Double immunofluorescent microscopy of normal and apoptotic unpermeabilized/unfixed Jurkat cells was performed using monoclonal anti-CD46 antibody and annexin V FITC. Fluorescent images confirmed that CD46 was evenly distributed (albeit distributed in small clusters) on control cells with no staining for exposed PS. In contrast, strong PS exposure as indicated by the annexin V staining was found to colocalize with CD46 staining (Fig. 5A). To confirm further that CD46 capping into PS-enriched blebs was shed from the surface of apoptotic cells, microparticles were isolated by ultracentrifugation and analyzed by Western blot. We confirmed that CD46 was reduced in apoptotic total cell lysates ($n = 2$) and as a result of being shed in the form of microparticles from the apoptotic cells (Fig. 5*B*). When the apoptotic cells were incubated with NHS as a source of complement (for 1 h), we found a strong C1q signal associated with the microparticle preparation. To ascertain whether there was a correlation between complement activation and loss of cell surface CD46, dual fluorescence flow cytometry analysis for expression of CD46 and C3b was performed. The loss of CD46 revealed stronger C3b opsonization of cells when exposed to complement (as indicated by a monoclonal anti-C3b neoepitope) (Fig. 5*C*).

The Loss of CD46 Is a Universal Hallmark of Caspase-dependent Cell Activation—Several more model cell lines were UV light-treated to ascertain whether the rapid loss of CD46 at the cell surface is a unique and universal feature of caspase-dependent cell activation. Without exception, we found a robust loss of CD46 staining on 13 human cell lines from different tissue sources (TABLE FOUR; n = 2). The loss of CD46 correlated with the level of apoptosis as indicated by the annexin V staining analyzed by FACS and the PARP staining on fixed cells (data not shown).

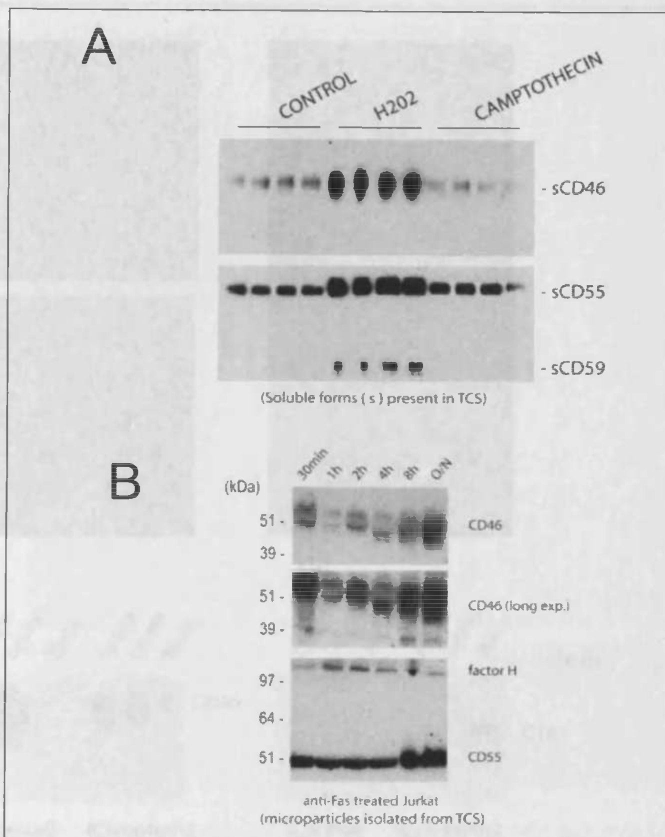

FIGURE 4. **Apoptotic Jurkat cells released CD46 in microparticle-like structures during the time course of programmed cell death.** *A* and *B*, Jurkat cell culture supernatants were separated into a soluble (s) (*A*) and vesicle-enriched fractions (*B*) (see "Materials and Methods") and analyzed by Western blot using affinity-purified rabbit polyclonal antibodies against CD46, CD55, and factor H. We used BRIC229 mouse anti-human CD59.

To confirm the observation that clustering of CD46 into microparticles is a caspase-dependent mechanism engaged in dying cells and not an artifact observed for the human Jurkat T lymphocyte cell line, a number of cells were stained for CD46 and caspase 3 by immunofluorescent microscopy (Fig. 6). In Jurkat cells treated with camptothecin, we confirmed that CD46 expression was dramatically reduced in cells stained for cleaved caspase 3 and PARP (Fig. 6, *a–f*). Although viable cells displayed a strong uniformed CD46 staining (Fig. 6*a*, *arrowhead*), only a weak cytoplasmic staining was observed on apoptotic cells (Fig. 6*d*, *arrowhead*). The apoptotic machinery is also utilized for a wide variety of tasks during development and, for instance, during the "individualization" process of maturing male germ cells, removal of senescent keratinocytes, and the differentiation of megakaryocytes into platelets (50–52). First, human spermatozoa treated with the A23187 ionophore to induce acrosomal reaction were shown to lose CD46 at their acrosomal surface, which correlated with activated caspase 3 and cleaved PARP stainings (Fig. 6, *g–i*). This observation was confirmed by Western blot analysis, which further indicated that CD46 in microparticles was released in the supernatant of treated cells (data not shown). Following growth factor withdrawal, we found that primary cultures of human keratinocytes produced caspase 3-positive particles that were highly enriched for CD46. No staining for cleaved caspase 3 was observed on viable keratinocytes strongly stained for CD46. Most inter-

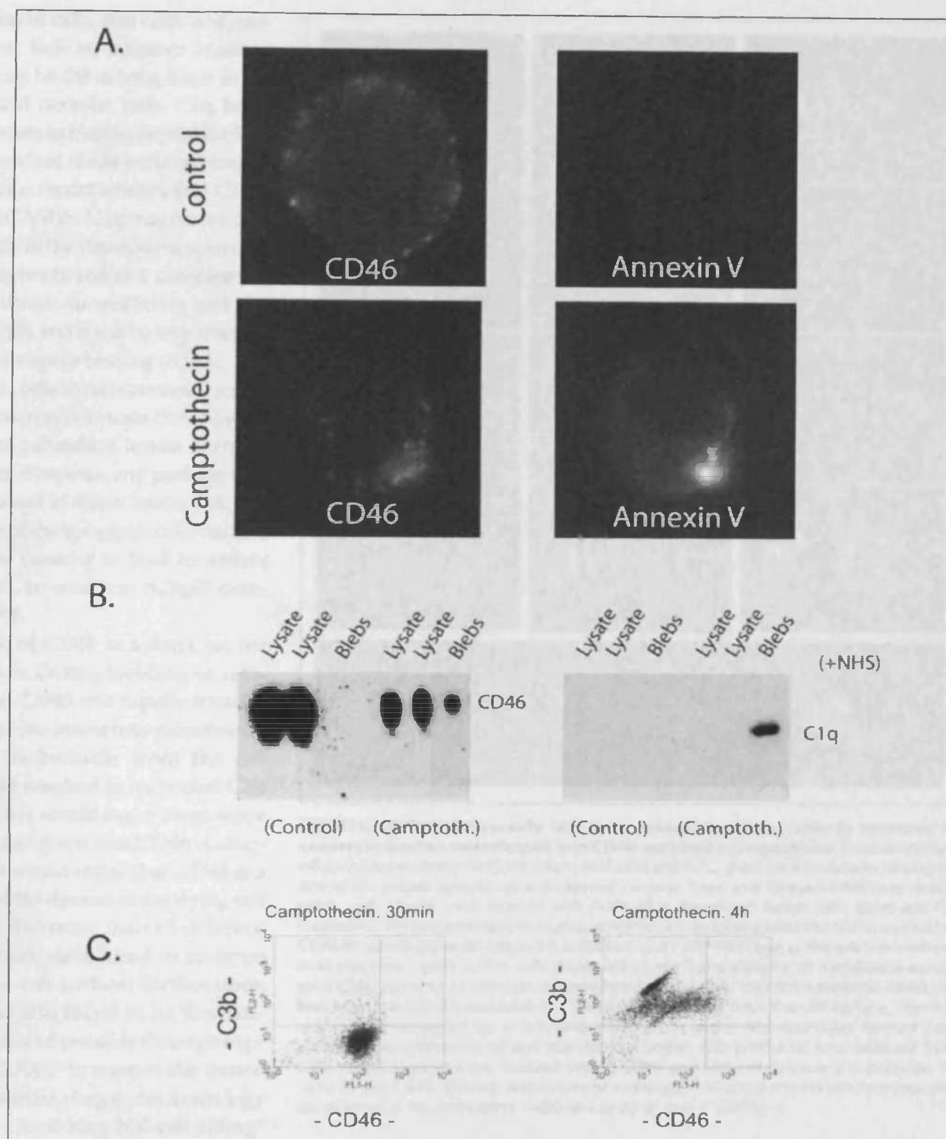

FIGURE 5. **CD46 is clustered to apoptotic blebs where phosphatidylserine exposure and complement activation are prevalent.** *A*, double immunofluorescence staining of unpermeabilized Jurkat cells using the mouse anti-CD46 (11CS clone) and annexin V-FITC prior to (*control*) and after camptothecin-treatment (2 h). *B*, total cell lysates and bleb cell lysates were prepared from control and apoptotic cells (camptothecin (*Camptoth.*) *4 h followed by incubation with NHS 1:4 for 1 h) and analyzed by Western blot for CD46 and complement C1q*. *C*, double FACS analysis of camptothecin-treated cells (30 min and 4 h post-treatment) incubated with NHS and stained using mouse anti-C3b neo antibody and rabbit anti-CD46.

estingly, a unique CD46 distribution was observed on keratinocytes with cytoplasmic and cell membrane distribution particularly at a point of contact between cells (Fig. 6*j*, *arrowhead*). This distribution is a canonical fingerprint of adhesion molecules generally involved in homotypic interaction (*e.g.* PECAM1, alias CD31). Unfortunately, no CD31 staining was detected on keratinocytes to confirm the possible colocalization with CD46 (data not shown). Megakaryocyte cell lines have been shown recently (52) to produce platelets by a novel compartmentalized form of caspase-directed cell differentiation. The CMK-derived platelets stained for cleaved caspase 3 were highly enriched with CD46 (Fig. 6, *m–p*). These platelets were also weakly stained for NA as indicated by DAPI staining.

DISCUSSION

Cells undergoing apoptosis display a number of eat me flags. Some are relatively well characterized, such as the exposure of PS normally restricted to the inner membrane leaflet or poorly characterized, such as changes in surface sugars detected by phagocyte lectins. Other eat me signals have also been defined for their capac[illegible]nd adhesive bridging molecules, such as innate immune defense collagen molecules MBL, surfactant proteins SP-A and SP-D, and complement C1q (53). The key role of C1q has been confirmed by comprehensive studies demonstrating that C1q-deficient animals were severely impaired in the clearance of apoptotic cells (11, 54). Our data further argue for a central role of C1q in the early detection of membrane-bound NA on apoptotic cells long before they expose the canonical PS eat me signal. DNA has long been known as a nonimmune activator of the complement pathway through its interaction with both the collagen-like region and the globular region of C1q (46). The precise region of C1q involved in DNA binding might be a matter of controversy (12, 15, 55), but a more recent and elegant study by Palaniyar *et al.* (56) highlights that all major defense collagens (SP-D, MBL, and C1q but not SP-A) are involved in binding to carbohydrate (pentose sugar-based anionic phosphate) polymers such as free DNA and RNA. Our paradigm does not exclude the newly described role of IgM in engaging the activation of the complement classical pathway on apoptotic cells (33–35).

C1q is one of the rare complement components not to be synthesized by hepatocytes, but its scavenging function should be credited to other cell

types such as macrophages, fibroblasts, epithelial cells, glial cells, and neurons (36). In immunoprivileged tissues that lack an adaptive immune response (hence IgM), we argue that C1q may be the driving force in the recognition and the demise of apoptotic and necrotic cells. C1q has a unique multimeric structure and has been shown to bind to several PAMPs and PPAMPs such as the prion agent and amyloid fibrils accumulating in the brain of demented patients (18, 36, 57). Our report stresses that C1q is also involved in the selective recognition of ACAMPs. C1q may have a dual role in the clearance of apoptotic cells: activator of the classical complement pathway to promote opsonization by C3 fragments and as a complement receptor-like molecule. Macrophages were shown to synthesize and also display C1q as a type II cell surface molecule (58), and it will be important to ascertain the direct contribution of C1q as a receptor binding to NA.

Recent data suggest that uptake of apoptotic cells involves two sequential steps: an initial tethering event followed by macropinocytosis (59). Macrophages "tether" apoptotic cells using several redundant innate immune receptors such as CD14, CD36 and scavenger receptors, and perhaps C1q expressed at the cell membrane may be involved in this scenario (14, 59). The second step that involves the engulfment of the apoptotic cells has also been linked to C1q but this time through its capacity to bind to surface calreticulin (also known as the collagen-tail C1q receptor, cC1qR) complexed to the endocytic receptor protein CD91.

Our data stress for the first time the role of CD46 as a don't eat me signal involved in innate immune recognition. During blebbing or zeiosis of cells dying by apoptosis, we found that CD46 was rapidly translocating from cytoplasmic/nuclear and membrane stores into membrane-bound bodies (apoptotic blebs) budding individually from the cell surface. As expected, the reduction in CD46 resulted in increased C3b deposition on apoptotic cells, which ultimately would make them more appetizing to macrophages. In this context and given that CD46 is ubiquitously expressed on all nucleated cells, we would argue that CD46 is a key and novel example of a SAMP to control the demise of the dying cell by the innate immune system. We showed that more than 15 different human cell lines and primary human cell cultures induced to undergo apoptosis were rapidly losing CD46 at the cell surface; furthermore, Crry, the CD46-like protein in rodents, was also found to be dramatically reduced in several apoptotic cell models and possibly through capping into blebs.[3] Is CD46/Crry a universal SAMP to control the innate immune recognition by professional and amateur phagocytes in analogy to major histocompatibility complex class I inhibiting NK cell killing? Regrettably, we don't know, and *in vivo* experiments along these lines are now highly warranted. CD46 genetic defects in humans or Crry experimental deficiencies are associated with severe pathologies, including hemolytic uremic syndrome and embryonic lethality (60, 61). We do not know whether the immunoregulatory properties of CD46/Crry are exclusively dependent on their capacity to control complement opsonization. Crry can protect from innate immune recognition in a complement-independent manner, stressing the plausible role of a CD46/Crry counterreceptor (CD46R) yet to be identified (62). In analogy to the CD47/SIRPα or the CD200/CD200R paradigms, it will be important to ascertain whether canonical innate immune cells (macrophages, neutrophils, mast cells, and dendritic cells) express the putative immunoregulatory CD46R. Preliminary FACS experiments using a CD46-human IgG4 Fc fusion protein indicated a strong specific binding to THP1, HL60, and PMN cells.[4] No specific staining above background was obtained with the human IgG4 control protein. Affinity chromatography experiments will help to delineate the nature of the binding molecule(s) and whether CD46R has classical ITIM motifs.

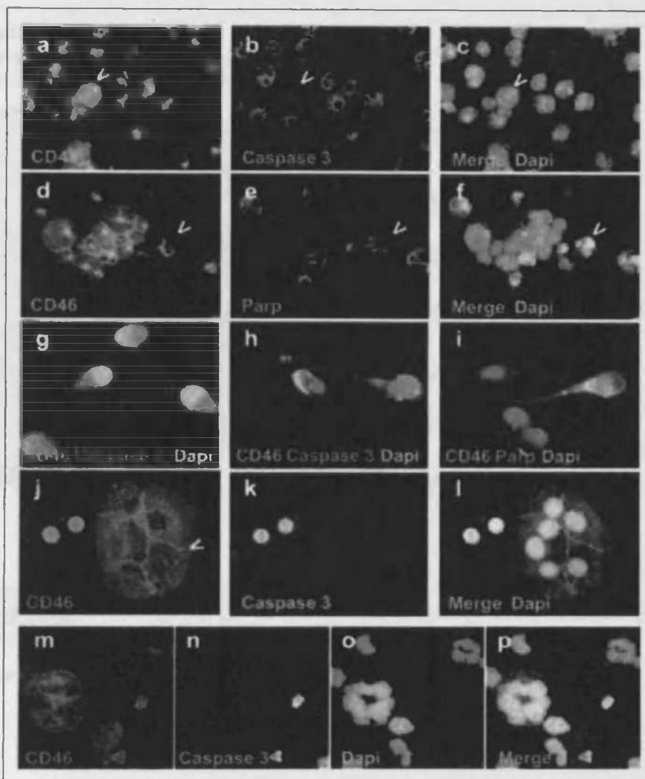

FIGURE 6. **CD46 is universally lost from several nucleated cells in response to caspase induction and released into CD46-enriched microparticles.** Double immunofluorescent staining for CD46 (clone MEM258 and FITC, *green*) and hallmarks of caspase activation (rabbit polyclonal anti-cleaved caspase 3neo and cleaved PARPneo, rhodamine, *red*). Nuclei were labeled with DAPI, *blue*. Apoptotic Jurkat cells (post anti-Fas treatment, 4 h) and activated human spermatozoa (ionophore treatment) showed loss of CD46 in correlation with caspase 3 activation and PARP cleavage. *a*, the *white arrowhead* indicates that viable Jurkat cells displayed strong intracellular and membrane-associated CD46 staining. In contrast (*d*, *arrowhead*), it was clear that the apoptotic Jurkat cell had highly reduced expression of CD46 with a total loss from the cell surface. Spermatozoa were untreated (*g*) or ionophore-treated (*h* and *i*). Microparticles formed from senescent keratinocytes (*j*) and platelet-like bodies (52) produced and released from CMK megakaryocytes (*m*) showed strong CD46 and cleaved caspase 3 expression. Of note, strong CD46 staining was observed at the point of contact between keratinocytes (*arrowhead*, *j*). Magnification $\times 400$ (*a–f* and *j–p*) and $\times 1000$ (*g–i*).

Consistently, CD46 capping into blebs was (i) associated with a caspase-dependent cell maturation processes of sperm and platelet precursor cells; (ii) taking place on senescent keratinocytes; (iii) independent of complement opsonization at the cell surface; (iv) relying on caspase-dependent activation events upstream of caspase 3; and finally (v) released from the cell surface as part of a microparticle together with NA, PS, C1q, iC3b opsonin, and factor H.

We would argue that these microparticles could diffuse out and provide "appetizers" to the surrounding macrophages to prepare for a bigger meal, the apoptotic corpses. Several eat me signals are carefully arrayed and highly enriched on these microparticles such as NA, PS, C1q, and iC3b, but interestingly, they could engage immunosuppressive pathways as recently described for iC3b docking onto CR3 (63, 64). The role of CD46 (and factor H) in these microparticles remains unclear apart from ensuring that C3b is converted to iC3b. The clearance of these microparticles by the neighboring cells is of paramount importance before they are captured by antigen-presenting cells (*e.g.* dendritic cells). Failure to do so could be associated with systemic lupus erythematous pathologies that are linked to autoantibodies against several entities present in these microparticles (NA, C1q, and fH) (65–67).

[3] K. Elward and P. Gasque, unpublished observations.
[4] K. Elward, C. L. Harris, and P. Gasque, unpublished observations.

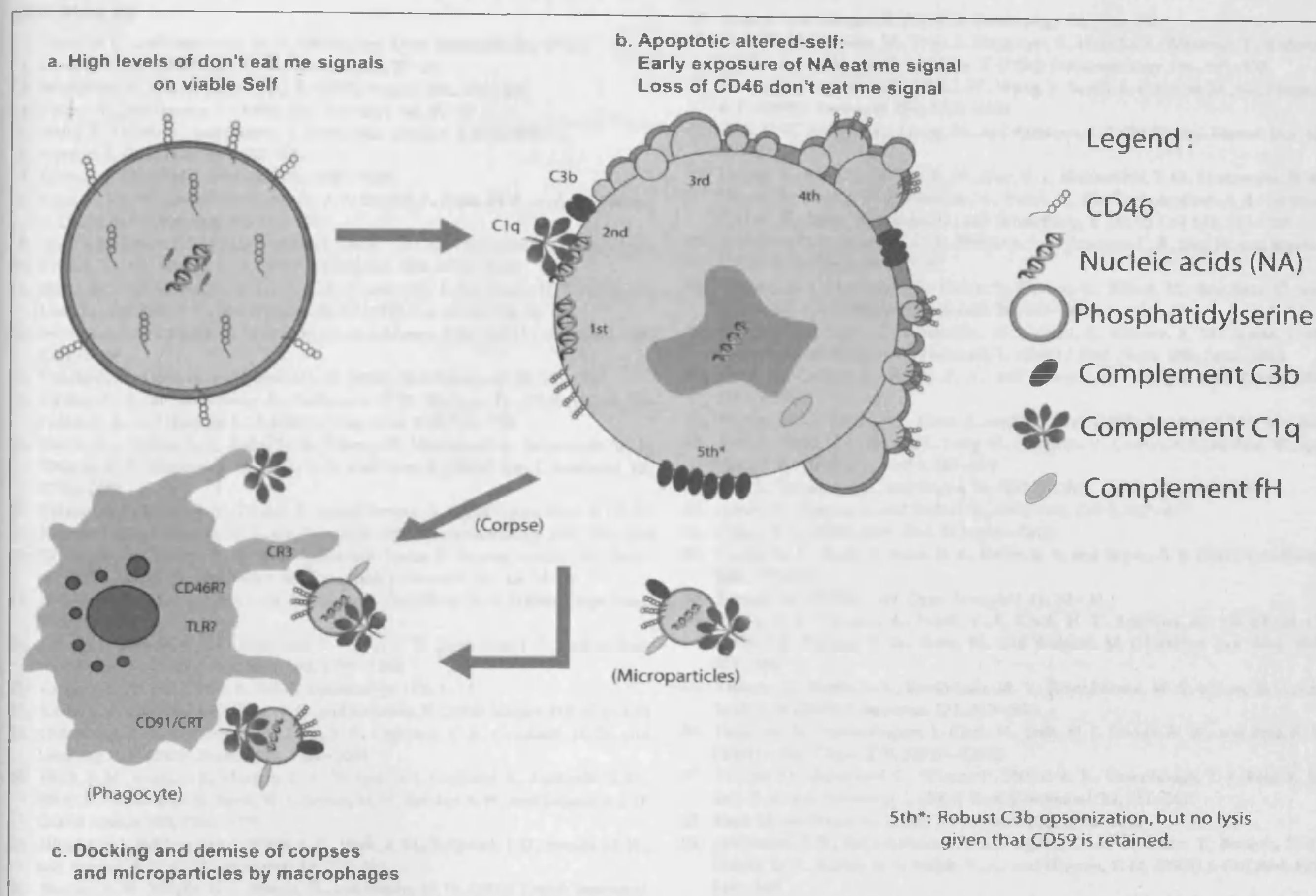

FIGURE 7. **Innate immune recognition and the safe demise of altered self; the NA and CD46 universal model.** Although apoptosis is initiated by many physiologic and pathologic stimuli, all apoptotic cells undergo a similar sequence of morphological and biochemical events. Our data underlines the role of two novel and universal key signals: membrane-bound nucleic acids as an eat me or altered self-signal, and CD46 as a don't eat me or missing self-signal. Proposed sequence of events to initiate the decoding of altered self-phenotype. *a*, viable cells express CD46 (found abundantly on all nucleated cells) and other regulators of the innate immune complement system as well as retain PS on the inner leaflet of the plasma membrane. *b*, in sharp contrast, apoptotic cells rapidly expose NA in the outer leaflet of the plasma membrane long before PS exposure and promote activation of the complement system (C1q selective binding to NA). Critically, we found that apoptotic cells retained high levels of glycosylphosphatidylinositol-anchored CD55 and CD59, hence preventing MAC formation. However, because CD46 is rapidly shed from the cell surface into microparticles, the apoptotic cell will gradually lose the capacity to control complement C3b opsonization ("missing self"-paradigm). The microparticles (enriched for CD46, NA, and C1q/C3b, and factor H) are likely to diffuse out and dock onto professional and amateur phagocytes to instruct the safe demise of the apoptotic corpses by mechanisms that remain poorly characterized. Phagocyte recognition could involve different receptors for PS, C1q (CD91/calreticulin,CRT), C3b/iC3b (complement receptor type 3, CR3), and other possible receptors for CD46 and NA, yet to be identified. Membrane-bound C1q could act as an NA receptor. Furthermore, Toll-like receptors have been reported to decode nonself-NA, but it is unknown whether they are also involved in sensing altered self-NA.

Most surprisingly, CD46 capping into blebs and budding from the apoptotic cells was not affected by the level of complement opsonization at the cell surface but was a caspase-dependent apoptotic event. Researchers in the spermatogenesis field have known for some time that apoptosis plays an important role in removing abnormal sperm (51), and perhaps the loss of CD46 will promote complement-dependent removal by neighboring Sertoli cells or even by epithelial cells of the vagina/uterus to control possible pro-inflammatory response against this natural foreign particle. Recent data in *Drosophila* led to the idea that developing spermatozoa use the apoptotic machinery to selectively dissipate unneeded portions of their cytoplasm (membrane blebbing), a process termed individualization (50). Our human sperm and megakaryocyte data clearly indicate that CD46 capping to caspase-positive blebs is part of a physiological process independent of complement activation and highlight a more general (and perhaps primary/ancestral) role of CD46/Crry in membrane blebbing and budding of microparticles from the cell surface.

In conclusion, the role of complement promoting the safe disposal of altered self has received bad publicity with the wisdom that it would do more harm than good. Complement is perhaps the only canonical innate immune system with the intrinsic capacity to handle eat me and don't eat me signals as well as "come and get me" signals in the form of complement C3a and C5a anaphylatoxins. Our original findings support the role of novel and universal eat me signal (*i.e.* NA interacting with C1q) which will drive and tailor the level of complement opsonization on apoptotic and necrotic cells (Fig. 7). Furthermore, our data stress the emerging role of a novel and universal don't eat me signal (*i.e.* CD46/Crry). Ongoing experiments in our laboratory will help also to delineate the role of C3a in the nonphlogistic removal of apoptotic cells.

Acknowledgments— We greatly thank Prof. Vaclav Horejsi (Institute of Molecular Genetics, Academy of Sciences, Prague, Czech Republic), Prof. John Atkinson (Washington University School of Medicine, St. Louis, MO), Dr. Eric Rubinstein (INSERM U602, Villejuif, France), Dr. M. Fontaine (INSERM U519, Rouen, France), and Prof. Otto Gotze (Department of Immunology, Gottingen, Germany) for kindly providing valuable reagents used in this study. We also thank Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN) and Dr. C. J. S. Edgell (University of North Carolina) for providing cell lines used for our experiments. Fig. 7 was kindly designed by Claudine Vitry.

CD46 Controls Innate Immune Recognition of Apoptotic Cells

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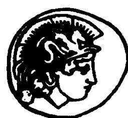

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Molecular Immunology 40 (2003) 85–94

Molecular Immunology

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Review

"Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system

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Abstract

A full innate immune system (e.g. complement system, scavenger receptors, Toll-like receptors (TLR)) has been described in the CNS and is thought to be an extremely efficient army designed to fight against invading pathogens and toxic cell debris such as apoptotic cells and amyloid fibrils. The binding of soluble or secreted innate immune molecules on pathogen-associated molecular patterns (PAMPs) as well as apoptotic cell-associated molecular patterns (ACAMPs) provide several "eat me" signals to promote the safe disposal of the intruders by professional and amateur phagocytes. These patterns are deciphered by receptors (pattern recognition receptors, PRRs; e.g. CR3) that control phagocytosis and associated inflammatory response depending on the meaning of these signals. Importantly, in order to avoid excessive collateral damage of surrounding cells, it is increasingly evident that "don't eat me" signals (coined herein as self-associated molecular patterns, SAMPs; e.g. complement regulatory proteins, CD200) are of paramount importance to signal a robust anti-inflammatory response and promote tissue repair. Further knowledge of the innate immune response in the CNS will greatly help to delineate the novel therapeutic routes to protect from CNS inflammation and neurodegeneration.

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Keywords: Complement; Innate immunity; Apoptosis; Phagocytosis; Neuroinflammation; Brain repair; Neurodegeneration

1. Introduction: innate immune competent cells of the CNS

In tissues other than the CNS, immune responses involving both the innate immune system and the adaptive immune response (T cell, B cell, antibody) have pivotal roles in the efficient clearance of pathogens. However, the brain is isolated from immunosurveillance and the limitation of an adaptive immune response in the CNS has been attributed to the intricate nature of susceptible neuronal networks and is thought to derive from an evolutionary adaptation (Gasque et al., 2000; Schwartz et al., 1999). However, it is critically important that the brain has the capacity to recognise and start to generate a local innate immune response against potential intruders expressing "eat me" signals. Moreover, mounting evidence indicates that the injured CNS (e.g. stroke, Alzheimer's disease, AD) requires an innate immune intervention in order to limit damage and initiate tissue repair with the clearance of apoptotic cells and other toxic debris (myelin debris, amyloid fibrils) (Barnum, 2002; Gasque et al., 2000; Nguyen et al., 2002; Wyss-Coray and Mucke, 2002). To this aim, it is critical that the glia and neurones have the capacity to discriminate between non-self, toxic materials and self and eliminate apoptotic bodies to limit further the local inflammation. In some instances the glial immune response will lead to massive recruitment of peripheral macrophages and reactive microglia, which are potentially threatening to the CNS (Perry et al., 1993). However, there is a growing body of evidence that neuronal cells can express specific secreted and membrane-bound "don't eat me" signals to control adverse phagocytosis and induce macrophages towards a restorative inflammatory response with the production of anti-inflammatory cytokines and growth factors involved in tissue repair (see herein). The aim of this mini review is to highlight the recent development regarding the expression and the role of the neuronal innate immune response in health and disease. We will introduce the role of the different cellular and molecular actors orchestrating the local brain immune response involved in the clearance of pathogens and apoptotic cells leading to tissue repair. Although microglia, the so-called 'professional' macrophages (about 10% of the total brain

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doi:10.1016/S0161-5890(03)00109-3

cell population) have been proposed to be the most important immune glial cells in the CNS (Perry et al., 1993, p. 80), astrocytes (50–60% of the brain cell population) could nevertheless be the most strategic glial cells for the initiation and progression of CNS innate immune functions, due to their location at the blood–brain barrier, a probable port of entry for most of the pathogens, and their prompt reactivity following tissue damage (Dong and Benveniste, 2001). In addition, most of the ventricular system and the choroid plexus in contact with the cerebrospinal fluid (CSF) is lined by ependymal cells (epithelial-like cell) and despite the fact that little is known about their innate immune activities, it is increasingly reported that these cells too are involved in the CNS immune response (Martino et al., 2001). Last but not the least, a sub-population of neurons have recently been characterized as important innate immune cells involved not only in phagocytic activities but also in restoring the immuno-privileged status of the brain while promoting tissue repair (Flugel et al., 2000).

The overall paradigm is that the brain can express numerous immune molecules of the innate immune system "a primitive and ancient system of host defence" with the spontaneous capacity to recognise and remove selectively 'non-self' components accumulating in an injured brain and subsequently, to nurture normal 'self' cells by clearing toxic cell debris and stimulating the release of growth factors.

2. "Eat me" signals of pathogens and dying cells (apoptotic and necrotic cells)

In agreement with the concept originally presented by Medzhitov and Janeway (1997), it is now known that a plethora of innate immune molecules are expressed by activated professional (peripheral macrophages, microglia) and amateur brain cells to recognise pathogen-associated molecular patterns (PAMPs), that are shared by large groups of pathogens and conserved products not subject to antigenic variability (for review, see Gasque et al., 2000). These PAMPs are microbial structures, which upon interaction with elements of the host innate immune system, trigger the initiation of host protective responses with the clearance of the pathogen by phagocytic cells. Only recently is more attention being focused on phagocytosis and innate immunity in development (Franc et al., 1999; Imler and Hoffmann, 2002; Johnson et al., 2003). New and exciting findings highlight the role of phagocytosis as an important process in tissue homeostasis. It is well established that programmed cell death, or apoptosis, is an integral part of the development of all metazoans and that dying cells are removed very early on. Remarkably, the cellular changes (proteins, lipids, carbohydrates) that accompany the recognition and subsequent phagocytosis of apoptotic cells and pathogens appear very similar (Savill et al., 2002). The emerging paradigm is that innate immunity has also been involved in the clearance of foreign, potentially dangerous and toxic entities such as apoptotic cells. In analogy to PAMPs, we and others have proposed that innate immune molecules recognise apoptotic cell-associated molecular patterns (ACAMPs) expressed de novo by cells undergoing programmed cell death (Franc et al., 1999; Gasque et al., 2000; Gregory, 2000).

Ultimately, 'professional' and 'amateur' phagocytes recognise PAMPs and ACAMPs through specific soluble and membrane-bound pattern recognition receptors (PRRs; e.g. collectins, phagocytic receptors), which lead to clearance of the different target cells (Gregory, 2000; Parnaik et al., 2000). Thus, in analogy to the specificity of the adaptive immune response, innate immunity is extremely selective and has divided the universe into innocuous self cells and potentially noxious substances ranging from pathogens and toxic cell debris and essentially according to their particular 'non-self' signatures. Most of the reported PRRs are expressed by brain cells and particularly in disease conditions (Gasque et al., 2000). Although some redundancy must exist to maintain tissue homeostasis, it is also evident that different signalling events mediated through the engagement of one or more of these receptors dictate the final glial innate immune response. On the one hand, it is essential that glial cells encountering a pathogen (e.g. fungus such as *Candida albicans*) are stimulated to produce pro-inflammatory cytokines and chemokines to initiate the recruitment of competent phagocytes and possibly engaging a more efficient acquired immune response. On the other, it is logical to consider that the phagocytosis of toxic cell debris and apoptotic neurons in ischaemic brain (stroke) should stimulate the glial cells to produce anti-inflammatory cytokines as well as growth factors involved in tissue repair.

Recognition of necrotic cells is more complicated because the markers of necrosis have yet to be characterised in detail. During necrosis, intracellular components that are normally hidden inside the cell (e.g. nuclear factor, High mobility group 1 protein, HMG1) are released and induce strong inflammation. Again, a delicate balance must exist which will either promote tissue repair or enhance further tissue damage.

3. Secreted forms of PRRs interacting with PAMPs and ACAMPs

Several structurally and functionally distinct classes of PRRs evolved to recognise PAMPs as well as ACAMPs. Secreted PRRs (also known as bridge molecules) bind to their target and flag them for destruction either by the complement system or by phagocytosis.

3.1. Soluble defence collagens and engagement of phagocytic receptors

One group of secreted PRRs that have been shown to recognise PAMPs have been collectively named "defence collagens" (Tenner, 1999). In general but not always, the

globular carboxy-terminal domain of these proteins recognise relatively broad categories of molecules (e.g. PAMPs) and the collagen-like amino-terminal domain links the invading organism to powerful effectors of the immune system (PRRs; e.g. phagocytic receptors). Soluble members of the defence collagens include C1q (the recognition component of the complement classical pathway, see below) and the collectins (mannan binding lectin, MBL; and lung surfactant protein A, SPA).

Complement (C) C1q mainly initiates the classical pathway by binding to Fc-region of immunoglobulins (Ig) that are engaged in immune complexes (Ag–Ig complexes). However, it has become clear that C1q can also bind to a wide variety of agents, including C reactive protein (CRP), serum amyloid protein (SAP), DNA, LPS, and more importantly, some bacterial, fungal and virus membranes, to mediate activation of the C system leading to the clearance of the target (Gewurz et al., 1993). We, and others, have found that C1q mRNA is not detectable in the liver and the lung but is expressed in spleen, thymus, heart and brain (Dietzschold et al., 1995; Morgan and Gasque, 1996). It has been shown that C1q is particularly abundant in areas of tissue damage and is produced by glial and neuronal cells. Interestingly, it has been shown that C1q can bind spontaneously to hypoxic neurones, to amyloid fibrils and to myelin proteins (MBP, MOG) suggesting that C1q is an important soluble PRR involved in the clearance of cell debris and toxic components in the CNS (for review, see Gasque et al., 2000). More recently, several lines of evidence suggest that C1q has an important role in the clearance of apoptotic cells. Several seminal studies have shown that C1q can bind directly and selectively to surface blebs of apoptotic cells (e.g. keratinocytes, endothelial cells, T cells) chiefly leading to the activation of the classical pathway (Korb and Ahearn, 1997; Mevorach et al., 1998; Nauta et al., 2002; Navratil et al., 2001; Ogden et al., 2001). The target structure on apoptotic cells involved in the C1q binding remains to be defined although recent data has suggested that activation of phospholipase A2 during apoptosis promotes the exposure of membrane lysophosphatidylcholine, which is subsequently recognised by natural IgM antibodies to initiate C1q binding and engaging the classical pathway (Kim et al., 2002). Deficiency of an early component of the classical C pathway (e.g. C1q) has been shown to regularly produce autoimmunity in man, especially systemic lupus erythematosus (SLE), a systemic autoimmune disorder characterised by autoantibodies to nuclear antigens (Bowness et al., 1994). Gene targeted C1q deficient mice also developed a lupus-like disease characterised by antinuclear autoantibodies and proliferative glomerulonephritis with immune deposits and multiple apoptotic bodies (Botto et al., 1998; Taylor et al., 2000).

When activated on a cell surface, C3 becomes covalently bound (opsonised) on the cell surface as C3b, which is subsequently cleaved to yield a very stable fragment, iC3b. There is well-documented evidence that CR3 (CD11b/CD18) and CR4 (CD11c/CD18; also known as the p150, 95 antigen) are involved in the phagocytosis of targets opsonised with C3b and iC3b fragments (Ehlers, 2000; Mevorach et al., 1998). Perhaps more importantly, the binding of phagocytes by way of CR3 recognition, either of natural microbial surface components, such as β-glucan, LPS, lipophosphoglycan and other as yet undefined structures, or of iC3b, is the critical event leading to the elimination of pathogens, toxic debris and apoptotic cells (Ross, 2002). A CR3-like molecule has recently been described in invertebrates and CR3 is now considered a key receptor in innate immunity and legitimately joins the ranks of the host PRRs such as CD14 and the macrophage mannose receptor (MMR) (for review, see Ehlers, 2000). Microglia within the parenchyma and Kolmer cells of the choroid plexus were reported to express abundant levels of CR3 and CR4 (Gasque et al., 2000). The interactions of CR3 or CR4 with different extracellular matrix molecules (such as fibronectin, laminin and collagen) might contribute further to the clearance mechanisms. Of note, ligation of iC3b-opsonised cells to C receptors (CR3 and CR4) has been confirmed not to be a pro-inflammatory effect (Aderem and Underhill, 1999).

MBL and SPA/D are important constituents of the innate immune system since they have the capacity to recognise sugar moieties and initiate killing of a large variety of pathogens, such as Gram+bacteria, Gram−bacteria, yeasts, HIV, mycobacteria and parasites (Holmskov et al., 1994; Lu et al., 2002). They are characterised by the presence of a lectin or carbohydrate recognition domain (CRD) of 115–130 amino acids involved in the binding to pathogens in a calcium-dependent manner. They have a similar repertoire of recognition to macrophage mannose receptor and binds directly to sugar moieties present on bacteria, yeasts, parasites and mycobacteria (Fraser et al., 1998). The collagen domain is thought to be involved in the binding of MBL and SPA to defence collagen 'C1q' receptors (see below). Interestingly, an interaction between SPA and Toll-like receptor 4 (TLR4) was demonstrated illustrating the remarkable cross-talk between several innate immune systems to promote the efficient recognition and clearance of intruders (Guillot et al., 2002). MBL is a soluble protein expressed so far only by hepatocytes while SPA is expressed by lung alveolar type II cells. Interestingly, several human astrocyte cell lines were shown recently to express MBL albeit at low levels (Kuraya et al., 2003). It remains to be tested whether MBL as well as SPA are indeed expressed in vivo and particularly in disease conditions associated with tissue damage. Although MBL and SPA are essential in the elimination of pathogens, their role in the clearance of apoptotic cells has been largely overshadowed (Nauta et al., 2002; Ogden et al., 2001; Vandivier et al., 2002). In fact, none of the well-known universal cell surface components of apoptotic cells (e.g. phosphatidylserine, PS) have been linked specifically with any of the known collectins, although sugar moieties at the apoptotic cell surface may yield important ACAMPs (Gregory, 2000). Moreover, the

overall change in carbohydrates at the cell surface of apoptotic cells such as the increased expression of galactose and *N*-acetylglucosamine or decreased expression or clustering of sialic acid contents may overall affect innate immune recognition and the final control of the inflammatory response (for review, see Savill, 1997). Thus, it is possible that lectins expressed by phagocytes and binding to exposed carbohydrates on apoptotic cells could promote clearance mechanisms. In addition, low levels of sialic acids on a target cell may affect the binding of soluble and membrane-bound PRRs (C regulatory protein factor H (fH) and siglecs, see below). Overall, demonstrating specific exposure of such sugars by apoptotic cells has proved problematic.

Monocytes that have adhered to surfaces coated with C1q (or MBL or SPA) display a 4–10-fold enhancement of ingestion of targets suboptimally opsonised with Ig or C (Tenner, 1999). Several candidate defence collagen receptors (C1qRp, CR1, calreticulin, gC1qRp) have been described but their role in C1q binding and the clearance of pathogen/apoptotic cells remains to be fully ascertained (for review, see McGreal and Gasque, 2002). C1qRp (also known as the AA4 antigen in rodents and renamed CD93) is the antigen recognised by a pro-adhesive monoclonal antibody called mNI-11 and antibodies against CD93 but recent data failed to confirm direct C1q binding activity (McGreal et al., 2002). In the CNS, CD93 is found abundantly on microglia, endothelial cells and a subset of neurons particularly in disease conditions (Gasque et al., unpublished observations). CR1 (C receptor type 1, CD35) is found on circulating monocytes (including microglia) and neutrophils but the major site of expression is B lymphocytes. CR1 is a multifunctional receptor both in its ligand specificity and in the C regulation activities. As a receptor, CR1 can bind to C1q and other C opsonins (C4b, C3b, iC3b) and MBL and as such, has been involved in signalling to phagocytosis (Nicholson-Weller and Klickstein, 1999). Several studies support a role for cell surface collagen C1q receptor (cC1qR; also known as calreticulin, CRT) (Eggleton et al., 1998). CRT belongs to the family of heat shock proteins, the most abundant and ubiquitous soluble intracellular proteins. Though CRT does not have a transmembrane domain, it seems to mediate phagocytosis of the apoptotic cells through association with CD91 (Ogden et al., 2001; Vandivier et al., 2002). A universal 33 kDa protein interacts with the globular head of C1q and logically has been termed gC1qR. This protein is located in mitochondria suggesting that gC1qR is not per se a cell surface receptor (Ghebrehiwet et al., 2001).

3.2. Soluble pentraxins and associated phagocytic receptors

Pentraxins are a superfamily of conserved proteins that are characterised by a cyclic multimeric structure. The classical short pentraxins, C-reactive protein and serum amyloid P component, are acute-phase proteins produced in the liver in response to inflammatory mediators (Gewurz et al., 1995; Steel and Whitehead, 1994). They regulate innate resistance to microbes and the scavenging of cellular debris (e.g. chromatin and small nuclear ribonucleoproteins) and extracellular matrix. Both short pentraxins have been found associated with toxic cell debris such as amyloid plaques but further experiments are warranted to confirm whether glial and/or neuronal expression can occur in the CNS (McGeer et al., 2001; Yasojima et al., 2000). Short pentraxins can bind to apoptotic cells and in analogy to experimental C1q deficiency, SAP knockout mice also manifest autoimmune disease in association with delayed degradation of chromatin (Bickerstaff et al., 1999; Familian et al., 2001; Gershov et al., 2000). It has recently been shown that the long pentraxin (PTX3) produced by endothelial cells, fibroblasts, adipocytes, monocyte/macrophages and dendritic cells does also bind to C1q as well as selected micro-organisms such as *Aspergillus fumigatus* to mediate a strong anti-fungal innate immune response (Breviario et al., 1992; Garlanda et al., 2002; Nauta et al., 2003; Rovere et al., 2000). In the mouse brain, PTX3 expression, presumably by glial cells, was induced in response to intra-cerebroventricular injection of LPS. Opsonisation of apoptotic cells with pentraxins can lead to the binding of C1q and subsequent activation of C but pentraxins may also directly promote uptake of apoptotic cells through Ig receptors (FcγR) (Mold et al., 2002). For instance, it has been proposed that the interaction of CRP and SAP with the different FcγRs (CD32, CD64 and possibly CD16) may promote phagocytosis of the target cell but careful experiments are urgently warranted to confirm this paradigm (Bharadwaj et al., 1999; Stein et al., 2000). A possible receptor for PTX3 on macrophages has not yet been characterised.

4. Membrane forms of PRRs interacting with PAMPs and ACAMPs

Several innate immune receptors which bind directly to pathogen molecules, including scavenger receptor type A (SR-AI/II) and more recently CD14 (LPS receptor), have also been implicated in the phagocytosis and clearance of apoptotic cells (for review, see Gregory, 2000; Savill et al., 2002). The ability of apoptotic cells to reverse their membrane polarity also gives them an overall polyanionic charge that makes them a good target for SRs (Platt et al., 1996). CD14 has recently been reported to interact with Intercellular adhesion molecule, ICAM3 exposed on the apoptotic cells via a region that might be identical to the LPS binding domain (Moffatt et al., 1999). Interestingly, both receptors are expressed by quiescent and reactive microglia. Macrophage mannose receptor has a similar repertoire of recognition to MBL and is able to recognise a wide range of Gram + bacteria and Gram − bacteria, yeasts, parasites and mycobacteria (Stahl and Ezekowitz, 1998). MMR is a transmembrane protein that has five domains: the amino-terminal cysteine-rich region, a domain containing a fibronectin type

II repeat, a series of eight tandem lectin-like carbohydrate recognition domains, and a transmembrane domain followed by a short cytoplasmic tail. The recognition of sugar moieties expressed by pathogens is restricted to the CRDs and only CRD4 and CRD5 contain the residues required for carbohydrate and calcium binding. The role of the MMR in the clearance of apoptotic cells is unknown. Although it was previously thought that the expression of MMR was restricted to tissue macrophages, it is clear that MMR is also expressed on immature dendritic cells, subsets of endothelial cells, smooth muscle cells and more importantly by glial cells (astrocytes and microglia) (Linehan et al., 1999).

The innate immunity field is fuelled with reports concerning the roles of Toll-like receptors in host defence (Medzhitov and Janeway, 2000). In contrast, very little is known about the plausible role of TLRs in promoting phagocytosis of apoptotic cells. Interestingly, degradation of the extracellular matrix occurring at sites of inflammation results in low molecular weight polysaccharides of hyaluronic acid (sHA) which can bind to phagocytes expressing TLR4. Moreover, it has been argued that endogenous ligands of TLR4 (e.g. heparan sulphate and hyaluronan) released from the cell surface may be able to signal phagocytosis by macrophages.

The vitronectin receptor ($\alpha v\beta 3$: CD51/CD61) co-operates with CD36 to bind the glycoprotein thrombospondin (TSP) which acts as a molecular bridge and allows the binding of macrophages to apoptotic cells (Savill, 1997). The ligand(s) recognised by TSP at the surface of the apoptotic cell is yet to be defined. Interestingly, PfEMP1, a high molecular mass malarial protein expressed on *Plasmodium falciparum* parasitised erythrocytes, has been shown to interact with TSP and CD36 and may behave as a protective PAMP to instruct binding to microvascular endothelial cells and vascular occlusion which, all in all, may confer protection from humoral and cellular innate immune assaults (Baruch et al., 1996).

Phosphatidylserine residues are the generic evolutionary conserved ACAMPs. It is important to note that the PS receptor (PSR) as well as SR-B1 play a critical role in the clearance of apoptotic cells following the exposure of PS to the outer leaflet of the cell membrane (Fadok et al., 2000). To date, it is unknown whether the PSR is involved in binding to PAMPs.

5. Molecules involved in silencing the innate immune response: roles of "don't eat me" signals and innate immunoregulatory cytokines

The immune mechanisms described above are so far mainly responsible for enhancing innate immune responses. Silencing immune responses to limit possible immunopathology is equally important (Medzhitov and Janeway, 2002). The apoptotic process needs to be tightly regulated as too much cell death and particularly of vulnerable 'self' cells may lead to pathology, including developmental defects, autoimmune diseases or neurodegeneration. We have highlighted above that innate immune molecules can determine by which mechanism dying cells are cleared and, probably even more importantly, the response of the phagocytosing cell. Critically, innate immune cells face an important paradox, namely that seemingly identical armory and integrating signalling pathways are involved to discriminate between a dangerous intruder (pathogen) by mounting a strong pro-inflammatory response while the clearance of apoptotic cells or toxic cell debris seem to engage a rather non-phlogistic response (Savill et al., 2002). It is increasingly evident that additional ligand/receptor interactions play a critical role to counterbalance overshooting innate immune responses to control unnecessary disposals. Interestingly, in analogy to "eat me" signals, several "don't eat me" signals (or self-associated molecular patterns, SAMPs, terminology coined herein for the first time) may be involved as ligands engaging inhibitory PRRs to modulate the innate immune response. Conceptually, this hypothesis is an important paradigm shift and it offers a new insight into the molecular switch which governs the phagocyte response to promote either a pro-inflammatory or instead, an anti-inflammatory response (see comprehensive illustration, Fig. 1). For instance, SAMPs must have important features that make them instrumental in the control of the innate immune response: first, SAMPs is expressed by almost all host 'self' cells, and not by pathogens. Second, SAMPs are membrane-bound or secreted molecules that control the level of humoral and/or cellular innate immune responses. Third, the interaction between SAMPs from the host cells and novel inhibitory PRRs expressed by macrophages engage immuno-regulatory activities to maintain the non-phlogistic response and promoting tissue repair. To date, a small array of possible SAMPs have been characterised and include inhibitors of the C system (e.g. fH), OX2/CD200, CD31, CD47 and sialic acids binding to siglecs. The hypothesis that self patterns must exist to control the humoral and cellular innate immune responses is in line with the well-documented concept that host cells express high levels of MHC class I molecules to avoid killing activities by natural killer (NK) cells (Cerwenka and Lanier, 2001). The concept of missing self (e.g. virus infected cell) was proposed to explain why NK cells preferentially kill target cells that express few or no MHC class I molecules on the cell surface. As MHC class I is constitutively expressed on all nucleated cell and is often down-regulated as a result of viral infection or cellular transformation, recognition by NK cells of a missing self ligand allows them to dispose of the target.

Recognition of missing self is not unique to NK cell function and another well-known example of the use of this strategy is the regulation of the innate humoral and cellular C system. To avoid this self-destructive tendency, host cells use a wide armamentarium of regulatory molecules (C inhibitors), which inhibit assembly of either the C3-cleaving enzymes or the formation of the membrane attack complex

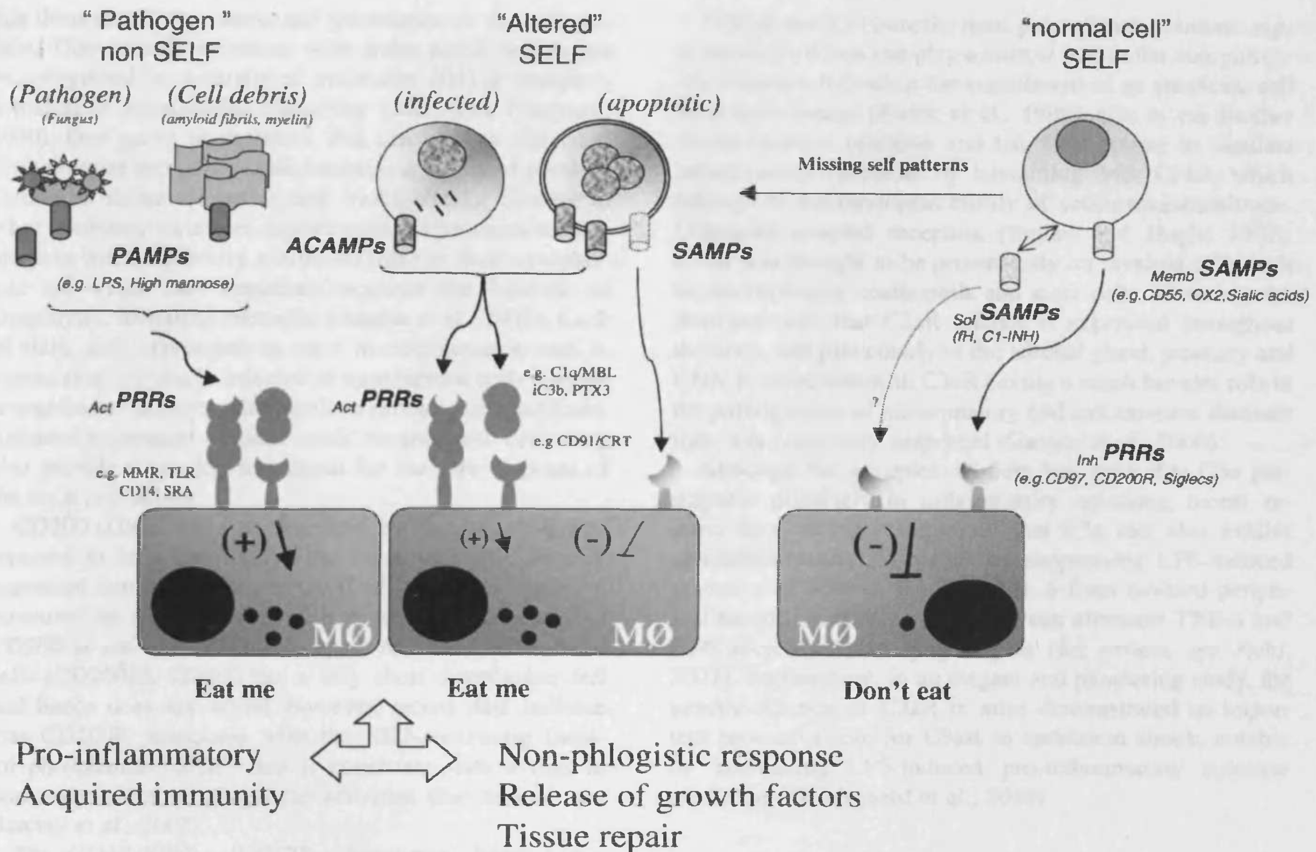

Fig. 1. Roles of "eat me" and "don't eat me" signals in the innate immune responses. The cellular and humoral innate immune system is an efficient army to fight invading pathogens, to clear toxic debris while preserving normal self cells. The difference between a harmful intruder and a dying cell is often subtle and it is therefore imperative that a code of conduct is operating. Professional phagocytes (microglia) and to a lesser extent amateurs (astrocytes, neurones) are programmed to eat but receive inhibitory or 'repulsive' signals expressed by normal self cells as a matter of safe guard. Remarkably, these "don't eat me" signals (SAMPs), either expressed at the cell membrane (e.g. CD55) or secreted and found loosely attached to the cell surface (e.g. complement factor H), are recognised by inhibitory PRRs expressed by phagocytes. The self-immune synapse is critical to maintain the phagocyte peaceful behaviour. On the contrary, apoptotic cells expressing low levels of SAMPs (missing self-paradigm described originally by Janeway; Medzhitov and Janeway, 2002) and yet displaying de novo ACAMPs will instruct rapid disposal of the corpses to limit the release of toxic and pro-inflammatory cell debris. Activating PRRs can exist as soluble bridge molecules which in turn can bind to cell surface receptors, or as membrane-bound receptors directly involved in recognition and binding to ACAMPs. Interestingly, apoptotic cells expressing ACAMPs and pathogens displaying PAMPs have been known to engage similar PRRs but yet promoting either anti-inflammatory or pro-inflammatory responses, respectively. Such sharp but yet elusive dichotomy may now be explained on the basis that apoptotic cells expressed ACAMPs and SAMPs which, following binding to $_{inh}$PRRs and $_{act}$PRRs, respectively, instruct a non-phlogistic phagocytic response (see main text). Interestingly, toxic cell debris such as myelin or amyloid fibrils may express ill-defined PAMPs and hence, promoting a strong pathological inflammatory response.

(MAC). As pathogens lack these inhibitors, activation of the C cascade can proceed on their surfaces and results in lysis or phagocytosis of microbial intruders. Similarly, as self cells progress to altered self (apoptotic cells), down-regulation of C inhibitors at the cell surface (low sialic acid content lowering fH binding; loss of membrane C inhibitors such as CD46, K. Elward, personal observation) can lead to moderate and limited C opsonisation (C3b, iC3b) to promote phagocytosis. C1 inhibitor (C1inh), C4b-binding protein (C4bp), factor H, factor I (fI), S protein (Sp) and clusterin are all soluble C inhibitors secreted and released in the fluid phase. The other C inhibitors are expressed on the cell membrane and include CR1, membrane cofactor protein (MCP, CD46), decay accelerating factor (DAF, CD55) and CD59 (see comprehensive review, see Morgan and Meri, 1994). From a SAMP standpoint, fH, C1inh, clusterin, CD46 and CD55 seem to fit the bill given that they are broadly expressed and extremely important in the control of C activation on self cells. Moreover, since CD55 is a ligand for CD97 on macrophages it is tantalising to speculate that CD55–CD97 interactions could play an important role to control phagocytosis (Hamann et al., 1996). This idea can be readily tested and experiments to test whether other C regulatory proteins bind to cell surface receptors on phagocytes are highly warranted.

Another example of a molecular signal that functions as a marker of normal self is the carbohydrate structures

that decorate glycoproteins and glycolipids on the cell surface. They usually terminate with sialic acids, which can be recognized by a variety of molecules (fH) or receptors involved in intracellular signalling (Meri and Pangburn, 1990). One group of receptors that can bind to sialylated glycoproteins and glycolipids comprise a family of proteins known as siglecs (Crocker and Varki, 2001). Similar to other inhibitory receptors, siglecs contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail which may negatively regulate the function of phagocytes, including microglia (Angata et al., 2002). Lack of sialic acid expression on most microorganisms, and in some cases, on virally infected or transformed cells may be recognised as missing self signals to promote phagocytosis. Reduced expression of sialic acids on apoptotic cells may also provide a missing self signal for the safe disposal of the toxic cell debris.

CD200 (OX2) was first described by Barclay et al, and reported to be a member of the Ig superfamily, broadly expressed and particularly in the CNS, though its function remained an enigma. To date, it is well-documented that CD200 is the ligand for a receptor restricted to myeloid cells (CD200R). CD200 has a very short cytoplasmic tail and hence does not signal, however, recent data indicate that CD200R associates with the SH2-containing inositol phosphatase (SHIP) that is consistent with a role in down-regulation of phagocyte activities (for review, see Barclay et al., 2002).

The CD47–SIRPα (CD172a) interaction bears many similarities to that of the CD200–CD200R interaction and is particularly involved in the down-regulation of macrophages through interaction of CD172a with the tyrosine phosphatases SHP1 and SHP2 (Brown and Frazier, 2001). CD47 is also known as integrin-associated protein as it was found to form *cis* interactions with αvβ3 integrin, which is a receptor for vitronectin (S protein). Moreover, CD47 also interacts with TSP suggesting a possible role in the regulation of apoptotic cell clearance.

CD31 prevents phagocyte engulfment of viable cells by transmitting "detachment signals" whereas its function is switched to a pro-adhesive signal on apoptotic cells to promote ingestion by dying cells (Brown et al., 2002).

All above examples illustrate the common features of the missing self strategy of immune recognition. Specialised markers of self are expressed constitutively on normal healthy cells of the host and, by engaging inhibitory PRRs, prevent phagocytosis of these cells and perhaps phagocytosis and presentation of antigens derived from healthy cells by dendritic cells. Although the missing self strategy is an efficient way to distinguish normal self from non-self or from altered self, it is not resistant to fraud. Indeed, in multiple examples of 'stolen identity', pathogens acquire or express genes encoding self markers (e.g. virus expressing C regulators, CD200-like molecules) and thus are relatively protected from detection and destruction by host (for review, see Barclay et al., 2002).

TGF-β and IL-10 are the most potent innate immuno-suppressive cytokines and play a critical role in the non-phlogistic response following the engulfment of an apoptotic cell by a macrophage (Fadok et al., 1998). C3a is yet another innate immune cytokine and has been shown to regulate inflammatory functions by interacting with C3aR, which belongs to the rhodopsin family of seven-transmembrane-G-protein coupled receptors (Ember and Hugli, 1997). C3aR was thought to be present only on myeloid cells such as macrophages, eosinophils and mast cells. However, the demonstration that C3aR mRNA is expressed throughout the body, and particularly in the adrenal gland, pituitary and CNS, is consistent with C3aR having a much broader role in the pathogenesis of inflammatory and autoimmune diseases than was previously suspected (Gasque et al., 2000).

Although the accepted wisdom has been that C3a participates positively in inflammatory reactions, recent reports have strongly suggested that C3a can also exhibit anti-inflammatory properties by suppressing LPS-induced secretion of TNF-α, IL-1β and IL-6 from isolated peripheral blood mononuclear cells and can attenuate TNF-α and IL-6 secretion from lymphocytes (for review, see Kohl, 2001). Furthermore, in an elegant and pioneering study, the genetic deletion of C3aR in mice demonstrated an important protective role for C3aR in endotoxin shock, notably by attenuating LPS-induced pro-inflammatory cytokine production (Kildsgaard et al., 2000).

6. Brain innate immune response: emphasis on the role of the C system

There is a growing body of evidence that brain cells can generate a full innate immune system to kill pathogens, dispose of toxic cell debris and yet be relatively well protected from direct or bystander lysis or phagocytosis through expression of soluble and membrane SAMPs (Gasque et al., 2000). It is clear that disruptions in innate immunity predispose humans to infections (meningitis) but also to SLE-like pathologies due to the failure to promote the clearance of apoptotic cells. However, this is a double-edged sword scenario since strong innate immune responses in the CNS are likely to be important factors in the pathology of degenerative disorders leading to neuronal loss and chronic inflammation and possibly autoimmune disorders such as multiple sclerosis (MS) (Barnum, 2002; Wyss-Coray and Mucke, 2002).

Alzheimer's disease is the commonest cause of dementia and is a multifactorial syndrome rather than a single disease. Senile (neuritic) plaques and neurofibrillary tangles (NFT) comprise the major neuropathological lesions particularly in limbic and association cortices. Neuritic plaques contain extracellular deposits of amyloid-β protein (βA4) as abundant amyloid fibrils intermixed with non-fibrillar forms of this peptide and also contain degenerating axons and dendrites (neurites). Several groups have clearly demonstrated

the presence of C proteins in senile amyloid plaques and NFTs in AD brains using immunohistochemical techniques (for comprehensive review, see Barnum, 2002; Gasque et al., 2000; Wyss-Coray and Mucke, 2002). By contrast, immunohistochemical staining for two alternative pathway proteins, fB and P, has not been observed in the AD brain. Interestingly, C1q immunostaining was co-localised to nearly all neuritic plaques, while no staining was detected in diffuse plaques. It has since been shown in vitro that C1q can bind directly to fibrillar but not soluble βA4 resulting in the activation of the classical pathway as seen in AD brains (Tenner, 2001). Other molecules associated with AD lesions such as SAP and CRP are known to interact with the collagen part of C1q and could also contribute to activation of the C cascade. Taken together, these data would suggest that C activation, at least in the early stages of these neurological diseases, could play an important and beneficial role in phagocytosis and clearance of otherwise toxic molecules. This elegant paradigm is further supported by recent data describing that inhibition of the C cascade increased, rather than decreased, βA4 deposition and neurodegeneration in APP transgenic mice (Wyss-Coray and Mucke, 2002). It is important that cell debris is removed efficiently to prevent further elicitation of the local inflammation. Furthermore, pioneering studies have recently shown that active or passive vaccination of a transgenic mouse hyperexpressing mutant human APP using the βA4 peptide caused accelerated clearance of neuritic amyloid plaques from the mouse brain and reduced the extent and progression of the AD-like pathology (Schenk et al., 1999). It was proposed that microglia expressing a high level of immunoglobulin Fc receptors were able to phagocytose the Ig–βA4 complex. The role of C was not investigated in this model although, from data discussed above, it is likely that increased C biosynthesis and classical pathway activation were taking place. Therefore, the possibility of glial cells expressing C receptors to phagocytose the opsonised antibody–amyloid complex coated with C is an interesting and attractive hypothesis that remains to be tested.

7. Conclusion

The essence of innate immunity is the detection of patterns that are unique to infectious organisms and noxious substances, to induce clearance of the intruders and it also dictates the conduct of the subsequent immune response either to promote a strong inflammatory response to engage acquired immmunity or the opposite, to favour tissue repair. C is widely accepted to constitute this critical link between the innate immune response involved in the selective recognition and clearance of potentially noxious substances, whether they are derived from the host following injury (apoptotic cells, toxic cell debris) or following an infectious challenge (microbial agents) and the interface with the acquired immune response. The identification of the cellular and molecular elements of the innate immune recognition discriminating between 'self' and 'non-self' have been the object of extensive studies but one burning question remains: how do these elements translate into the modulation of the response either towards pro-inflammatory or anti-inflammatory outcomes? Experiments along these lines are now highly warranted and providing unique knowledge to ascertain the possibility of manipulating the local innate immune response towards tissue repair (e.g. in AD) while avoiding the initiation of an acquired autoimmune response (e.g. in MS pathology).

Acknowledgements

This work was supported by the UK Medical Research Council (MRC).

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