Complement Receptor 1 in microglia; Implications for Alzheimer's disease

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Declaration

I, Helen Crehan, confirm that the work presented in thesis is my own, unless stated otherwise.

Flow cytometry was carried out in collaboration with Ms Jessica Estanislau.
Paraffin embedded tissue was processed and prepared by Dr Cynthia Lemere, PhD.

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Abstract

Recent genome wide association studies in Alzheimer's disease have highlighted the importance of the complement cascade in the pathogenesis of Alzheimer's disease. However, the cellular and molecular roles of these complement proteins are not fully understood. Microglia express complement receptors and the activation of specific receptors may increase Aβ clearance and reduce/prevent neurodegeneration. The work presented in this thesis was aimed at investigating the contribution of Complement receptor 1 (CR1), the second most significant hit in GWAS studies, on microglia to neuronal damage.

To explore the consequences of blocking CR1 to microglial-neuronal interactions, primary rat microglia were treated with a CR1 functional blocking antibody together with microglial activators for 24 h. It was found that microglia displaying an activated phenotype demonstrated an increase in CR1 expression. Activation of microglial CR1 was found to be detrimental to neurons and this correlated with an increase in microglial intracellular superoxide generation, nitric oxide (NO) production, tumor necrosis factor-α (TNFα) and interleukin-1 β (IL-1β) secretion. Amyloid-β 1-42 ($A\beta_{1-42}$)-treated microglia displayed an increased ability to phagocytose dextran beads following antibody blockade of CR1 but a decreased capacity to phagocytose fluorescent-tagged $A\beta_{1-42}$. CR1 immunoreactivity was investigated by immunohistochemistry in AD and control human post-mortem brain tissue. A higher level of CR1 immunoreactivity was found in areas of high Aβ plaque burden in AD brain tissue. A difference in CR1 expression on red blood cells between individuals was

measured by flow cytometry. Together, these results indicate that microglial CR1 plays a role in the neuronal death observed in AD and investigating this further may provide a possible strategy to control neurotoxicity in the AD brain.

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Abbreviations

Aβ	amyloid-β
ABC	avidin-biotin complex
AD	Alzheimer’s disease
AGE	advanced glycation end product
AMC	aged-matched control
APLT	ATP-dependent aminophospholipid translocase
APP	amyloid precursor protein
APS	ammonium persulfate
Apo	apocynin
ApoE	Apolipoprotein E
Ara-C	cytosine furanoarabinoside
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
CD59	complement defence 59
CGC	cerebellar granule cells
CNS	Central nervous system
CSF	cerebrospinal fluid
C3	complement 3
COX	cyclooxygenase
COX-2	cycleoxygenase-2
Crry	CR1-related protein Y
DAB	diaminobenzine

DHE	dihydroethidium
DIV	days *in vitro*
DMEM	Dulbecco's Modified Eagle's Medium
dsRNA	double-stranded ribonucleic acid
EA	ethacrynic acid
EBSS	Earl's Balanced Salts Solution
ECL	enhanced chemiluminescence
EDTA	ethylene tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EOAD	early onset Alzheimer's disease
ERK	extracellular-signal-regulated kinase
eNOS	endothelial nitric oxide synthase
FBS	foetal bovine serum
GPI	glycophosphinositol
GSH	glutathione
GSSG	glutathione disulphide
GWAS	genome-wide association studies
H_2O_2	hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HPLC	high performance liquid chromatography
HRP	horseradish peroxidise
ICC	immunocytochemistry
IF	immunofluorescence

IFN	interferon
IHC	immunohistochemistry
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
L-Arg	L-arginine
LCRs	low-copy repeats
LHR	long homologous repeat
LME	*L*-leucine-methyl-ester
LOAD	late-onset Alzheimer's disease
LPS	lipopolysaccharide
MAC	membrane attack complex
MAPKs	mitogen-activated protein kinase
MCB	monochlorobimane
MEM	Minimum Essential Medium
MGCM	microglial conditioned medium
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
mTNFα	membrane TNFα
NaOH	sodium hydroxide
NBT	nitroblue-tetrazolium
$NF_{K}B$	nuclear factor $_{K}B$
NGF	nerve growth factor
mtNOS	mitochondrial nitric oxide synthase

NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NFT	neurofibrillary tangle
NT	neurotrophin
$O_2^{\cdot -}$	superoxide anion
$ONOO^-$	peroxynitrite
PBS	Phosphate buffered saline
PDL	poly-dextro lysine
PET	positron emission tomography
PI	propidium iodide
PI-3K	phos-phoinositide 3-kinase
PNS	Peripheral nervous system
PS	phosphatidylserine
PTK	protein tyrosine kinase
PVDF	polyvinylidene difluoride
RAGE	receptor for advanced glycation end product
RBC	red blood cell
RISC	ribonucleic acid induced silencing complex
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SBTI	soybean trypsin inhibitor

SC	serum containing
SCR	short consensus repeat
SCR	short consensus repeat
SDS-PAGE	sodium dodecly sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SF	serum free
SH2	src-homology 2
siRNA	small interfering ribonucleic acid
smFRET	single-molecule fluorescence resonance energy transfer
SOD	superoxide dismutase
ssRNA	sequence-specific ribonucleic acid
sTNFα	soluble TNFα
TACE	TNFα converting enzyme
TBS	tris-buffered saline
TBS-T	tris-buffered saline with tween 20
TEMED	tetramethylethylenediamine
TLRs	Toll-like receptors
TNF	tumour necrosis factor
Trks	tyrosine kinase receptors
XO	xanthine oxidase
1400W	1400W dihydrochloride

Chapter 1

Introduction

Introduction

1.1 Cells of the Central Nervous System

The mammalian brain and spinal cord constitute the central nervous system (CNS), which is made up of a 1:1 ratio of neuronal to non-neuronal cells at 86 billion each (Azevedo et al. 2009). Non-neuronal cells collectively known as glia, support, maintain and protect neurons and it is these neuronal-glial interactions that will play a fundamental role in our understanding of AD progression.

1.1.1 Neurons

There are at least 86 billion neurons in the brain and they are responsible for the transmission of chemically mediated electrical messages within the CNS and the peripheral nervous system (PNS). Neurons consist of the soma, typically 20 μm in diameter containing the nucleus and the majority of the organelles, branched projections called dendrites that receive the majority of electrochemical signals to the cell, and a branched axon which is responsible for the neuronal output of the cell (Fig 1.1). The axon extends from the axon hillock to the terminal bouton. It is at this terminal site where the axon comes into contact with dendrites from other neurons to pass information on to them. The axon hillock is where the membrane potentials are propagated from synaptic inputs, and the communication of this information to the terminal bouton of the same axon is called conduction. Neurons have a negative resting membrane potential of -70 mV, and are described as polarized. A neural signal is sent when the membrane potential reaches a voltage-dependent threshold, usually

about 10 mV more positive than the resting membrane potential, initiating an action potential resulting in a brief depolarisation of the cell to a value of approx. +40 mV. This is followed by repolarisation and a refractory period, which all occur under 1 millisecond (Purves et al. 2012; Bear et al. 2007).

As previously mentioned, the site at which the terminal bouton communicates with a dendrite or another cell type to pass information, is called the synapse. This specialised structure is composed of the pre-synaptic terminal, the post-synaptic terminal, the glial terminals and the synaptic cleft. When the action potential during depolarization reaches the terminal bouton, the pre-synaptic terminal in the case of a chemical synapse, releases chemicals known as neurotransmitters into the synaptic cleft mediating neurotransmission to the post-synaptic terminal. Neurons are also coupled through gap junctions known as an electrical synapse that can transmit information without the need for neurotransmitters (Hormuzdi et al. 2004).

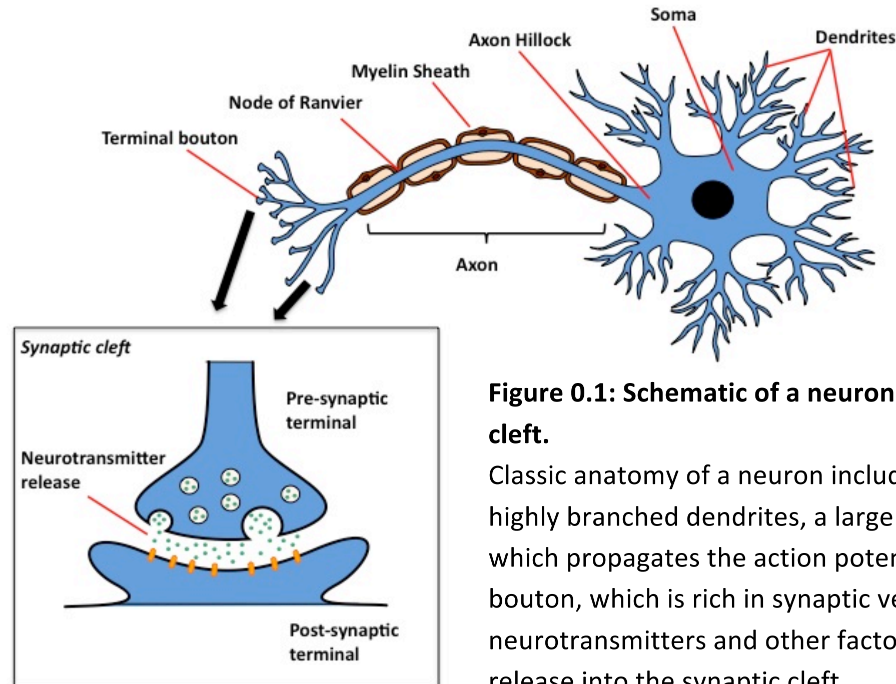


Figure 0.1: Schematic of a neuron and synaptic cleft.
Classic anatomy of a neuron includes the soma, highly branched dendrites, a large myelinated axon which propagates the action potential and a nerve bouton, which is rich in synaptic vesicles containing neurotransmitters and other factors ready for release into the synaptic cleft.

1.1.2 Cerebellar granule neurons

Recent studies have indicated that the cerebellum, not only controls voluntary movement and balance, but is also responsible for non-motor aspects of behaviour such as cognitive function (Ravizza et al. 2006; Strick et al. 2009). More than 90% of the neurons in cerebellum are granule neurons, and they make up the largest homogenous neuronal population of the mammalian brain (Contestabile 2002). Cerebellar granule cells (CGCs) develop postnatally, making them a good source of primary neurons to be used *in vitro* for the study of cell and molecular mechanisms of neurodegeneration. Typically the cerebellum was not thought to be affected by Alzheimer's disease (AD), but morphometric analysis of 11 subjects who died in the final stages of Alzheimer's disease demonstrated atrophy of the molecular and granule layers of the cerebellum,

which correlated with a 30% reduction of granule cells (Wegiel et al. 1999). This may suggest that cerebellar pathways play a role in AD.

The cerebellum is composed of three distinct layers (Fig. 1.2). At the bottom lies the granule cell layer, densely packed with granule cells along with golgi interneuron's. The middle layer, called the Purkinje layer, consists of a single row of Purkinje cell bodies. Finally the outermost layer, called the molecular layer, is composed of the branching Purkinje cell dendrites positioned at right-angles to parallel fibres and basket cell interneuron's. Excitatory information is transferred to Purkinje cells in the molecular layer from climbing fibres (originating from the inferior olive), and to granule neurons in the granule layer by mossy fibres (arising from cerebral cortex, spinal cord and vestibular system) (Purves et al. 2012).

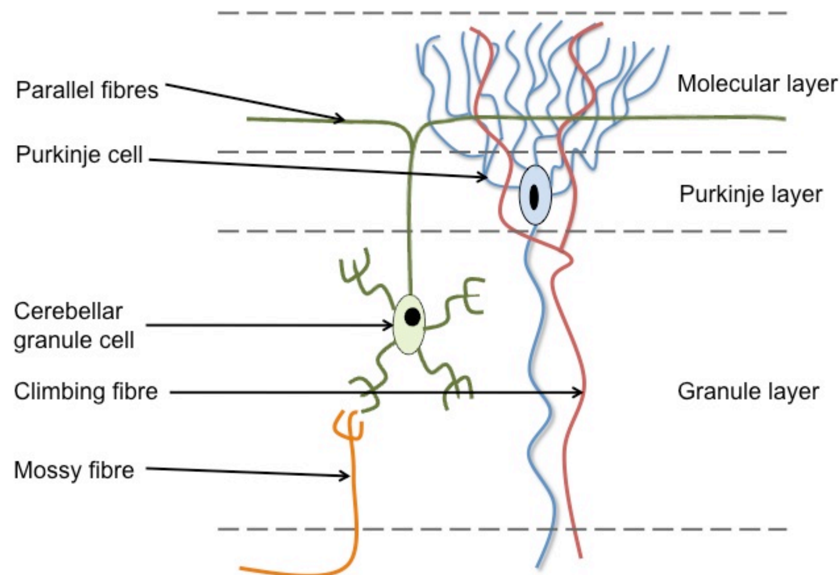


Figure 0.2: Circuitry of the cerebellum.
A longitudinal representation of the three distinct layers of the cerebellum shows the molecular layer, consisting of the dendritic projections of the purkinje cells, the purkinje layer, containing the purkinje cell bodies and the granule layer, containing the cerebellar granule cells. The mossy fibres provide excitatory signals for the granule cells and the excitatory signal from the climbing fibres are propagated into the dendrites of the purkinje cells.

1.1.3 Microglia

Origin

Microglia are a distinct population of cell in the CNS that were first described by the Spanish neuroanatomist, del Rio-Hortega in 1932 and represent 20% of the total glial population in the CNS. As previously mentioned, the 1:1 ratio of neuronal to glial cells in the brain would therefore suggest that that 10% of all brain cells are microglia (Azevedo et al. 2009). Microglia are the innate immune cells of the CNS and are derived from primitive haematopoiesis in the foetal yolk sac and migrate into the CNS at embryonic day 10.5 where by contrast, the macrophage lineage is derived from definitive haematopoises in the bone marrow (Ginhoux et al. 2010; Saijo & Glass 2011). In the presence of CNS insult, a large increase in microglia population is observed known as microgliosis (Reitz et al. 2012; Fellner et al. 2011), which in the past was thought to be partly supplied from bone-marrow derived progenitors through an impaired blood-brain barrier caused by neuroinflammation (Djukic et al. 2006; Flügel et al. 2001; King et al. 2009). This is argued however, by other studies that suggest the increased numbers of microglia are as a result of local proliferation (Lawson et al. 1992; Ajami et al. 2007).

Phenotype

<u>Ramified microglia</u>

"Resting" or ramified microglia are capable of extending their processes to analyse their surrounding environment allowing a fast response to pathogens or tissue

injury (Wilkinson & Landreth 2006). In this state they have a small soma, thin and highly branched, constantly moving processes that extend around to survey the surrounding area. Studies using *in vivo* two-photon laser-scanning microscopy recorded little movement of the soma of the resting microglia in the healthy brain but a continuous extension and retraction of the branched process at a speed that would suggest the brain is thoroughly scanned (Nimmerjahn et al. 2005; Davalos et al. 2005). As described there are no periods of microglial inactivity; it is of the general opinion that microglia move from a surveillance resting state to other states over the course of activation (Kettenmann et al. 2011).

Activated microglia

In response to inflammation these resting microglia transform into an 'activated' state altering the microglia into an ameboid morphology, including an enlargement of the soma, shortening of cellular processes and expression of cell-surface molecules including CD14, major histocompatibility complex (MHC) molecules and chemokine receptors and are able to multiply in number and gather around the site of injury (Verkhratsky & Butt 2007). In this activated state, microglia up-regulate the expression of cell surface activation antigens, pattern recognition receptors and secrete free radicals such as nitric oxide (NO) and reactive oxygen species (ROS) (Meda et al. 2001). In response to injury, microglia also express the immunological receptors such as

complement and cytokine receptors to recruit peripheral immune cells, to influence the pathologic process (Lee et al. 2002).

As close ‘relatives’ to the peripheral monocytes, activated microglia act as the macrophages of the CNS. Microglia play a phagocytic role in the CNS to engulf tissue debris and dead cells in an effort to restore homeostasis. Phagocytosis is the process by which a cell engulfs a solid particle with its cytoplasm, leading to the formation of a phagosome that will be internalized by a form of endocytosis (Kettenmann & Ransom 2013). In this state microglia take on an ameboid morphology with cytoplasmic hypertrophy, shortened or absent processes and cell-surface expression of CD68 (Streit et al. 1999). Triggering microglial phagocytosis is dependent on the particle requiring engulfing. Bacteria stimulate phagocytosis through Toll-like receptor signalling which promotes a pro-inflammatory response, involving IL-1, TNFα and NO production (Ravichandran 2003). Although this reaction is important to successfully eliminate the infectious agent, when exaggerated it can cause damage to the neighbouring tissue in the CNS. Invading microbes are further cleared through antigen presentation on the cell surface via MHC class II molecules, contributing to a recruitment of $CD4^+$ helper T cells (Mantegazza et al. 2013).

Apoptotic neurons can also trigger microglial phagocytosis through firstly release of ‘find-me’ signals to recruit the phagocyte to the proximity of the cell, followed by exposure of ‘eat-me’ signals on the surface of the cell to initiate engulfment (Ravichandran 2010). The dying neuron releases factors such as ATP, ADP and UTP

which mediate microglial chemotaxis and induce their migration towards the apoptotic neuron (Davalos et al. 2005). Once in the vicinity, the apoptotic neuron exposes an 'eat-me' signal, of which phosphatidylserine (PS) is the most universally observed and best studied (Ravichandran 2010). The localization of PS is maintained by an ATP-dependent aminophospholipid translocase (APLT), which can control the transport of PS from the outer to the inner leaflet of the plasma membrane in healthy cells (Zachowski et al. 1986). In a dying neuron, PS is translocated to the outer leaflet. The mechanism by which it does this is not well understood, but it has been associated with a loss of APLT activity and calcium-dependent movement of phospholipids across the membrane (Bratton 1997). PS is recognised by receptors on the phagocyte and it can bound directly by members of the TIM family; 1,3 and 4 (DeKruyff et al. 2010; Rodriguez-Manzanet et al. 2010; Wong et al. 2010), brain angiogenesis inhibitor 1 (Park et al. 2007) or Stabilin-2 (Park et al. 2008). It can also be bound indirectly via soluble bridging molecules such as milk fat globule EGF factor-8 (MGF-E8) to $\alpha_v\beta_3$ integrins on the phagocyte (Hanayama et al. 2002; Hanayama et al. 2004), Gas 6 and Protein S to Tyro-3-Axl-Mer family of receptors (Scott et al. 2001; Rothlin et al. 2007). Microglial phagocytosis of apoptotic neurons is not associated with a pro-inflammatory response but it is met with the secretion of anti-inflammatory cytokines transforming growth factor-β (TGFβ) and interleukin-10 (IL-10) (Savill et al. 2002) which is ideal in not provoking any further tissue damage.

1.2 Microglial mediators of neurotoxicity and neuroprotection

1.2.1 Cytokines

Cytokines comprise a diverse group of small polypeptides ranging between 8-30 kDa in size, which work as signalling molecules to assist in the execution of cellular activities. Cytokine production is rapidly upregulated in response to tissue injury, infection or inflammation due to their important role in tissue repair (Smith et al. 2012). Depending on the action carried out in the peripheral tissue, they can be grouped as either pro-inflammatory or anti-inflammatory cytokines. As mentioned, microglia are constantly surveying their environment and react fast to signs of tissue injury or pathogen infiltration, and cytokine production is one of the microglial responses. These include interferons (IFN), interleukins (IL), tumour necrosis factors (TNF), chemokines and growth factors (Allan & Rothwell 2001). One of the most potent stimuli for release of cytokines such as TNF-α, IL-1β and Il-6 from microglia is bacterial endotoxin lipopolysaccharide (LPS), which is derived from the outer cell wall of gram-negative bacteria (Olson & Miller 2004) and such cytokine release is mediated by mitogen-activated protein kinases (MAPKs), protein tyrosine kinases and transcription factors such as nuclear factor $_{K}$B ($NF_{K}B$) (Qin et al. 2005). Chronic microglial production of IL-1, IL-6, TNFα and IFNγ have received considerable attention recently for their roles in neurodegeneration (Panitch et al. 1987; Hüll, Strauss, et al. 1996; Collins et al. 2000; Simi et al. 2007; Shaftel et al. 2008). Cytokine production does not entirely depend on microglia though, as astrocytes are implicated as another source of cytokine production involved in CNS insult (Li et al. 2011). An association between

microglial cytokine production and AD has been supported by a number of studies ranging from post-mortem identification of pro-inflammatory cytokines near AD lesion sites (Griffin et al. 1995; Hüll, Berger, et al. 1996) to genetic studies linking polymorphisms in genes encoding TNFα and IL-1β to disease progression (Di Bona et al. 2008; Di Bona et al. 2009).

1.2.2 Chemokines

Chemokines belong to a highly conserved superfamily of secreted proteins of about 8-14 kDa in size that can be classified into 4 main subfamilies: CXC, CC, CX3C and XC. They are small cytokines that bind to G protein-coupled receptors causing conformational changes such as the separation of the G protein heterodimer into its α and βγ subunits initiating several parallel signalling pathways. 6 of the 40 chemokine receptors have been identified in the human CNS (CCR2, CCR3, CCR5, CXCR2, CXCR3 and CXCR4) with microglia among the cell types that have been identified as a source of chemokines in the brain (Cartier et al. 2005). They can be classified by their function as either inflammatory or homeostatic chemokines, with expression of inflammatory chemokines attracting immune cells to the site of inflammation in response to pro-inflammatory cytokines, and constitutively produced homeostatic chemokines involved controlling the migration of cells in normal tissue maintenance or development (Sallusto et al. 2000). Chemokine receptors and an increased presence of their ligands have been detected in a number of brain regions such as the hippocampus, cortex, cerebellum and striatum in the AD brain with a couple (CCR3 and

CCR5) exhibiting an elevated expression on reactive microglia (Xia et al. 1998; Cartier et al. 2005).

1.2.3 Neurotrophins

Microglia are also a source of neurotrophic factors which are a family of proteins including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophins 3 and 4 (NT-3/4) that all bind separately to specific tyrosine kinase receptors (Trks) to enhance neuronal survival (Hempstead 2006; Allen et al. 2011). Since the 1980's the Neurotrophic Factor Hypothesis has linked NGF and BDNF to AD with studies demonstrating an increase measurement of NGF in the AD brain (Crutcher et al. 1993; Scott et al. 1995) and neuroprotective effects of BDNF in animal models of AD (Nagahara et al. 2009).

1.2.4 Nitric oxide

Nitric oxide (NO) is a small signalling molecule synthesized by a group of enzymes known as nitric oxide synthases (NOS), of which there are four: neuronal NOS (nNOS) and it's isoform mitochondrial NOS (mtNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Guix et al. 2005). NOS catalyzes the synthesis of NO from L-arginine in the presence of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) with citrulline as a co-product.

NOS

nNOS and eNOS are Ca^{2+}calmodulin-dependent constitutively expressed enzymes with short-lasting release of picomoles of NO but iNOS is Ca^{2+}calmodulin-independent with long-lasting release of nanomoles of NO (Alderton et al. 2001). Although not typically expressed in the brain following immunological insult or inflammatory stimulation, iNOS is expressed in microglia (Murphy 2000). NO has a number of physiological roles from diffusion and signalling to cytotoxic effects. iNOS mediated NO production causes toxicity through inactivation of the mitochondrial respiratory complex I causing apoptosis in the target cell (Brown & Borutaite 2004). NO has been reported to stimulate cyclooxygenase-2 (COX-2), an inducible cyclooxygenase (COX) isoenzyme thought to be involved in the pathogenesis of Alzheimer’s disease (Shin et al. 2007), through an increase in the production of pro-inflammatory prostaglandins (Salvemini et al. 1993). NO’s reported toxic effects are however not likely to be mediated by NO directly, they are more inclined to be due to it’s oxidation products (Dimmeler et al. 1992; Mohr et al. 1994). Evidence suggests that it is the reaction of NO with another free radical, the superoxide anion ($O_2^{\cdot-}$), to produce peroxynitrite ($ONOO^-$) can be attributed for most of the cellular damage (Pacher et al. 2007).

1.2.5 Superoxide

Superoxide is a compound that contains $O_2^{\cdot -}$, one of the reactive oxygen species (ROS). $O_2^{\cdot -}$ is produced from molecular oxygen O_2 in the presence of NADPH oxidase, COX, xanthine oxidase (XO) and NOS, and is a highly reactive species with the potential to damage cells. Microglia express three isoforms of NADPH oxidase, Nox1, Nox2 and Nox4, therefore triggering the production of $O_2^{\cdot -}$ in microglia (Mead et al. 2012a). There are also antioxidant mechanisms in place such as superoxide dismutase (SOD), which catalyses the dismutation of $O_2^{\cdot -}$ into O_2 and hydrogen peroxide (H_2O_2) which is further catalyzed into water and oxygen by catalase and glutathione (Kamata & Hirata 1999; Klabunde 2011) (Fig. 1.3).

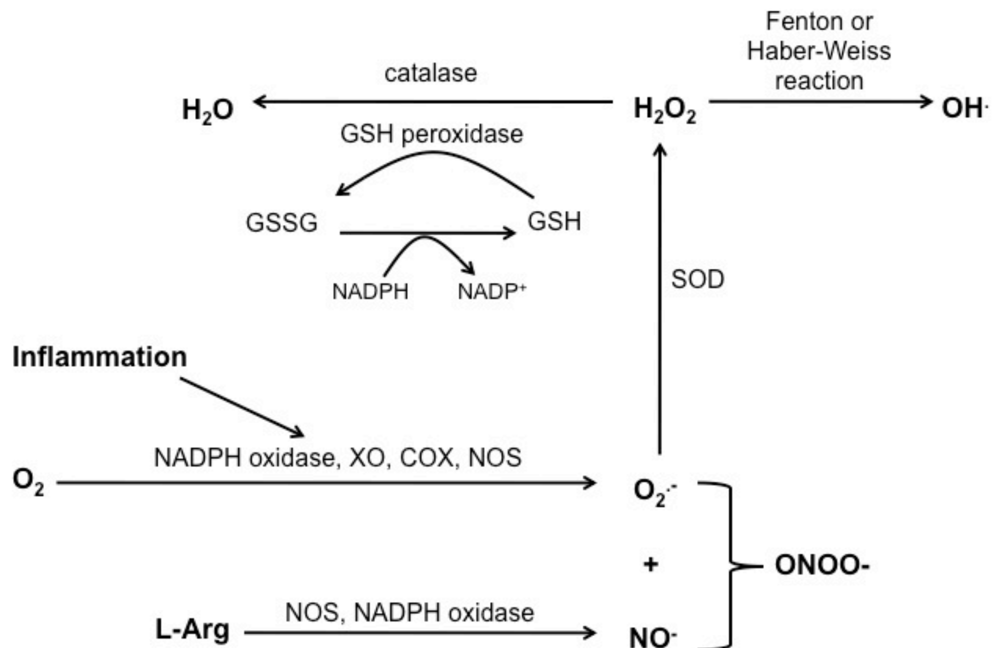


Figure 0.3: Metabolic pathways of reactive oxygen species.
Reactive oxygen species (ROS), such as $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$ are generated through several pathways in the cell. Nitric oxide synthase (NOS) catalyses the conversion of L-arginine (L-Arg) to nitric oxide (NO) in the presence of NADPH oxidase. NADPH oxidase, xanthine oxidase (XO), cycloxygenase (COX) and NOS contribute to the generation of the superoxide anion ($O_2^{\cdot -}$) from molecular oxygen (O_2). The products of these two reactions, $O_{2.}^{-}$ and NO^{-} can react to form highly toxic peroxynitrite ($ONOO^{-}$). Superoxide dismutase (SOD) converts $O_{2.}^{-}$ into hydrogen peroxide (H_2O_2) which is mostly degraded to water (H_2O) by catalase and glutathione (GSH) peroxidase. H_2O_2 also produces a highly reactive radical, $OH^{\cdot}$ by the Fenton or Haber-Weiss reactions.

As previously mentioned $O_2^{\cdot-}$ quickly binds to NO which also has an unpaired electron to form the highly toxic $ONOO^-$. Peroxynitrite oxidises proteins and other macromolecules and also causes nitration of tyrosyl residues in proteins (Torreilles et al. 1999), therefore nitrosyl residue detection was used in a number of studies to investigate a link between oxidative stress and a contribution to AD pathogenesis. Region specific accumulation of these residues, with 5 to 8-fold greater concentrations than in cognitively normal subjects have been seen in the AD brain (Hensley et al. 1998). There has also been evidence for the presence of nitrotyrosine in neurofibrillary tangles (NFTs), one of the hallmarks of AD (Good et al. 1996) further implicating oxidative stress in AD pathogenesis.

1.2.6 Glutathione

Glutathione (GSH) is an anti-oxidant that is produced by the cell to maintain the redox state of the cell. It does this by serving as an electron donor to other unstable molecules such as ROS, thereby converting it to its oxidised form glutathione disulfide (GSSG), of which can be reduced back by glutathione reductase using NADPH as an electron donor (Kamata & Hirata 1999; Couto et al. 2013). GSH eliminates H_2O_2, previously generated from $O_2^{\cdot-}$ by SOD, and converts it to H_2O (Fig. 1.3). This redox balance in the cell is important to maintain the health and survival of the cell. An age-related decline of GSH in rodents, has been found linked to a downregulation of the enzyme required for GSH synthesis, glutamate cysteine ligase (Liu 2002). Together with the significant decrease in GSH observed in red blood cells of male AD patients

suggests a possible contributor of GSH levels with AD pathology (Honglei Liu et al. 2004).

1.3 Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia accounting for 50-60% of all cases (Blennow et al. 2006). According to the World Alzheimer's Report 2010 there are currently 35.6 million people worldwide living with the condition and this is estimated to rise to 65.7 million people by 2030 (Alzheimer's Disease International, 2010). In the United Kingdom alone this presents a huge socioeconomic burden, with AD costing the UK economy £23 billion per year (Alzheimer's Research Trust, 2010).

AD typically involves slow progressive memory degeneration and deterioration of other cognitive domains. Plaques composed of amyloid β (Aβ) and tangles of hyperphospholyated tau are the universally accepted neuropathological hallmarks of the disease (Blennow et al. 2006). Senile plaques are primarily composed of Aβ in its fibrillar form which can lead to a number of pathologies resulting in neuronal dysfunction and neurodegeneration (Deshpande et al. 2006). Aβ undergoes a number of conformational changes prior to fibrillization which can also be toxic at lower nanomolar concentrations (Sakono & Zako 2010) and these soluble Aβ species, consisting of forms such as monomers, oligomers and protofibrils, have been found in the cerebrospinal fluid (CSF) of AD patients (Georganopoulou et al. 2005). It is important to note that different conformations of Aβ can contribute to the

pathogenesis of AD though distinct mechanisms (Deshpande et al. 2006). The formation of Aβ through endopeptidase cleavages (discussed later) of the amyloid precursor protein (APP) result in a number of Aβ species being formed. Aβ species ending at position 40 being are the most abundant ($A\beta_{40}$) but the slightly longer $A\beta_{42}$ is the principle species deposited in the AD brain (Selkoe 2001; Murphy & LeVine 2010). Aside from the C-terminal variants ($A\beta_{40}$ and $A\beta_{42}$), N-terminally truncated species such as pyroGlu-3 Aβ and pyroGlu-11 Aβ, have also been frequently found in the AD brain (Frost et al. 2013). It should also be noted that neurodegeneration has been shown to occur within AD regions: the hippocampus, temporal lobe, frontal lobe and parietal lobe, regardless of Aβ deposition suggesting that other features of AD pathology may be important during the preclinical stages of the disease (Wirth et al. 2013).

Death of the patient typically occurs between 3 to 9 years after diagnosis (Querfurth & LaFerla 2010). Alzheimer's disease can be divided into early-onset AD (EOAD) and late-onset AD (LOAD), a classification that is usually distinguished by the presence of the first disease symptoms before or after the age of 65 respectively (Kowalska 2004). There are both non-genetic and genetic risk factors for the development of AD. Increasing age is the greatest known risk factor for the development of AD, which along with cerebrovascular disease and type II diabetes mellitus have been reported as non-genetic risk factors for the disease (Luchsinger et al. 2001; Lindsay et al. 2002; Reitz & Mayeux 2014).

Familial EOAD is typically inherited in an autosomal dominant fashion and three genes have been found to harbour AD causative mutations: the amyloid precursor protein, *presenilin 1* and *presenilin 2* genes (Bekris et al. 2010). Until recently the genetics of LOAD were not well understood and the ε4 allele of Apolipoprotein E (*ApoE*) was the only confirmed risk factor (Chartier-Harlin et al. 1994). However, recent genome-wide association studies (GWAS) have indicated common genetic variations in *CLU*, *CR1*, TREM2, *PICALM*, *ABCA7*, BIN1, *EPHA1*, *CD33, CD2AP* and the *MS4A* gene cluster as additional risk factors for the development of LOAD (Harold et al. 2009; Lambert et al. 2009; Hollingworth et al. 2011; Naj et al. 2011; Guerreiro et al. 2012). The finding of complement receptor 1 (*CR1*) and *CLU* by GWAS suggests a central role for complement in AD pathogenesis.

1.4 Complement receptor 1 gene

CR1 is found on chromosome 1 at the locus 1q32 in a genetic cluster of complement related proteins (Weis et al. 1987). Membrane-bound complement regulatory proteins have evolved from the same structural and functional domain, the short consensus repeat (SCR), comprised of roughly 60 amino acids each which are further grouped into four long homologous repeats (LHRs) (Jacobson & Weis 2008). Four alleles of different sizes are known and the variation is due to genetic duplications and deletions (Krych-Goldberg et al. 2002). This is a process thought to arise as a result of imprecise chromosome crossing over (Holers et al. 1987). The different alleles are known by different names depending on the group studying them. The smaller alleles

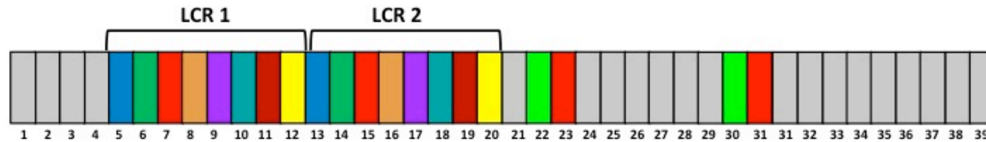
are known in ascending order of size as CR1-C or CR1-F’ (160kDa); CR1-A or CR1-F (190kDa); CR1-B or CR1-S (220kDa); and CR1-D (250kDa).

The relative frequencies of the four alleles vary only slightly between populations (Wong 1990). The most frequent alleles are CR1-A/F and CR1-B/S. In Caucasians these alleles have frequencies of 0.87 and 0.11 respectively. This is different to 0.82 and 0.11 in African Americans, 0.89 and 0.11 in Mexicans (Moulds et al. 1996) and 0.916 and 0.084 in Asian Indians (Katyal et al. 2003). The remaining two alleles, CR1-C/F’ and CR1-D are infrequent in all populations (Eikelenboom & FC C. Stam 1982; Wong et al. 1983; Moulds et al. 1996). The relative scarcity of the smallest and largest alleles is likely due to population selection effects which can confer a survival advantage, such as resistance to *Mycobacterium tuberculosis* (Noumsi et al. 2011).

Although the differences between these alleles seem large, the functional differences in the resultant proteins are apparently subtle. The increased number of LHR regions means that the larger alleles (CR1-B/S and CR1-D) have an additional C3b/C4b binding site (Fig. 1.4). The smaller CR1-C/F’ allele has only one of these binding sites and although the functional implication of this is unclear there seems to be some sort of loss of function associated with this allele (Wong 1990; Liu & Niu 2009). Brouwers *et al*., (2011) showed an association between one of the isoforms of CR1, CR1-B/S, and AD risk. CR1-S contains an extra binding site for C3b/C4b therefore

suggesting increased complement activity and AD risk in these carriers (Brouwers et al. 2011).

A
CR1A/F – L allele



B
CR1B/S – H allele

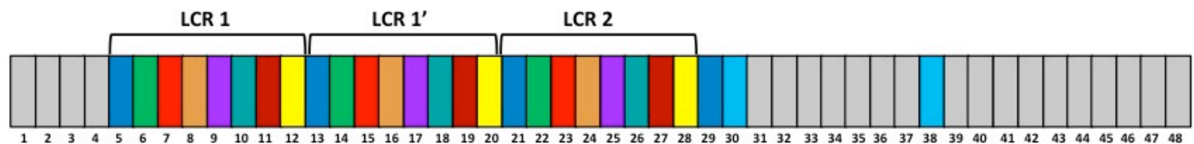


Figure 0.4: Schematic showing the exon structure of the major CR1 alleles.
Analogous exons are represented by the same colour. Previously, different names have been used to describe the major isoforms of CR1. (A) The exons of the CR1-A allele otherwise known as the F isoform or the L allele. This CR1 coding region includes low-copy repeats (LCRs), LCR 1 and LCR 2. (B) The exons of the CR1-B allele otherwise known as the S-isoform, or the H allele. This allele contains the LCRs, LCR1, LCR2 and additional LCR1’.

1.5 Species homology of CR1

Human and mouse CR1 are produced differently however we know that there are similarities between both, such as mouse CR1 containing six N-terminal SCRs which are homologous to 1-6 of human CR1 SCRs. The expression and distribution of CR1 in humans and rodents contrast because human CR1 is encoded by a separate gene to human CR2, but murine CR2 encodes both CR1 and CR2 (Kurtz et al. 1990; Molina et al. 1990). However the protein sequence and function of the murine specific gene, CR1-related protein Y (Crry), displays more likeness to human CR1 than mouse CR1 (Killick et al. 2012). In addition, the structure of rat CR1 may be closer to human CR1 than

mouse as demonstrated in a study using a nucleotide probe for SCR 11-14 of human CR1 which showed that it hybridized to rat CR1 rather than mouse CR1 (Quigg & Sneed 1994).

1.6 The Complement system

The complement system is composed of a series of soluble and membrane-associated proteins present in the blood, which play a role in host defence through the identification, opsonisation and lysis of pathogenic targets (Morgan & Gasque 1996; Yasojima et al. 1999; Dunkelberger & Song 2010). Activation of complement leads to an enzymatic cascade whereby one protein promotes the sequential binding of the following protein (Sarma & Ward 2010). There are three pathways through which complement activation can occur: *classical, lectin* and *alternative* (Fig. 1.5). Although these pathways depend on different binding molecules for their initiation, they all ultimately lead to the production of complement 3 (C3) convertase which is responsible for the actions of complement (Murphy et al. 2008). The initiation of the classical pathway involves the binding of C1q, the first protein in the complement cascade, to an antigen-bound antibody complex (IgG or IgM) to either the pathogen surface or to the C-reactive protein bound to the pathogen leading to the generation of the protease C3 convertase through C4 and C2 cleavage (Dunkelberger & Song 2010). Lectin pathway activation involves carbohydrate binding proteins such as mannose-binding lectin (MBL) or Ficolin binding to carbohydrate elements present on

the surface of pathogens, further leading to the production of C3 convertase (Sørensen et al. 2005).

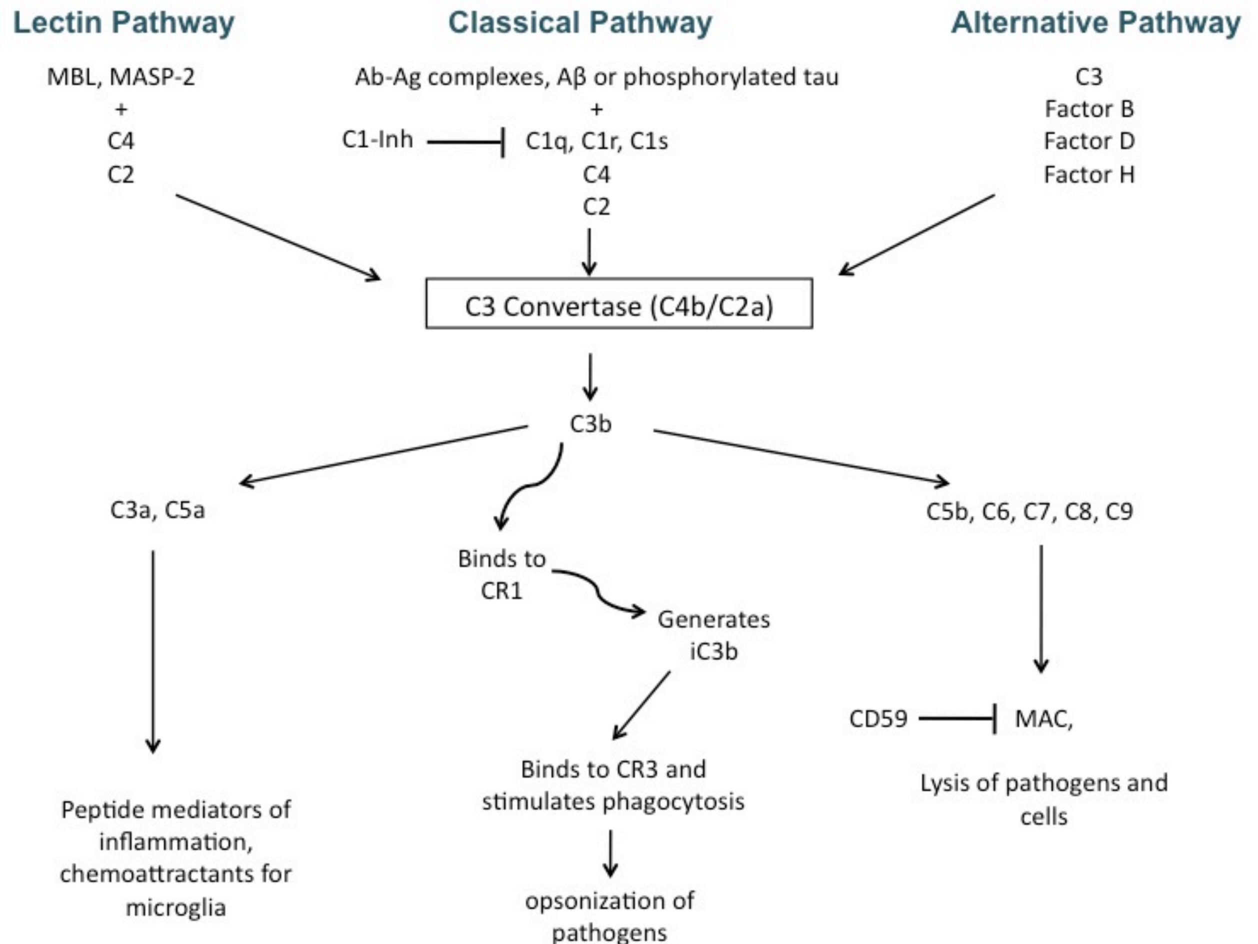


Figure 0.5: Pathways activating and inhibiting complement.

The three complement activation pathways converge at the formation of the enzyme C3 convertase (or C4b/C2a), activation of which leads to the formation of C3b, the ligand for complement receptor 1 (CR1, also known as CD35). Activation of the complement pathways can ultimately lead to the release of inflammatory mediators, opsonisation of pathogens, and the membrane attack complex (MAC). The C1 complex of the classical complement pathway is comprised of C1q, C1r and C1s. The endogenous complement C1 inhibitor/C1-esterase inhibitor (C1-Inh), which regulates the activation of the C1 complex, is decreased in AD. C5b, C6, C7, C8 and C9 form the MAC complex in the alternative complement activation pathway. CD59, an endogenous regulator of the MAC complex, is decreased in AD whilst C9 may be increased. Levels of Factor H, a regulatory glycoprotein of the alternative complement cascade, may also be perturbed in AD.

The third pathway, the alternative pathway, is different in that there is a constant low level of activation due to the spontaneous hydrolysis of C3 to C3(H_2O) and this forms C3 convertase through the cleavage of Factor B by Factor D and positively and negatively regulated by the protein properdin and factor H (Qu et al. 2009). C3 convertase, the protease formed by all three complement pathways, further binds to the pathogen surface to cleave C3, generating C3b which serves as a ligand for complement receptor 1 (CR1) (Liu & Niu 2009). CR1 does not have enzymatic activity, however, along with factor-H, a co-factor for plasma factor I, it is able to convert C3b to iC3b (Tuveson et al. 1991; Holers 2014).

1.7 Complement and Alzheimer's disease

Although the recruitment of phagocytes and the destruction of pathogenic targets by complement activation are in the main beneficial, under certain conditions, these processes may prove harmful. Classical markers of immune-mediated damage have been identified in AD brains including major histocompatability complex class I (MHC-I) and II (MHC-II) positive microglia (McGeer et al. 1987; Tooyama 1990; Perlmutter et al. 1992), glial cells expressing inflammatory cytokines (Griffin et al. 1989) and the acute phase protein α1-antichymotrypsin (Abraham 1988). Early studies identified complement proteins of the classical pathway in AD brains (Eikelenboom & FC C. Stam 1982) and subsequent studies established the presence of all of the native complement proteins as well as their activation products C4d, C3d, and the membrane

attack complex (MAC) in AD brain (Yasojima et al. 1999). However, the lack of classical immune complexes, lead to the search for other complement activators. Rogers *et al*., (1992) demonstrated that Aβ was able to bind and activate C1q, the classical complement cytolytic pathway, and that this occurred in the absence of antibody, and, furthermore, that such complement activation was localised to areas of AD pathology. C1q was subsequently shown to be intimately associated with Aβ plaques (Afagh et al. 1996). Studies have shown that $Aβ_{42}$ can bind to erythrocyte CR1 through a C3b-dependent mechanism suggesting that there may be peripheral clearance of Aβ occuring in the body (Rogers et al. 2006). Although the primarily identified function of CR1 on erythrocytes is to bind complement tagged particles for their removal through the liver and spleen, it has been shown that PDZ domains in CR1's cytoplasmic tail bind to PDZ domains of the scafolding protein Fas-associated phosphate 1 (FAP-1), suggesting a means by which CR1 can interact with the cytoskeleton (Ghiran et al. 2008a). The search for antibody-independent activators of the complement pathway continued with the investigation of tau, the primary protein component of neurofibrillary tangles. Shen *et al*., (2001) demonstrated complement activation by neurofibrillary tangle material extracted from AD brains, and furthermore by human recombinant tau. The ability of Aβ plaques and neurofibrilliary tangles, classical hallmarks of AD, to activate the complement pathway may contribute towards an explanation for low levels of inflammation observed throughout the disease course. Although most research on complement activation in AD has focussed on the classical pathway, alternative pathway activation also occurs, since the presence of mRNA of

the essential alternative pathway element, factor B, has been observed in the frontal cortex of the AD brain (Strohmeyer 2000).

1.7.1 Membrane attack complex

The membrane attack complex (MAC) is a macromolecular complex consisting of a number of complement components; C5b, C6, C7, C8 and several C9 molecules whose function is to allow the influx of Ca^{+} though its ring-like structure resulting in lysis of the target cell (Kim et al. 1987). Most cells express complement defence 59 (CD59) and this provides protection against MAC as the glycophosphinositol (GPI)-anchored membrane protein prevents the complete assembly and insertion of the complex into the membrane (Meri et al. 1990). The hippocampus and frontal cortex of AD patients display significantly less CD59 expression compared with non-demented control brains (Yang et al. 2000) although evidence for changes in CD59 expression in AD remain inconclusive(McGeer et al. 1989; McGeer et al. 1991).

1.7.2 C1q

C1q is the initiating protein of the classical complement cascade that is known to be expressed in AD brains (Eikelenboom & FC C. Stam 1982; Afagh et al. 1996). The role of C1q in AD has been experimentally addressed in studies using animal models deficient in the protein. One such study involved the crossing of C1q-deficient mice with a Tg2576 mouse model which exhibits an age-dependent increase in Aβ, dystrophic neurites and activated glial cells (microglia and astrocytes) (Fonseca et al.

2004). These authors reported that the number of activated glia surrounding Aβ plaques was lower in the C1q-deficient mice compared with the AD mouse model. In addition there was a reduction in the loss of synaptophysin and MAP2 compared with Tg2576 control mice (Fonseca et al. 2004), leading to the conclusion that C1q may have a harmful effect on the integrity of the neuron through initiating an inflammatory response. C1q-deficient mice also exhibited reduced retinal synapse elimination in mouse models of glaucoma, leading to proposals that C1q mediates synapse loss in other neurodegenerative diseases (Stevens et al. 2007). Sárvári *et al.*, (2003), investigating the effects on hippocampal cells of the C1 complex inhibitor, C1-Inh, showed that inhibition of C1q protected hippocampal cells from Aβ-induced complement lysis. Neurons in the hippocampus and in the cortex are more vulnerable to complement-mediated damage as they are low in the complement inhibitors which usually protect host tissue from complement lysis (Singhrao et al. 2000), but conversely are abundant sources of complement (Terai et al. 1997). Since these are the two brain areas which correlate with AD pathology, this may explain why analysis of CSF of AD patients indicated significantly lower C1q levels compared with control CSF and decreased levels of C1q correlate with a diminished cognitive function (Smyth et al. 1994).

1.8 Complement sources and functions

Complement can enter the brain via a compromised blood-brain barrier (BBB). Increasing evidence suggests BBB dysfunction is an early event in AD (Gay & Esiri 1991;

Alafuzoff et al. 1987; Claudio 1995; Fiala et al. 2002; Minagar & Alexander 2003; Bowman & Quinn 2008). This may potentiate the triggering of detrimental brain parenchymal signalling cascades by blood components including serum factor proteins such as albumin and fibrinogen (Hooper et al. 2009; Piers et al. 2011), as well as complement. Additionally the presence of MAC component mRNA and proteins in the AD brain has been reported, suggesting a possible CNS origin of synthesis (Shen, Li, et al. 1997; Van Beek et al. 2000; Walker et al. 1995). Neurons are an abundant source of complement proteins (Terai et al. 1997), and the expression of complement protein mRNA has been observed on murine astrocytes and microglia (Davoust et al. 1999) and in post-mortem derived human CNS microglia (Walker et al. 1995). Furthermore, astrocytoma cell lines, astrocytes, and oligodendroglial cells have also been shown *in vivo* and *in vitro* to produce complement proteins, indicating glial cells as another potential source of complement within the brain (Gasque et al. 1993; Walker et al. 1998; Hosokawa et al. 2003; Rutar et al. 2011).

The role of complement in the elimination of pathogens by phagocytic recruitment and opsonisation occurs through binding with complement receptors (Frank & Fries 1991; McGreal & Gasque 2002).

1.9 Microglia and Alzheimer's disease

Microglia, resident in normal brain as sentinel cells (Raivich 2005; Vilhardt 2005; Kettenmann et al. 2011), become reactive in AD (Pocock et al. 2002). In AD, microglia

surround damaged or dead cells, clear cellular debris and predominate around Aβ plaques (Fetler & Amigorena 2005). Microglia proliferate around neurons prior to their loss in murine models of AD (Fuhrmann et al. 2010). A positron emission tomography (PET) study detecting both activated microglia and an increase in amyloid load, correlated the increase in activated microglia with cognitive impairment (Edison et al. 2008). Microglia in animal models of AD show reactivity before obvious amyloid plaque deposition (Rodríguez et al. 2010), indicating an early, "silent " (preclinical and asymptomatic) response of microglia may occur in AD by as yet unconfirmed triggers. These may include amyloid oligomers and hypoperfusion (Ferretti & Cuello 2011; Huang et al. 2002), but also complement. Complement activation and activated microglia are early neuropathogical events in AD brains (Eikelenboom et al. 2012), and microglial responses show similarity to the peripheral immune system reaction of the macrophage. Activation products of the early complement components C1, C4, and C3 are found within neuritic plaques but there is little evidence of late complement components C7 and C9 or of MAC in the neuropathological lesions in AD brains (Eikelenboom & Veerhuis 1996). This finding leads to the suggestion that in AD the complement system does not act as an inflammatory mediator through MAC formation, but through the actions of the early complement products which fuel the inflammatory responses, leading to neurotoxicity (Eikelenboom & Veerhuis 1996).

It is thus increasingly the accepted dogma that inflammation can actively cause neuronal damage and ultimately death of the neuron ultimately driving the

progression of AD (Block & Hong 2005; McGeer & McGeer 2013). Recent findings that the responsiveness of the innate immune system is higher in offspring with a parental history of late-onset AD, indicates heritable traits for AD that are related to inflammatory processes (Eikelenboom et al. 2011). Furthermore the correlation of higher serum levels of certain acute-phase proteins with cognitive decline or dementia, provide additional evidence for the early involvement of inflammation in AD pathogenesis (Eikelenboom et al. 2011).

Microglial reactivity is generally beneficial but the prolonged and progressive nature of the microglial response in AD can promote neurodegeneration. Pathogenic input to microglia, including the enhanced deposition of Aβ peptides, can result in the production of excessive free radicals, pro-inflammatory cytokines, complement proteins and glutamate (Davenport et al. 2010; Morales et al. 2010). Amyloid precursor protein (APP) is an integral membrane protein whose cleavage by two enzymes, β- and γ-secretase, results in the formation of Aβ (Velliquette et al. 2005) which can behave as a microglial activator (Barger & Harmon 1997). Additional evidence supporting the view that microglia are active mediators of AD includes the use of synthetic Aβ in the investigation of microglia-plaque interactions. The 10-16 amino-acid domain of human Aβ is critical for microglial activation and binding (Giulian et al. 1996). A number of receptors have been implicated as essential for microglial-Aβ interaction, including CD14 which is required for the recognition and destruction of Aβ damaged neurons by microglia (Bate et al. 2004), and a cell surface receptor complex

comprised of the scavenger receptor CD36, α6-β1 integrin and CD47 (Koenigsknecht & Landreth 2004). This complex is responsible for the induction of phagocytosis and subsequent internalisation of Aβ through a mechanism distinct from the classical phagocytic mechanisms (Koenigsknecht & Landreth 2004).

Consequences of the attenuation of inflammation in AD are seen clearly in animal studies. Craft et al., (2004) demonstrated that inhibition of glial inflammation in an animal AD model resulted in reduced neurotoxicity. Advanced glycation end product (AGE) accumulation is accelerated in AD as it accumulates on plaques, and AGE-positive neurons and glia both increase with age and dramatically so with AD progression (Lüth et al. 2005). Activation of the receptor for AGE, (RAGE), on microglia with one of its ligands, such as AGE or Aβ, results in the release of pro-inflammatory mediators (free-radicals and cytokines) (Berbaum et al. 2008). It has even been shown that a combination of both these ligands (AGE and Aβ) can lead to an enhanced microglial inflammatory response (Gasic-Milenkovic et al. 2003). These data taken together with similar findings have promoted the search for therapeutics designed to modify these pathways. Presenilins function as part of the γ-secretase protein complex which together with β-secretase, cleaves APP resulting in the formation of Aβ (Cole & Vassar 2008). One of the major causes of AD is mutations in presenilins 1 and 2.The loss of presenilin function in presenilin-conditional knockout mice resulted in an up-regulation in inflammatory markers, and including C1q, suggesting that presenilins may control complement mediated inflammation (Beglopoulos et al. 2004).

1.10 Aims and Objectives

Microglia are increasingly recognised as important mediators in AD, playing opposing roles in the pathogenesis of the disease both eliminating β-amyloid aggregates via phagocytosis and causing inflammation and as such releasing neurotoxic factors, thereby killing neighbouring neurons (Cameron & Landreth 2010; Krabbe et al. 2013). The recent genome-wide association studies linking polymorphisms of CR1 to AD (Lambert et al. 2009; Carrasquillo et al. 2010; Corneveaux et al. 2010; Zhang et al. 2010) have directed this research to investigate its possible contribution to AD disease pathogenesis. Here, our main aim is to try and elucidate the roles of CR1 signalling and how it is has an influence on microglial phenotype following activation with neuroinflammatory markers. This will be performed using basic *in vitro* culture models with pharmacologic manipulation where required. This thesis also aimed to address the question of whether CR1 expression showed correlation in the human brain and this was performed through analysis of human post-mortem brain tissue from AD patients. By the end of this thesis it is hoped that the following questions will have been answered:

- Does CR1 play a role in microglial phagocytosis during a neuroinflammatory state?
- Can modulating CR1 affect the neurotoxic role of microglia in neuroinflammation?
- Can microglial CR1 influence neuronal toxicity?
- Does CR1 expression vary within regions of the human AD brain and does it correlate to Aβ plaque burden.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Animals and Materials

Sprague Dawley rats were bred and reared in house from stock animals from Charles River UK Ltd. (Kent, UK). All chemicals were purchased from Sigma-Aldrich (Dorset, UK), unless otherwise stated. Foetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium (MEM) were obtained from Invitrogen (Paisley, UK). Tissue culture plastic-ware was obtained from Triple Red (Buckinghamshire, UK) and coverslips were obtained from Scientific Laboratory Supplies (Nottingham, UK). Phosphate buffered saline (PBS) powder was purchased from ICN Biomedicals (Maidenhead, UK). Anti-CD35 functional blocking antibody, C3b receptor was from antibodies-online.com (Atlanta, USA), anti-iNOS was from BD Biosciences (Oxford, UK), anti-NeuN was from Millipore (Oxforshire, UK), anti-CD35 antibodies raised against purified human C3bR were from Hycult Biotech (Uden, the Netherlands) and the E11 antibody (ab25) from Abcam (Cambridge, UK). The remaining CR1 antibodies were a kind gift from Dr Anne Nicholson-Weller and Dr Ionita Ghiran (see Table 3). The Aβ antibodies R1282 and 3A1 were both kind gifts from Dr Dennis Selkoe and Dr Brian O'Nuallian respectively (see Table.3). The mouse non-immune IgG antibody was from Abcam (St Louis, MO, USA), rabbit non-immune IgG antibody was from Invitrogen (Paisley, UK), anti-NeuN antibody was from Merck Millipore (Oxfordshire, UK), Cleaved caspase-3 antibody was from Cell Signaling

Technology (MA, USA), dextran beads were from Molecular Probes, Invitrogen (Paisley, UK), β-Amyloid (1-42) HiLyte Fluor™ 488-labeled was from AnaSpec (Fremont, CA, USA), Vectashield mountant for fluorescence was from Vector (Peterborough, UK), enhanced chemiluminesence reagent (ECL) was from Amersham Pharmacia (Buckinghamshire, UK). Alexa-fluor 488 and Cy3 IgGs were from Life Technologies (Paisley, UK). ON-TARGETplus Crry siRNA and Cyclophilin B control siRNA was purchased from Thermo-Biosciences (San Jose, CA, USA). Flow Cytometry reagents and consumables were from Becton Dickinson (BD) Biosciences (San Jose, CA, USA). Quantikine Rat TNFα and IL-1β Immunoassay kits were from R&D Systems (Abingdon, UK). Anti-β-actin and all other reagents were from Sigma (Dorset, UK).

Table 2.1 outlines the compounds used to treat cells, the concentration at which these compounds were used, and the supplier.

Compound	**Description**	**Final Concentration**	**Supplier**
LPS	Microglial activator through binding TLR4	1 µg/mL	Sigma L2762 Serotype: 026:B6 (Dorset, UK)
CR1 blocking antibody Clone: J3D3	Functional CR1 blocking antibody which binds to purified C3b receptor	2 µg/mL	antibodies-online.com ABIN234603 (Germany)
$A\beta_{1-42}$	Activate a tyrosine-kinase based signaling response resulting in production of neurotoxic secretory products, proinflammatory cytokines and ROS	20 nM	Sigma A-9810 (Dorset, UK)
1400W Dihydrochloride	Highly selective inhibitor of inducible nitric oxide synthase (iNOS)	100 µM	Tocris 1415 (Bristol, UK)
Thalidomide	Selective inhibitor of tumor necrosis factor α (TNFα) synthesis	10 µg/mL	Tocris 0652 (Bristol, UK)
Phorbol myristate acetate (PMA)	NADPH oxidase activator	10 ng/mL	Sigma P1505 (Dorset)
apocynin	NADPH oxidase inhibitor	10 µM	Calbiochem 178385 (Nottingham, UK)

Table 2.1. Compounds used for treatment of BV2 cells, primary microglia and CGCs.

2.2 Methods

2.2.1 Primary cell culture preparation and treatment

2.2.1.1 Rat Cerebellar granule neurons

Cerebellar granular cultures (CGC) were prepared as described by (Evans & Pocock 1999), with modifications. Animals were sacrificed through cervical dislocation and decapitation in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom). Cerebellar from five day old Sprague Dawley rat pups were collected on ice into a buffer consisting of 153 mM $Na^{+}Cl$, 4 mM K^{+}, 1.5 mM Mg^{2+}, 139 mM Cl^{-}, 10 mM PO_4^{2-}, 1.5 mM SO_4^{2-}, 14 mM glucose, and 50 µM BSA (pH, 7.4). The cerebellar were chopped finely with a sterile razor blade and collected into the buffer containing 0.5 mg/ml trypsin and incubated for 5 minutes at 37°C with gentle shaking every 1-2 min to catalyse the enzymatic reaction. Neutralization of the extracellular matrix digestion was then performed by addition of 20 mL of a buffer containing 8 µg/mL soybean trypsin inhibitor (SBTI) and 8 U/ml DNAase. Following several inversions to ensure the enzyme was fully neutralised, the suspension was centrifuged at 65 x g for 5 min (Eppendorf Centrifuge 5804R). The supernatant was discarded, and the pellet was triturated until a smooth suspension was formed, using three flamed Pasteur pipettes with progressively narrowed ends, in 3 ml triturating medium (buffer containing 3 mM Mg^{2+} and SO_4^{2-} supplemented with 50 µg/mL SBTI and 50 U/ml DNAase). Perikarya were collected by underlying the supernatant with 5 ml 4% BSA in Ca^{2+}-free Earle's Balanced Salts Solution (EBSS) and centrifuging for 5 min at 100 x g.

The pellet was resuspended in warm CGC medium MEM containing 10% fetal bovine serum, 20 mM KCl, 30 mM D-glucose, 2 mM L-glutamine, 25 mM $NaHCO_4$, 50 U/ml penicillin, 50 µM streptomycin, and 6 µg/ml ampicillin) at a density of 8^6 cells/ml. Cells were plated on 13 mm diameter poly-Dextro-lysine (PDL) -coated coverslips at a density of 8×10^5 per coverslip. Additional CGC medium was not added until at least 1 hour after plating to give time for the perikarya to adhere. After 24 hours, the medium was changed and supplemented with 10 µM cytosine furanoarabinoside (Ara-C) to prevent further glial proliferation. Our laboratory has previously shown that the cultures then contain <5% microglia. Ara-C was added to all CGC cultures used in this thesis. The cultures were kept at 37°C in a humidified atmosphere of 5% CO_2. Cultures were viable for experimentation after 6 days in vitro (DIV).

2.2.1.2 Rat hippocampal neurons

Mixed cultures of hippocampal neurons and glial cells were prepared as described previously with modifications, from 2-4 day old Sprague-Dawley rat pups (Vaarmann et al. 2010). Animals were sacrificed through cervical dislocation and decapitation in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom). Hippocampi were removed into ice-cold phosphate buffered saline (PBS; in mM: 140 NaCl, 5 KCl, 25 Na_2HPO_4, 2.9 $Na_2HPO_4.2H_20$, 11 glucose, plus 0.2% bovine serum albumin (BSA), pH 7.4). The tissue was chopped finely with a sterile razor blade and collected in buffer containing 0.25% trypsin and incubated for 5 min at 37°C in a water-bath with occasional shaking of the tube. The cells were centrifuged at 65 g

for 5 min and washed in 3 ml DMEM with 10% FBS, supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO, Carlsbad, CA). Centrifugation and washing steps were repeated and this was followed by careful tituration using a flame Pasteur pipette until cloudy. The cells were centrifuged at 65 g for 5 min, the supernatant was discarded and the resulting pellet was suspended in 1 ml DMEM. For quantification of cells, 10 µl of the cell suspension was analysed by counting 4 quadrants of a haemocytometer, equating to the number of cells present in 0.1 µl of the suspension volume. Cells were plated on PDL-coated coverslips and cultured in DMEM. Cell density per well was calculated so that the microglia were initially plated in a volume of 100 µl to provide optimum adherence conditions. After 1 h in an incubator at 37°C in a humidified atmosphere with 5% CO_2, culture medium well volume was increased to 500 µl and plates were returned to the incubator and left for approximately 24 h before being gently washed with pre-warmed media to remove debris. Cultures were maintained at 37°C at 5% CO_2 and were viable for experimentation at 7 DIV.

2.2.1.3 Rat microglia

Primary microglia were isolated from 5 day old Sprague Dawley rat pups by a modification of methods previously described (Slepko & Levi 1996; Kingham et al. 1999). Animals were sacrificed through cervical dislocation and decapitation in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom). Brains were transferred into ice-cold phosphate-buffered saline, on ice (137 mM NaCl,

5.37 mM KCl, 5.65 mM $NaH_2PO_4.H_2O$, 13.3 mM $Na_2HPO_4.7H_2O$), supplemented with 11.1 mM D-glucose, 0.02% BSA, 100 units/ml penicillin, 100 µg/ml streptomycin, and 3 µg/ml ampicillin. The solution was made up in filtered, UV-treated dH_2O, pH 7.4. Brains were then gently homogenised using 10 – 12 strokes of a Potter-Elvehjem tissue grinder with a Teflon pestle (VWR, Leicester, UK). The smooth homogenate was split and transferred to two 50 ml tubes (no more than 4 brains per tube) and centrifuged at 500 *g* for 5 min (Eppendorf Centrifuge 5804R). The supernatant was discarded and the pellet was resuspended in 10 ml 70% Percoll. A gradient was then produced by overlaying the cells containing 70% Percoll solution with 10 ml of 30% Percoll solution. Addition of the overlaying 30% solution was done very gently, so as not to disturb the layer below. The Percoll gradient was then centrifuged at 1250 *g* (Eppendorf Centrifuge 5804R) with no acceleration or breaking for 50 min to allow separation of the microglia from the other brain tissue, which could be collected at the 70%/30% Percoll gradient interface.

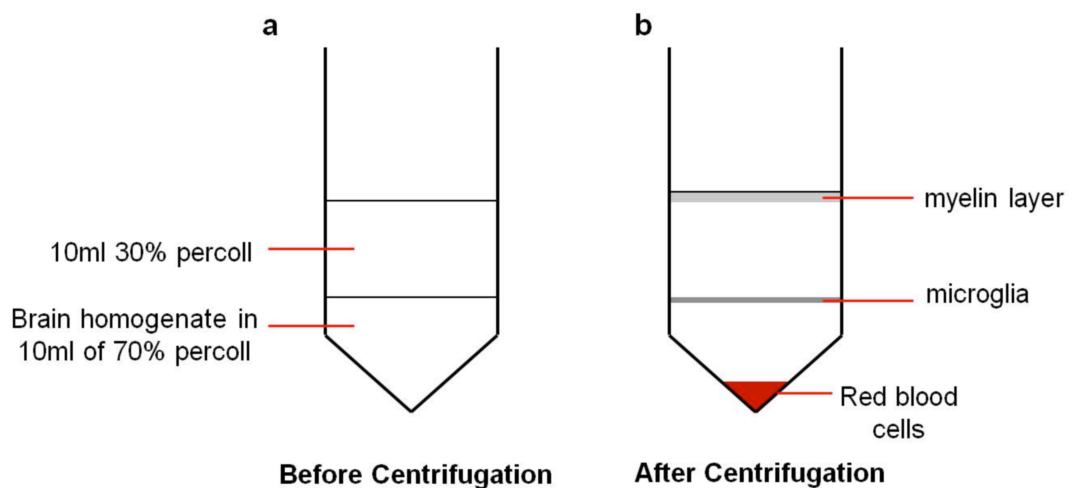


Figure 2.1: Microglial preparation using a percoll gradient.
(a) The brain homogenate is resuspended in 70% Percoll, which is overlaid with 30% percoll. (b) Following centrifugation at 1250 x g for 50 minutes, the myelin layer is removed and microglia are separated from the homogenate and migrate to the 30%/70% Percoll interface.

Microglia were collected and washed in 50ml sterile PBS to allow for the dilution of the Percoll concentration. The solution was then centrifuged at 500 *g* (Eppendorf Centrifuge 5804R) for 5 min. The resulting pellet was resuspended in 1 ml of pre-warmed medium (Minimal Essential Medium - MEM) supplemented with 10% FBS, 20 mM KCl, 30 mM D-glucose, 2 mM L-glutamine, 25 mM $NaHCO_4$, 50 U/ml penicillin, 50 µg/ml streptomycin, and 6 µg/ml ampicillin). As previously described, cells were quantified using a haemocytometer (see hippocampal method).

Microglia were cultured in the same medium as the CGCs cultures to enable microglial conditioned medium (MGCM) to be added to the CGCs as a treatment. It was previously observed in the laboratory that primary microglia did not adhere to tissue culture plastic so cells were always plated on sterile 13 mm glass coverslips at a density of 1 x 10^5/well in 24 well plates. Cell density per well was calculated so that the microglia were initially plated in a volume of 100 µl to provide optimum adherence conditions. After 1 h in an incubator at 37°C in a humidified atmosphere with 5% CO_2, culture medium well volume was increased to 500 µl and plates were returned to the incubator and left for approximately 24 h before being washed 3 times in pre-warmed medium to remove significant debris. Finally, serum-containing (SC) medium was replaced with serum-free (SF) medium (MEM supplemented with 20 mM KCl, 30 mM D-glucose, 2 mM L-glutamine, 25 mM $NaHCO_4$, 50 U/ml penicillin, 50 µg/ml streptomycin, and 6 µg/ml ampicillin), due to observations made previously in the laboratory that the cultures appeared more ramified and responded better to stimuli.

The cultures were left to rest for a further 3 h before treatment. Microglia were used within 48 h of isolation.

2.2.2 BV2 cell culture

The BV2 cell line was a kind gift from Dr Claudie Hooper (MRC Centre for Neurodegenerative Research, Institute of Psychiatry, Kings College London, UK) and was originally obtained from Dr FS Tzeng (Department of Life Sciences, National Cheng Kung University, Taiwan). BV2 cells are a mouse microglial cell line, immortalized by the inclusion of a v-raf/v-myc oncogene carrying retrovirus J2 (Blasi et al. 1990). The cells retain the morphological and phenotypical characteristics of primary microglial cells in culture conditions, and LPS treatment of BV2 microglia induces the up-regulation of inflammatory genes also seen following LPS treatment of primary microglia, suggesting that BV2 microglia respond in the same way as primary microglia to activating stimuli, however BV2 activation is less pronounced (Lund et al. 2006). BV2 microglia were maintained in T175 culture flasks (Appleton Woods, Bucks, UK) in DMEM with 10% FBS, supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cryopreservation of cells was routinely performed to increase longevity of the cell line. The BV2 microglia, at a density of 1 x 10^6/ml were suspended in Sigma freezing medium (DMEM, 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 10% DMSO – to prevent membrane rupture) and stored at -80°C. Once required, a frozen vial of cells was thawed and added to pre-warmed BV2 medium in a T25 culture flask. The cells

were maintained at 37°C in a humidified atmosphere with 5% CO_2 and medium was replaced after 2-3hrs to remove the cytotoxic components of the freezing medium. For experimental procedures and passaging, BV2 microglia were harvested from the culture flasks enzymatically by incubation for 5 minutes at 37°C with ~ 10 ml of 0.5 mg/ml trypsin (1:10 dilution of 5 mg/ml stock; Sigma). Neutralisation of the trypsin was performed by adding 10ml of BV2 medium to the flask. The cells were then pelleted by centrifugation at 3645 g for 5 minutes at room temperature and resuspended in 5 ml of fresh warm medium. As previously described, cells were quantified using a haemocytometer (see hippocampal method). The BV2 cell line was used for Western Blotting and superoxide live imaging (Figs. 3.6 (d), 3.8, 3.9). The cells were routinely plated on 6 well plates at a density of 2 x 10^5/well. Cultures were exposed to SF medium (DMEM, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin), and left to rest for at least 2 hr before treatment.

2.2.3 Cell treatments

2.2.3.1 Direct treatments of cultures

Primary microglia and the BV2 microglial cell line were routinely plated 24 h prior to treatment and the cell medium was changed to serum-free media at least 2 h before treatment to ensure cells were down-regulated and in a resting state. Microglia, BV2s, CGCs and hippocampal neurons were activated with LPS and $A\beta_{1-42}$ alone or in combination with CR1 blocking antibody for 1-48 hrs. The $A\beta_{1-42}$ was previously tested and found to be endotoxin free and after which was pipetted into aliquots and stored

at -80°C. The aggregation state of the peptide is unknown and possible experiments to determine this are discussed in the future work section of this thesis.

CR1 blocking antibody, clone J3D3, is a monoclonal antibody to CD35 (CR1) (tested to be endotoxin free) which has been shown to bind with high affinity to CR1 on erythrocytes, polymorphonuclear leukocytes and lymphocytes to inhibit CR1 mediated decay of cells (Cook et al. 1985). To inhibit TNFα synthesis prior to activation with the above treatments, cultures were pre-treated with Thalidomide 1 h before. Inhibition of iNOS activity was performed in the same manner by pre-treating cultures 30 minutes before activation with 1400W dihydrochloride. Superoxide activation via NADPH oxidase was induced by administration of PMA to LPS cultures for 24 h. Inhibition of this induction was performed by co-treatment of cultures with apocynin.

All compounds with concentrations used to treat cultures are provided in Table 2.1. The concentrations selected are justified in the corresponding result chapters and were determined by concentration dependent analysis, from previous experiments in the laboratory or from the literature.

2.2.3.2 Microglial conditioned medium administration to neuronal cultures

CGCs were treated with MGCM to assess survival following the release of toxic or protective factors from microglia after modulation of CR1, and also to investigate the signalling pathways involved in neuronal survival or death. Microglia were plated in 24 well plates as described and treated as shown in Table 1 for 24 h and the medium

was collected into Eppendorf tubes and placed immediately on ice. MGCM was centrifuged at 15,800 g at 4°C for 5 minutes to remove significant debris and immediately snap frozen with dry ice, followed by storage at -20°C. During the MGCM assays, half of the medium already on the CGCs (250µL) was removed from each well, and replaced with 250µL of MGCM. Only half the media could be removed as CGCs release growth factors which are essential to their survival, therefore removing these fully would have a detrimental effect on neuronal survival and may also increase cell death in a way not attributed to the MGCM (Taylor et al. 2002). Following MGCM treatment, CGCs were imaged to assess cell death by nuclear morphology and propidium iodide staining.

2.2.4 Microglial depletion from neuronal cultures

Following previously described methodology (Morgan et al. 2004) depletion of microglia from CGC cultures was carried out using *L*-leucine-methyl-ester (LME) treatment for 24 h. LME is a lysosomatropic agent which was originally employed to selectively deplete macrophages (Thiele et al. 1983), however a number of research groups have used it to selectively deplete microglia from astrocyte or oligodendrocytes cultures (Giulian et al. 1993; Hewett et al. 1999). The methodology followed previously published studies from this laboratory (Morgan et al. 2004), with has been further adapted in the laboratory using a previously published methodology (Hamby et al. 2006). 25 mM LME had been demonstrated to be the optimum concentration with a significant decrease microglia number, yet no increased death seen in the CGC culture.

At 6 DIV CGCs were treated with 25 mM LME for 1 hr. Initially, LME was dissolved in culture medium, the pH was returned to 7.4 using 1 M sodium hydroxide (NaOH), and filter sterilised. LME concentration dissolved in the medium was equal to twice the concentration required. Half the culture medium was removed from each CGC-containing well and retained at 37°C, followed by administration of double concentrated LME to the CGC-containing wells, i.e. diluted 1:1 giving the desired concentration. The cultures were then incubated at 37°C in a humidified atmosphere with 5% CO_2 for 1 h. LME was removed from the cultures following two washes with pre-warmed CGC medium. The retained cell medium plus an equal volume of fresh CGC medium was then added to the corresponding wells. Finally, cultures were left to rest for 24 h, then fixed and analysed as previously described with IB_4 and DAPI staining. Control cultures were treated the same but CGC medium without LME was used instead.

2.2.5 Phagocytic assay

Treated microglia were incubated with 10 kDa TRITC-conjugated dextran beads (20 µg/mL) for 3 h or with 2 µM fluorescently-labelled HiLyte Fluor 488-$A\beta_{1-42}$ for 2 h at 37°C. Cells were then incubated with 5 µg/mL of the nuclear stain 2'-[epoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazol Hoechst-33342 (Hoechst-33342) for 10 min and then washed in PBS to remove loose beads and bead uptake was visualized by fluorescence microscopy. Fluorescence images were captured using a TRITC filter set on a Zeiss fluoresce microscope plus 40x Neoflaur objective (Zeiss Axioskop 2,

Oberkochen, Germany). A phase contrast image of the same field of view was also captured for quantification of total cell number. Data were collected from at least 3 fields of view per coverslip with each condition repeated on 3 coverslips in 3 separate experiments. Labelled cells were counted as a percentage of total cells per field. Control cells were incubated with beads alone at 37°C for all conditions or at 4°C, the latter as a binding control.

2.2.6 Assessment of apoptosis and cell death

Neuronal death was assessed by nuclear morphology and the incorporation of propidium iodide (PI) into necrotic and late apoptotic nuclei. Apoptosis is programmed cell death which is morphologically characterized by membrane blebbing and bright pyknotic nuclei, due to chromatin condensation (Kerr et al. 1972; Kroemer et al. 2009). These morphological features can be identified in cultures using the nuclear stain Hoechst-33342. This membrane permeable DNA stain binds preferentially to adenine-thymine (A-T) regions of the minor groove in the DNA, and can access the nucleus at all stages of cell survival and death, allowing for assessment of health. The necrotic and late apoptotic marker PI is not membrane permeable, and is only incorporated into the nucleus when the plasma membrane is compromised, which is not seen during early apoptosis and therefore allows for the distinction between the different types of cell death. Nuclear staining was performed on live, unfixed CGCs to determine the effects of both direct treatment and MGCM treatment on neuronal survival. Treated CGC's were incubated with 5 µg/mL PI for 30 min and 5 µg/mL Hoechst-33342 for 20 min as

previously described (Pinteaux-Jones et al. 2008). The apoptotic cells, which stained to show bright pyknotic nuclei and the necrotic cells, which stained red, were observed using the TRITC (red fluorochrome, 543 nm) and DAPI (blue fluorochrome 364 nm) filters on a Zeiss fluoresce microscope plus 40x Neoflaur objective (Zeiss Axioskop 2, Oberkochen, Germany). Cell counts were performed on a minimum of three fields per coverslip, three coverslips per treatment from three independent experiments.

2.2.7 Immunocytochemistry

Treated microglia were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; 154 mM NaCl, 1.84 mM KH_2PO_4, 9.81 mM $K_2HPO_4.3H_2O$, pH adjusted to 7.4 with further addition of $KH_2PO_4/K_2HPO_4.3H_2O$ as necessary) for at least 30 min at room temperature. Cultures were then washed three times with PBS and permeabilized with 100% ice-cold methanol for 20 min at -20°C followed by three further washes with PBS. Cells were then blocked with 4% normal goat serum (NGS) for 30 min at room temperature to combat non-specific binding of the secondary antibody. The choice of block was due to the species the secondary antibody was raised in. Cultures were incubated at 4°C overnight with primary antibody diluted in PBS, anti-iNOS (1:250), NeuN (1:500) or cleaved caspase-3 (1:500) in a dark, humidified atmosphere. Negative controls were performed in all experiments where the PBS was used to replace the primary antibody. Cultures were then washed three times with PBS and incubated at room temperature with the appropriate secondary antibody diluted in PBS for 2 h in the dark. Cultures were washed with PBS in the dark and incubated

with 200 µl of 1:1000 4',6-Diamidino-2-Phenylindole (DAPI) in PBS for 1 minute in the dark at room temperature. Finally cultures were washed once more with PBS, rinsed with ddH_2O and mounted with Vectashield on a glass slide. The edges of each coverslip were sealed to prevent the cells drying out and the slides were stored in the dark at -20°C until viewing on the Zeiss Axio 2 fluorescence microscope.

Antibody (source)	Species	Relevant Reactivity	Target	Dilution
pAb-iNOS (BD transduction lab 610333)	Rabbit	Rat, Human	Inducible nitric oxide synthase	1:250
mAb-NeuN (Millipore MAB377)	Mouse	Rat	Neuronal specific protein	1:500
pAb-Cleaved caspase-3 (Cell Signaling 9661)	Rabbit	Rat, Human	Activated caspase-3	1:500

Table 2.2: Summary of primary antibodies used in ICC experiments.

2.2.8 Superoxide production measurement

There are several compounds that can be used for the measurement of superoxide production however the focus of this study was on the production of intracellular superoxide as a signaling molecule therefore narrowing the method of measurement to a few. In this study, two probes were used to investigate this intracellular superoxide generation: dihydroethidium (DHE), which is oxidized to 2-

hydroxyethidium (2-Oh-E^+) by superoxide, and nitroblue-tetrazolium (NBT), which is reduced from a yellow liquid to a blue formazan precipitate $NBTH_2$ by superoxide. The measurement of superoxide through the use of these probes also carries with it the possibility of production of artifacts and so it is important to conduct measures to reduce false positives. Both methods of measurement used in this study have proven abilities in studying superoxide production, however they too have their negatives. For example, probes that are reduced by superoxide, such as NBT, can lead to false positive results due to possible superoxide production from redox cycling which is caused by the reaction of molecular oxygen with a partially reduced probe (Vásquez-Vivar et al. 1998; Spasojevic et al. 2000). Whereas, DHE, which uses the oxidative activity of superoxide, can also be oxidized by other ROS therefore making this method less specific to superoxide (Dikalov et al. 2007). In this thesis, all experiments were conducted both in the presence of activating stimuli alone and in the presence of the activating stimuli with apocynin, a nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) inhibitor which works by preventing the translocation and assembly of the enzyme thereby preventing the production of superoxide (Stefanska & Pawliczak 2008). Using this inhibitor would therefore reduce the incidence of false positive results.

2.2.9 Superoxide live cell staining using Dihydroethidium (DHE)

DHE is a superoxide sensitive probe used for the detection of intracellular superoxide. It functions by entering the membrane-bound organelles and following oxidation becoming positively charged and oxidized to 2-OH-E^+ which binds to DNA and fluoresces red (Peshavariya et al. 2007). Treated primary microglia or BV2 microglia were assessed for superoxide production by incubation at 37°C for 40 min with DHE (5 µM), and were counterstained with Hoechst-33342 as previously described (Mead et al., 2012). Evidence has shown that this red fluorescence is more readily produced with superoxide generating systems and that superoxide is the only biologically relevant oxidant that can react with DHE to form 2-OH-E^+ which makes this a highly attractive method to measure superoxide (Benov et al. 1998; Robinson et al. 2006; Zielonka et al. 2008). Duplicate conditions were treated with apocynin (10 µM) as previously optimized in our laboratory, to verify superoxide production was via NADPH oxidase activity. Coverslips for superoxide imaging were mounted in saline solution consisting of 153 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM TES, 5 mM $NaHCO_3$, 1.2 mM Na_2SO_4, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5 mM glucose, and observed immediately by fluorescence microscopy using the TRITC (red fluorochrome, 543 nm) and DAPI (to counterstain for total cell numbers) (blue fluorochrome 364 nm) filter sets on a Zeiss fluorescence microscope (Zeiss Axioskop 2, Oberkochen, Germany). The mean cellular fluorescence of approximately 50 cells/field, from three fields per coverslip, three coverslips per condition and from three independent experiments was determined using Image J software.

2.2.10 NBT detection of intracellular superoxide

NBT has long been used for the detection of intracellular superoxide (Baehner et al. 1976). Following exposure of NBT to the superoxide anion ($O_2^{\cdot-}$), it converts from a yellow liquid to a blue formazan precipitate, which can quantify superoxide production by measuring the absorbance of this precipitate at 630 nm (Choi et al. 2006). The NBT assay was optimized from published methodologies (Choi et al. 2006; Mead et al. 2012a). BV2 microglia were plated in 96 well plates at $1x10^4$ cells per well and were grown to 70% confluency before the medium was changed to serum and phenol red free media for 3 h before treatment. Cells were treated with PMA, which is a known activator of the NADPH oxidase by phosphorolation of the p47phox subunit through PKC activation (Tauber et al. 1989; Abramov et al. 2005). Cells were treated with 10 ng/ml PMA (Mead et al. 2012a) or 1 ug/ml LPS (Taylor et al. 2005) in the presence of 10 ug/ml NBT (Choi et al. 2006) for 4 h and 24 h and NBT reduction was measured by absorbance at 630 nm at each time point using a Tecan x flou4 plate reader. It was important that formazan precipitate production in BV2 cells treated with the positive control PMA was increased when compared to basal superoxide production in untreated BV2 cells incubated with NBT only.

2.2.11 Glutathione production measurement

Monochlorobimane (MCB) is extensively used for the detection of glutathione (GSH) in living cells. Following addition to cell medium, intracellular glutathione *S*-transferases form adducts with GSH that can then be fluorescently measured (Kamencic et al. 2000). Unlike other bimanes it has been shown to form an adduct exclusively with GSH (Fernández-Checa & Kaplowitz 1990). Ethracrynic acid (EA) is a potent inhibitor of glutathione *S*-transferases (Ploemen et al. 1993), therefore experiments were conducted both in the absence and presence of the potent inhibitor to reduce false positive results.

2.2.12 Glutathionine live cell staining

Treated primary microglia and BV2 microglia were assessed for glutathione production by incubation at 37°C for 45 min with MCB (5 µg/mL) and counterstained with PI. To confirm glutathione production, cells were incubated with 1 mM EA, a potent inhibitor of glutathione S-transferases for 10 min before and during incubation with MCB as previously described in our laboratory (Hooper et al. 2009). Coverslips for glutathione imaging were mounted in saline solution consisting of 153 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM TES, 5 mM $NaHCO_3$, 1.2 mM Na_2SO_4, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5 mM glucose, and observed immediately by fluorescence microscopy using the FITC (green fluorochrome 488 nm) and DAPI (blue fluorochrome 364 nm) filter sets on a Zeiss fluorescence microscope (Zeiss Axioskop 2, Oberkochen, Germany). The mean cellular fluorescence of approximately 50 cells/field, from three

fields per coverslip, three coverslips per condition and from three independent experiments was determined using Image J software.

2.2.13 Small interfering RNA (siRNA)

Ribonucleic acid (RNA), one of the information macromolecules, has a number of functions in gene coding, decoding, regulation and expression. RNA molecules are able to inhibit gene expression in a process known as RNA interference and small interfering RNA (siRNA) play a pivotal role in this. The mechanism involves the degradation of double-stranded RNA (dsRNA) into short fragments of about 21-25 base pairs in length known as siRNAs, by an enzyme specific to dsRNA called Dicer. After the fragment is unwound into single stranded RNA (ssRNA), the guide strand incorporates into a sequence-specific RNA endonuclease known as RNA induced silencing complex (RISC). This complex then binds to a complementary messenger RNA (mRNA) leading to their degradation and therefore reduced levels of protein translation, subsequently blocking the function of the gene (Strachan & Read 2004).

2.2.13.1 Small interfering RNA transfection

In this study nanoTherics magnet-assisted transfection technology (Magnefect nano II) was used. The system involves mixing biodegradable iron oxides coated with cationic molecules to biomolecules such as siRNA to form a biomolecule/nanoparticle complex, which would be then added to the cells to be transfected. The plate containing the cells and biomolecule/nanoparticle complex is placed on a magnet to

attract the complex to the cells at the bottom (see Fig. 2.2). The oscillation of the magnet array promotes endocytosis into the cell allowing for efficient transfection, avoiding cell membrane disruption.

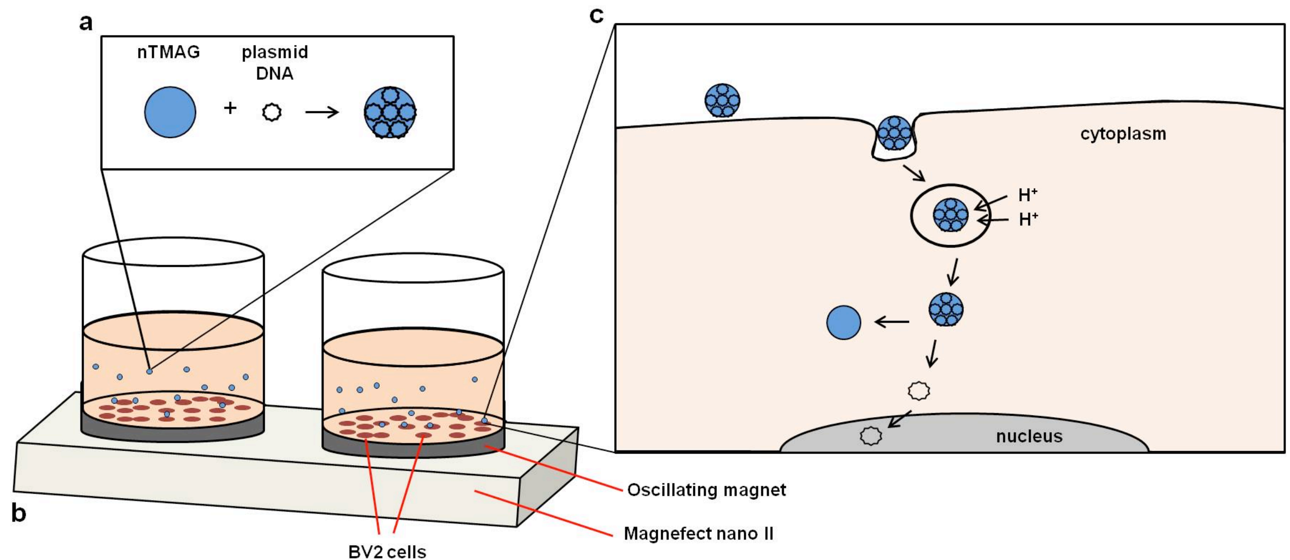


Figure 2.2: Overview of how transfection technology uses magnetic nanoparticles and oscillating magnetic fields to accurately and rapidly transfect DNA into BV2 cells.
(a) Incubation of the DNA with the magnetic nanoparticle coated with a positively charged polymer (nTMAG) in a 1:1 ratio for 30 min with vigorous shaking results in hundreds of plasmids condensing onto each nTMAG. (b) Magnefects-nano II's oscillating magnets pull nTMAG-DNA particle onto the cell surface. (c) The cells are stimulated by the oscillation motion of the particle to initiate endocytosis enhancing uptake of the nTMAG-DNA complex. The polymer coating facilitates the release into the cytoplasm where the DNA can then diffuse into the nucleus. Adapted from Nanotherics website.

2.2.13.2 Optimization

Initially, the transfection was optimized from published methodologies (Fouriki et al. 2010; Fouriki et al. 2012) with transfection of nos2 siRNA. A DNA/T-Mag bead concentration dependent assay (0, 0.5, 1, 1.2µl) was carried out on BV2 microglia plated at a number of densities (2.5 x 10^3 and 5 x 10^4 on 13mm PDL-coated coverslips)

to determine the optimum conditions for the experiment. Further optimization of this experiment was carried out by testing a range of displacement values (0.2, 0.5mm). The optimum conditions determined demonstrated inhibition of cells with the NOS2 siRNA expressing iNOS fluorescence within a favourable cell volume and were found to be: 1.2 µl DNA/T-Mag bead mix at 1:1 ratio at 2Hz and 0.5mm displacement for 1hr and BV2 density at 5 x 10^4 per well of a 24-well plate (Fig. 2.3).

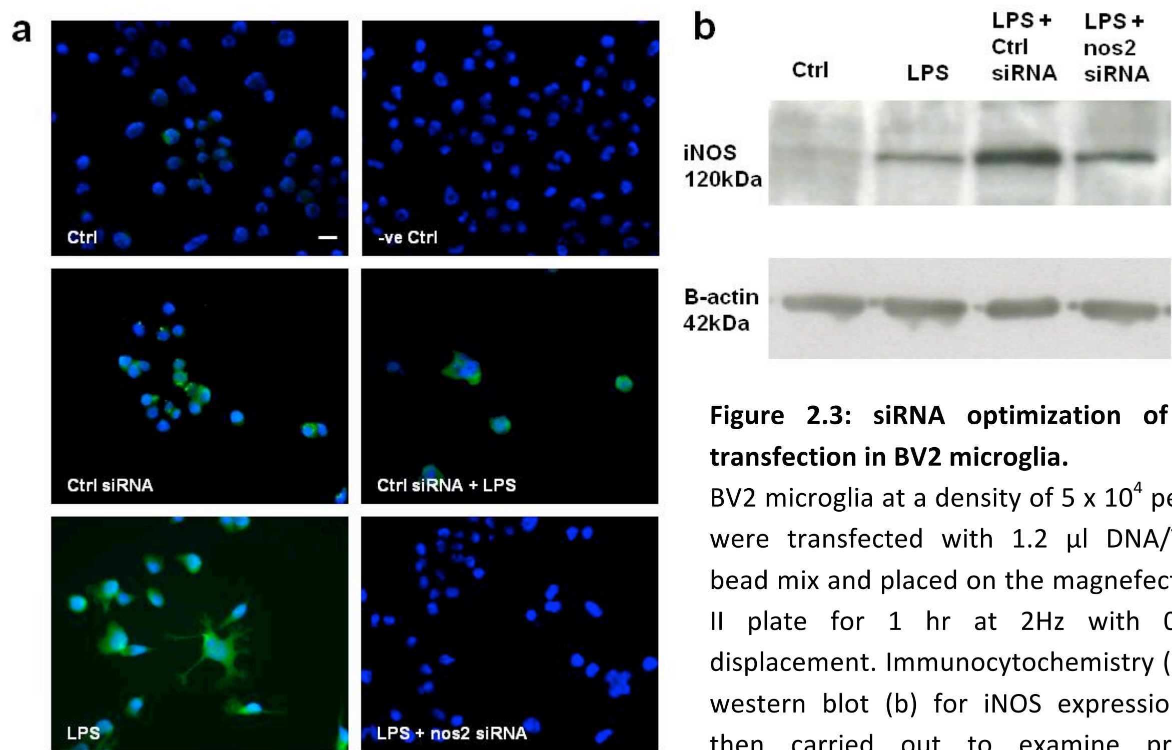


Figure 2.3: siRNA optimization of nos2 transfection in BV2 microglia.
BV2 microglia at a density of 5 x 10^4 per well were transfected with 1.2 µl DNA/T-Mag bead mix and placed on the magnefect nano II plate for 1 hr at 2Hz with 0.5mm displacement. Immunocytochemistry (a) and western blot (b) for iNOS expression was then carried out to examine protocol efficiency.

2.2.13.3 Crry siRNA transfection

BV2 microglia were grown on a 24-well plate at a density of 5 x 10^4 per well in D-MEM supplemented with 10% FBS, penicillin and streptomycin. ON-TARGETplus crry DNA (0.6 µM) (Dharmacon, CO, USA) and Polymag beads (Oz biosciences, Marseille, France) were added in a 1:1 ratio to 200 µl serum-free D-MEM per well and incubated

at room temperature for 30 mins with vigorous shaking. BV2 microglia were transfected with ON-TARGETplus cyclophilin B control siRNA (Dharmacon, CO, USA) as control. The DNA/bead mix was then added to the BV2 microglia and placed on a Magnefect nano II system (nanoTherics, Staffordshire, UK) at 2Hz for 1 h at 0.5 mm displacement in a tissue culture incubator at 37 ^{o}C in a humidified atmosphere with 5% CO_2. BV2 microglia were then incubated at 37 ^{o}C for 12 h prior to treatment with LPS (1 μg/ml) and $A\beta_{1\text{-}42}$ (20 nM) for a further 24 h.

2.2.14 Enzyme linked immunosorbent assays (ELISA) of TNFα, IL-1β and IL-6 release

The enzyme-linked immunosorbent assays (ELISA) is a method that was simultaneously developed by two groups in 1971 which is used for quantification of protein concentration in a given sample (Engvall & Perlmann 1971; Van Weemen & Schuurs 1971). This technique is useful for the quantitative analysis of functional cytokine release. Quantitative sandwich ELISA kits for the measurement of TNFα, IL-1β and IL-6 from R&D Systems were used for the concentration of TNFα, IL-1β and IL-6 released from treated microglia. The sandwich ELISA kit involves a primary monoclonal antibody bound to the surface of the well, which enables the antigen under investigation to bind directly onto the bound antibody (Belanger et al. 1973). The antigen is visualized by a second polyclonal antigen-specific antibody with a HRP conjugation, providing a concentration dependent signal that is amplified and visualized using a plate reader.

Microglia were treated as required and the media was collected from the cultures in sterile Eppendorf tubes centrifuged at 800 g in an Eppendorf 5415R benchtop centrifuge for 5 min at 4°C to pellet debris. Supernatant was removed and placed in fresh Eppendorf tubes and snap frozen on dry-ice and stored at -20°C until required. It is important to promptly prepare and store the sample to prevent significant degradation of the released cytokines.

The assay was performed according to the manufacturer's instructions, in 96 well plates precoated with a monoclonal rat TNFα/IL-1β/IL-6 specific antibody. 50 µl of assay diluents was added to each well followed by 50 µl of each of the internal kit standard rat TNFα/IL-1β/IL-6 samples of known concentrations, TNFα/IL-1β/IL-6 kit controls and media samples. After gentle mixing on an orbital shaker (Medgenix Diagnostics, Fleurus, Belgim) for 1 min at 60rpm, the samples were incubated for 2 h at room temperature followed by five washes with wash buffer. Samples were then incubated with 100 µl of rat TNFα/IL-1β/IL-6 conjugate, which consists of the second rat anti- TNFα/IL-1β/IL-6 antibody conjugated to horseradish peroxidase (HRP), for 2 h at room temperature, followed by five washes with wash buffer. 100 µl of substrate solution was added to the wells for 30 min in the dark at room temperature. The substrate solution reacts with the HRP conjugate and provides a luminescent signal. Finally, 100 µl of the stop solution (hydrochloric acid) was added to each well to terminate the colour reaction. The plate was gently shaken at 60rpm for 1 min and optical density was measured with a microplate reader at 450 nm with a reference filter at 540 nm to correct for optical imperfections. The plate optical density was

measured within 30 min of the addition of the stop solution. The concentration of TNFα/IL-1β/IL-6 in the cell medium samples was determined against a standard curve from the standard samples used on the same 96 well plate. Samples were run in duplicate from three independent experiments to allow for statistical analysis.

2.2.15 Western Blotting

Western blotting is a gel electrophoresis and blotting method used to separate and identify proteins of interest in a biological sample. This technique was used to determine the expression levels of different proteins of interest. Denaturing gels were used and the Western blotting protocol relied on the denaturation of all expressed proteins in the cell, of which single proteins could be identified by immunoblotting with antibodies directed against epitopes of the protein of interest.

2.2.15.1 Cell lysis for Western blotting

Cells were harvested into lysis buffer containing 20 mM Tris-acetate, 1 mM ethylene diamine tetraacetic acid (EDTA) to chelate calcium, 1 mM ethylene glycol tetraacetic acid (EGTA) to chelate magnesium, 10 mM of the phosphatise inhibitor sodium–β–glyerophosphate, 1 mM of the tyrosine phosphatise sodium orthovanadate, 5% glycerol to depress the solution's freezing temperature, 1% Triton X-100 membrane solubiliser, 0.27M sucrose, 1 mM of the protease inhibitor benzamidine, 4 µg/ml of the protease inhibitor leupeptin, 1 µM pf the phosphatase inhibitor microcystine LR and 0.1% of the reducing agent β-mercaptoethanol, pH 7.4 (Kingham and Pocock. 2000)

and incubated on ice for 10 min, before being centrifuged for 10 min at 14,000 g to pellet the nuclear and membrane fractions. Supernatants were transferred to new eppendorf tubes and all samples were stored at -20°C until used. The formulation of the lysis buffer is such that the buffer lyses the cells and also solubilises the membranes, in addition to preserving the proteins at the time of lysis by preventing phosphorylation, dephosphorylation and proteolysis.

2.2.15.2 Bradford assay

A Bradford protein determination assay (Bradford 1976) was carried out to determine the protein concentration of the cell lysate supernatants (SN) using bovine serum albumin (BSA) as a standard. In a 96 well plate, 1 µl of each sample was added into the wells in triplicate and 200 µl Bradford reagent was added onto this. Samples were shaken and left at room temperature for 5 min before the absorbance shift of the Coomassie dye towards the blue spectrum following the binding of protein was measured at 595 nm on a Tecan X Fluor 4 plate reader. The absorbance of each sample was compared to a standard curve of BSA of known concentrations, constructed on the same plate as the experimental samples. From this, the volume of the lysate required for 45 µg of protein on the SDS page gel derived, and this quantity of sample was then removed to another eppendorf tube. To these samples, an equal volume of sample buffer (2% SDS, 10% glycerol, 2.5% β-mercaptoethanol, 125 mM Tris/HCL (pH 6.8) and a few bromophenol blue crystals) was added to denature the protein, and aid loading and visualisation of the gel front. Samples were then boiled for 5 min before loading.

2.2.15.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Cell lysate protein was separated on Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels using a minigel apparatus (Biorad), with 10% resolver and 4% stacking gels. The resolver gel contained 3.33 ml 30% polyacrylamide, 2.5 ml resolver buffer (pH 8.8) (18.1 g Tris/HCL, 1 ml 10% SDS), 4.17 ml H_20, 50 µl 10% APS (fresh) and 5 µl tetramethylethylenediamine (TEMED). The stacker gel contained 1.33ml 30% polyacrylamide, 2.5 ml stacker buffer (pH 6.8) (6.05 g Tris/HCL, 1 ml 10% SDS), 6.17 ml H_20, 50 µl 10% Ammonium persulfate (APS) and 5 µl TEMED. The TEMED and APS were added last to aid in the setting of the gels. After the addition of the stacker gel a comb was placed in the top of the plate to form wells. The gels were placed in their holder into the tank and the wells were filled with running buffer (125 mM Tris/HCl, 1 M glycine, 0.01% SDS). The samples were loaded into the well based on the amount of sample containing 45 µg of protein as determined by the Bradford assay. Prior to loading the samples were diluted 1:1 in sample buffer (2% SDS, 10% glycerol, 2.5% mercaptoethanol, 125 mM Tris/HCl pH 6.8 and a few bromophenol blue crystals) and were boiled for 4 min to denature the protein. The molecular weight marker and samples were carefully added into the wells and the tank was filled with running buffer. The gels were run at 160V (constant voltage) until the dye front was near the end of the gel.

Following the separation of the proteins the gel was carefully removed from the glass plates and equilibrated for 20 minutes in cold transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.01% SDS). While the gel was equilibrating, a piece of

polyvinylidene difluoride (PVDF) membrane (Immobilon P) cut to the same size as the gel was activated in ice-cold methanol for 1 min. This was followed by 5 min in distilled water and approximately 20 min in cold transfer buffer. Two pieces of wadding and 3MM paper also cut to the size of the gel were pre-soaked in cold transfer buffer. After equilibration the gel was transferred to a cassette. The order of placement within the cassette was wadding, 3MM paper, gel, PVDF membrane, 3MM paper and wadding. The cassette was slotted into its holder and placed into the tank filled with cold transfer buffer, next to an ice pack. The transfer was run at 80V for 1 h to allow proteins to transfer onto the membrane. The proteins migrated from the negative anode to the positive cathode, and attached to the positively charged membrane. Following transfer, membranes were washed in Tween-20 Tris buffer saline (TTBS: 10 mM Tris HCL, 150 mM NaCl, 0.5% Tween-20, pH 7.4) for 10 min, and membranes were then blocked in 5% non-fat milk solution in TTBS for 1 h to prevent non-specific binding of the antibody. The membranes were then ready for immunoblotting.

2.2.15.4 Immunoblotting

After blocking with 5% non-fat milk solution, membranes were incubated with the primary antibody diluted in 5% non-fat milk as shown in Table 2.3. Typically membranes were incubated with the primary antibodies overnight at 4°C on an orbital shaker.

Primary Antibody (Source)	Dilution and incubation	Corresponding secondary antibody (Source)	Dilution and incubation
Rabbit anti mouse iNOS (BD transduction lab 610333)	1:5000 2 h room temp	Goat anti-rabbit IgG peroxidise (Sigma A-0545)	1:2000 1 h room temp
Anti-CD35 (E11) (Abcam ab25)	1:500 overnight at 4°C	Rabbit anti-mouse IgG HRP (Santa Cruz 358914)	1:1000 1 h room temp
Anti-CD35 (31R) (Hycult biotech HM2107)	1:100 overnight at 4°C	Rabbit anti-mouse IgG HRP (Santa Cruz 358914)	1:1000 1h room temp
Mouse anti rabbit beta actin (Sigma A2066)	1:2000 2 h room temp	Rabbit anti-mouse IgG HRP (Santa Cruz 358914)	1:1000 1 h room temp

Table 2.3. List of primary and secondary antibodies used for Western blotting.

Following incubation with the primary antibodies (Table 2.3), the membranes were washed three times with TTBS and then incubated with secondary antibodies as shown in Table 2.3 for 1 h at room temperature on an orbital shaker. The membranes were then washed a further three times with TTBS and were exposed using enhanced chemiluminescence (ECL). Membranes were drained and placed in a tray containing an equal volume of each ECL solution and were incubated for 1 min. Membranes were then blotted on filter paper to remove excess ECL solution, and were wrapped in saran wrap and fixed in an X-ray cassette. In the dark, a piece of Amersham photographic film (GE Healthcare, UK) was placed onto the membranes to expose the protein bands. Photographic paper was then developed in developing solution and immersed in fixing solution before finally being rinsed in water. The films were dried and analysed and the

expression of each protein was quantified using densitometry (Image J). Western blots were performed three times and those presented here are representative. Blots were routinely re-probed for β-actin as a loading control to ensure equal loading of protein, which was also used for densitometry.

2.2.16 Immunohistochemistry

Human post-mortem tissue was collected in accordance with Institutional Review Board-approved guidelines at Brigham and Women's hospital. Blocks of brain tissue were fixed for ~2 h in 10% neutral buffered formalin. After fixation, the tissue was dehydrated and embedded in paraffin. 10-micron serial sections were cut, dried, and baked at 60°C for 1.5 h.

Sections were deparaffinized in two changes of Histoclear (National Diagnostics, Atlanta, GA) and rehydrated in graded ethanol solutions. Endogenous peroxidase activity was quenched by incubation of these sections in 0.3% hydrogen peroxidase in methanol for 10 min at room temperature. The sections were washed in water twice at 5 min each. Pretreatment and dilution testing was performed for each primary antibody and the experiment continued with the suitable combination, as shown in Table 2.4. Formic acid pretreatment consisted of the application of 88% formic acid to sections for 10 min at room temperature, followed by a 10 min water wash. Heat mediated antigen retrieval pretreatment consisted of heating sections in citrate buffer in a microwave (BioGenex, San Ramon, CA) until the solution came to a boil. The heat

level was then reduced to provide gentle cyclic boiling for an additional 5 min. Sections were cooled to room temperature and washed in water. After the pretreatment, all sections were blocked for 20 min in 10% horse serum in TBS (for CR1 antibodies). Sections were incubated with primary antibody in TBS overnight at 4°C. Following a 10 min wash in TBS, the horseradish peroxidase avidin-biotin complex system (ABC) (mouse Elite ABC kit; Vector Laboratories, Burlingame, CA) and diaminobenzine (DAB) (Sigma Immunochemicals, St. Louis, MO) were used to visualize the bound antibodies. The ABC staining allows for an improved sensitivity to the antibody through increasing the amount of enzyme molecules bound to the target antigen thereby amplifying the signal.

Sections were counterstained with hematoxylin, differentiated with acid alcohol, dehydrated, cleared in Histoclear, and mounted on coverslips with Permount (Fisher Scientific, Pittsburgh, PA).

Antibody	Target	Stock conc (mg/ml)	Dilution	Pretreatment on paraffin sections	Source
6B1	CR1 - LHR-D	0.465	1:100	Heat-mediated	Dr Anne Nicholson-Weller and Dr Ionita Ghiran
543	CR1 - LHR-C, D	0.6	1:100	Heat-mediated	
CR1 Rabbit pAb	CR1 - multi-epitope	3	1:100	Heat-mediated	
YZ1	CR1 - SCRs 5,6,7, of LHR-A,B,C	3	1:100	Heat-mediated	
IC7	CR1	0.8	1:100	Heat-mediated	
3C10	CR1	0.2	1:100	Heat-mediated	
E11	CR1 – LHR-C,D	0.8	1:100	Heat-mediated	abcam
Non-immune IgG1	IgG1	1	-	-	abcam
3A1	Aβ1-15, Aβ1-40	0.685	1:100	-	Dr Brian O'Nuallian
R1282	$A\beta_{1-40}$	-	1:1000	FA	Dr Dennis Selkoe

Table 2.4. List of primary antibodies used for Immunohistochemistry and flow cytometry.

For Immunofluorescence staining with 6B1, 543 and CR1 Rabbit pAb's, tissue sections or blood smears underwent heat-mediated/formic acid/methanol pretreatment as indicated in chapter 5. The sections/smears were pretreated with

0.1M Tris solution and then blocked in 2% goat serum in 0.1M Tris buffer for 5 min. The sections/smears were incubated with the primary antibodies at dilutions indicated in Table 2.4 overnight at 4°C. Following a rinse with 0.1M Tris buffer, the sections/smears were immersed in 2% goat serum in 0.1M Tris buffer for 5min. The sections/smears were incubated with the secondary antibody (1:1000) for 2 hr at room temperature in a moist chamber. The sections/smears were washed twice in 0.1M Tris buffer for 20 min and tissue sections were immersed in Sudan Black B for 10 min. Immersion with Sudan Black B reduces the autofluorescence background without interfering with the integrity of the brain tissue (Oliveira et al. 2010). Following washing steps in TBS the sections and smears were washed in water for 5 min, fixed in 10% formalin for 1 h at room temperature, washed in water for 20 min and mounted on coverslips with Vectashield with DAPI as a counterstain (Vector, Burlingame, CA). To quantify immunoreactivity, acquisition of images was performed in a single session using a QICAM camera (Q-imaging) mounted on an Olympus BX50 microscope.

2.2.17 Flow Cytometry

Flow cytometry is a technology used for the measurement and analysis of multiple characteristics of single cells, by passing thousands of cells per second through a laser beam and capturing the light that emerges from each cell as it passes through. This method is used for a range of applications including immunophenotyping and cell counting. The data gathered can be statistically analyzed using software to measure cellular characteristics such as size (by measuring forward scatter), phenotype

(by measuring fluorescence of bound antibodies) and complexity (by measuring side scatter). In this thesis flow cytometry was used to measure fluorescence to determine presence of CR1 and Aβ on red blood cells (RBCs). Fluorescence is described as the excitation of a fluorophore to a higher energy level followed by the return of that fluorophore to its ground state with the emitting of light. The energy of this emitted light is dependent on the energy level to which the fluorophore is excited.

The method used is this study was adapted from previously published work (Ghiran et al. 2008b). Briefly, 50 µl of whole blood cells were mixed with 500µl Hank's balanced salt solution (HBSS) followed by centrifugation at 65 g for 2 minutes. The washing step was repeated one time. 2µl of packed RBCs were mixed with 500 µl HBSS containing specific antibodies to CR1, Aβ or an IgG isotype control (Table 2.4) at a concentration of 5 µg/mL. The mixture was incubated for 5 mins at room temperature. This was followed by two washes with 200 µl HBSS followed by incubation with Alexa Fluor 488-congugated secondary antibodies specific for each primary antibody at a dilution of 1:1000 in HBSS. After incubation with secondary antibodies for 10 min, RBCs were washed once, resuspended in 400 µl HBSS, transferred to polystyrene round bottom FACS tubes and analyzed using a FACSCalibur flow cytometer (BD Biosciences). For each sample, 10,000 events were counted and analyzed using FlowJo software (version 10.0.6), which then provided the number of positive cells after gating on the IgG isotype control and the mean fluorescence intensity for each sample.

2.2.18 Statistical analysis

To assess statistical significance, experiments were performed in triplicate using three separate cell preparations. In imaging experiments, three coverslips were imaged per condition, and a minimum of six fields of view per coverslip were imaged and analysed. Where percentages were calculated, statistical analysis was performed on transformed data. Statistical analysis was performed using the GraphPad Prism software. Data was analysed using one-way ANOVA with Tukey multiple comparison post-hoc analysis whereby post-hoc comparisons were made between control, LPS and $A\beta_{1-42}$ and between the same conditions following the treatment of CR1 blocking antibody (i.e. CR1 block, LPS with CR1 blocking antibody and $A\beta_{1-42}$ with CR1 blocking antibody) and also between LPS and LPS with CR1 blocking antibody and between $A\beta_{1-42}$ and $A\beta_{1-42}$ with CR1 blocking antibody. Significance was stated as *$p<0.5$, **$p<0.001$, ***$p<0.001$. All data were expressed as mean ± the SEM.

Chapter 3

The role of CR1 in modulating

microglial phenotypes

3. The role of CR1 in modulating microglial phenotypes

3.1 Introduction and Summary of results

The association observed between the CR1 loci and age-related cognitive decline and plaque burden has been implicated in impaired clearance of Aβ plaques in the brain of AD patients (Chibnik et al. 2011). Although the recruitment of phagocytes and inflammatory mediators are intended to be beneficial, under certain conditions, these processes can prove harmful instead. Classical markers of immune-mediated damage have been identified in AD brains including MHC-I and MHC-II positive microglia (McGeer et al. 1987; Tooyama 1990; Perlmutter et al. 1992).

In normal brain, microglia become reactive, surround damaged or dead cells and clear cellular waste from the area to promote regeneration and repair (Fetler & Amigorena 2005). Microglia generally have beneficial effects, but their overstimulation can promote neurotoxicity due to pathogenic signals, including Aβ, resulting in the excess of free radicals, pro-inflammatory cytokines, complement proteins and glutamate (Morales et al. 2010). In AD, microglia display an early reactive phenotype (Lautner et al. 2011), and changes in the immune response are another risk marker for the development of AD (Jones et al. 2010).

Exposure of microglia to extrinsic C1q complement protein demonstrated a shift towards a pro-inflammatory phenotype, similar to that seen after the exposure of microglia to LPS, with a release in IL-6, TNFα, NO and an oxidative burst (Färber et al. 2009). This chapter investigates how modulation of CR1 signalling influenced the

microglial phenotype during exposure of activators complicit in microglial responses in AD.

This chapter provides evidence that microglial CR1 can elicit a neurotoxic effect which correlated with enhanced cytokine and superoxide production, but that blocking CR1 does not contribute a protective response by triggering glutathionine production or inhibiting iNOS induction. The data shows an increased ability of $A\beta_{1-42}$ treated microglia to phagocytose dextran beads following antibody blockage of CR1 but a reduced ability to phagocytose fluorescent $A\beta_{1-42}$.

3.2 Results

3.2.1 CR1 expression is increased after microglial activation; and antibody blockage of CR1 modulated microglial phagocytosis

β-amyloid is hypothesized to accumulate in late-onset AD at a normal rate however it's clearance is impaired (Mawuenyega et al. 2010). This dysregulated clearance and subsequent amyloid accumulation initiates the activation of the complement system (Rogers et al. 1992). The complement system acts in a number of ways to support host defence, including opsonisation of pathogens through phagocytes expressing complement receptors such as microglia (Tohyama & Yamamura 2006). With recent associations between the CR1 polymorphism and poor cognitive performance (Mengel-From et al. 2010) and MRI characteristics in AD (Biffi et al. 2010), it could be suggested that microglia mediate their phagocytic ability to clear Aβ through this receptor, which was therefore investigated here. Since there is controversy about the ability of microglia to express CR1, two different anti-CR1 antibodies were firstly tested on lysates from primary cultures of rat microglia through Western blotting. These cultures were ascertained to be >90% pure (by Isolectin B_4 fluorescence staining, Fig 3.1).

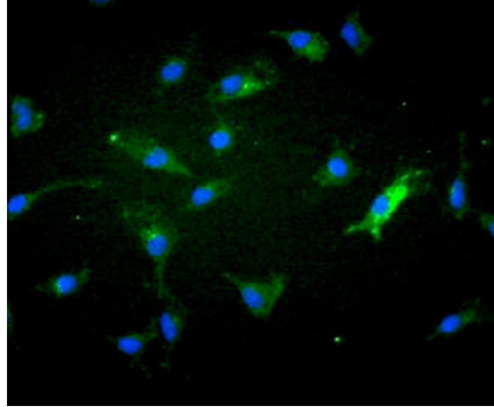


Figure 3.1: Microglia stained with Isolectin B4 (green) and DAPI (blue).
To confirm the purity of the microglial culture, cells were stained with microglial marker, Isolectin B_4 (2 μg/mL). Image courtesy of Dr Jennifer Pocock.

Fig. 3.2a shows the resultant Western blot incubated with anti-CD35 (E11) antibody and Fig 3.2b shows the resultant Western blot incubated with anti-CD35 (31R) antibody. In each case human lymphoblast cells, a kind gift from Dr Victoria Burchell, UCL, were also analysed as a positive control for each antibody due to their known CR1 expression (Rødgaard et al. 1991). In order to assess how CR1 signalling influences the pro-inflammatory phenotype associated with microglial responses in AD, the effects of activators such as LPS or $A\beta_{1-42}$ on microglial CR1 expression were investigated (Fig. 3.2). There was trend towards an increase in CR1 protein expression following activation of primary rat microglia with either LPS (1 μg/mL) or $A\beta_{1-42}$ (20 nM) for 24 h.

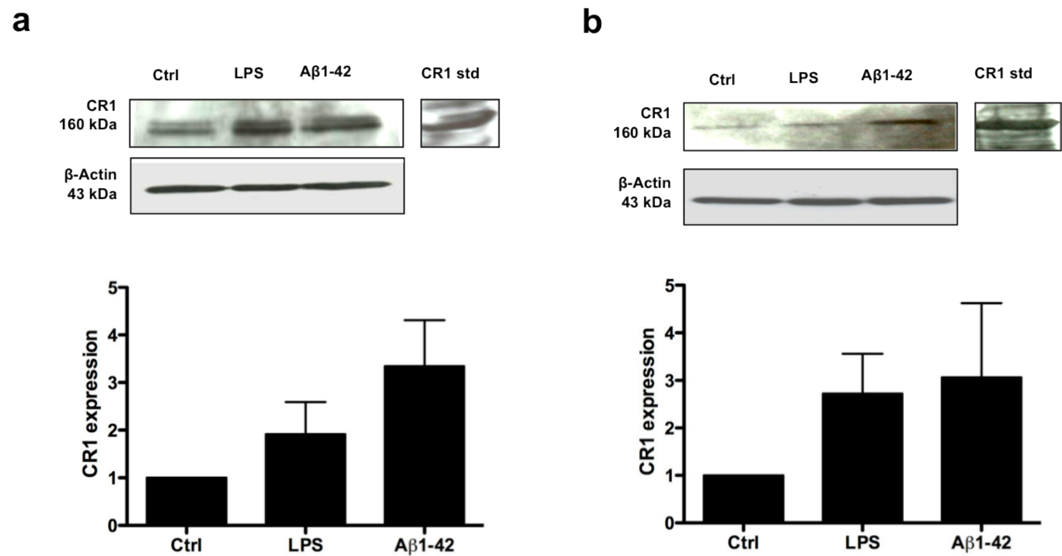


Figure 3.2: CR1 expression is increased in primary rat microglia (45 μg) treated with microglial activators LPS (1 μg/mL) and Aβ1–42 (20 nM).

Western blot with (a) antibody CD35 (E11) from Abcam and (b) with antibody CD35 (31R) from Hycult biotech. In each case a standard of human lymphoblasts (45 μg) was also analysed for each antibody as a positive control. Densitometry of expression relative to the loading control, β-actin and against control CR1 expression was performed on three separate blots. Data were analysed by one-way ANOVA and Tukey post-hoc tests.

In order to determine the functional consequences of CR1 up-regulation in microglia following LPS or $A\beta_{1-42}$ stimulation, a microglial phagocytic assay measuring uptake of dextran beads was performed. Exposure of primary rat microglia to either LPS (1 μg/mL) or $A\beta_{1-42}$ (20 nM) for 24 h, decreased dextran bead uptake (Fig. 3.3a and 3.3c) following bead incubation for 2 h. In the case of LPS treatment, phagocytosis of beads was not reversed by 24 h co-incubation with CR1 blocking antibody (1 mg/mL) and LPS (1 μg/mL). However, blocking CR1 reversed the observed $A\beta_{1-42}$ suppression of dextran bead phagocytosis back to control levels. Phagocytosis is a dynamic process

requiring energy (Kvarstein 1969; Peterson et al. 1977; Pratten & Lloyd 1984) and phagocytosis of dextran beads was significantly reduced when the assay was carried out at 4°C, thus eliminating binding or a superficial cell-association as an explanation for the results observed (Fig. 3.3b). The experiment was repeated measuring phagocytosis of HiLyte Fluor™ 488-labeled $A\beta_{1-42}$ (Fig. 3.4a and 3.4b) and the results exhibited a differential response compared with phagocytosis of dextran beads. Following antibody blockade of CR1 in microglia treated with LPS (1 μg/mL) or $A\beta_{1-42}$ (20 nM), there was a significant reduction in fluorescent $A\beta_{1-42}$ phagocytosis compared with LPS or $A\beta_{1-42}$ treatment alone. Again, conduction of the experiments at 4°C revealed little phagocytosis, therefore confirming the fluorescence observed was due to phagocytosis.

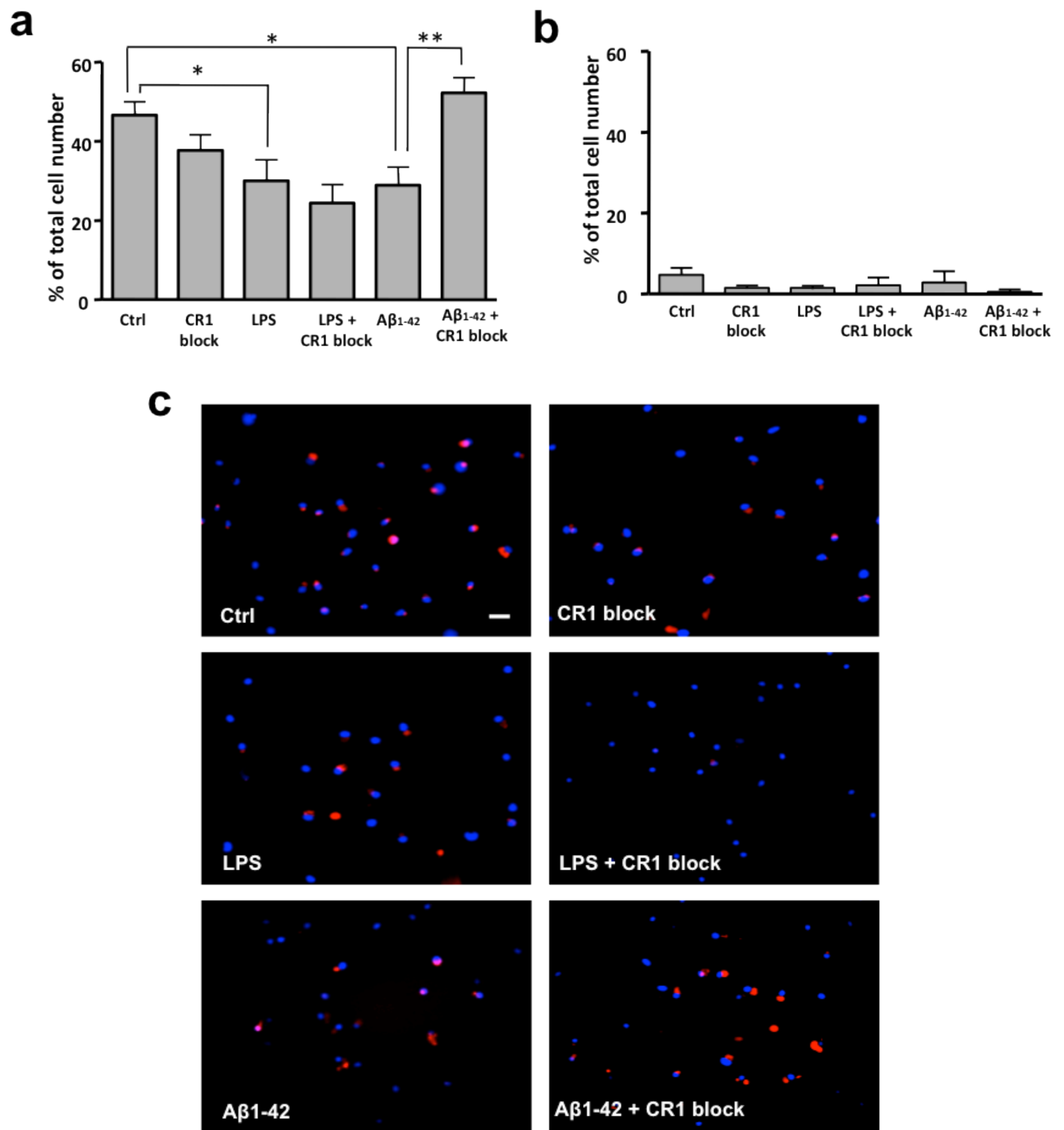


Figure 3.3: CR1 functional blocking antibody modulates microglial phagocytosis following treatment with Aβ or LPS.

Quantification and images of primary microglial phagocytosis of dextran beads (a,c) after direct treatment of microglia with Aβ1-42 (20 nM) or LPS (1 μg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL) for 24 h. (b) Quantification of primary microglial phagocytosis carried out as in (a) at 4 °C. Phagocytosis was measured as a percentage of the number of cells with a fluorescence signal to total microglial cell number/field of view from 3 fields per coverslip, 3 coverslips per condition, from 3 separate experiments. Data were analysed by one-way ANOVA ($\rho < 0.0001$) and Tukey post-hoc tests, $^{**}\rho < 0.01$ and $^{*}\rho < 0.05$. Data are shown as mean ± SEM.

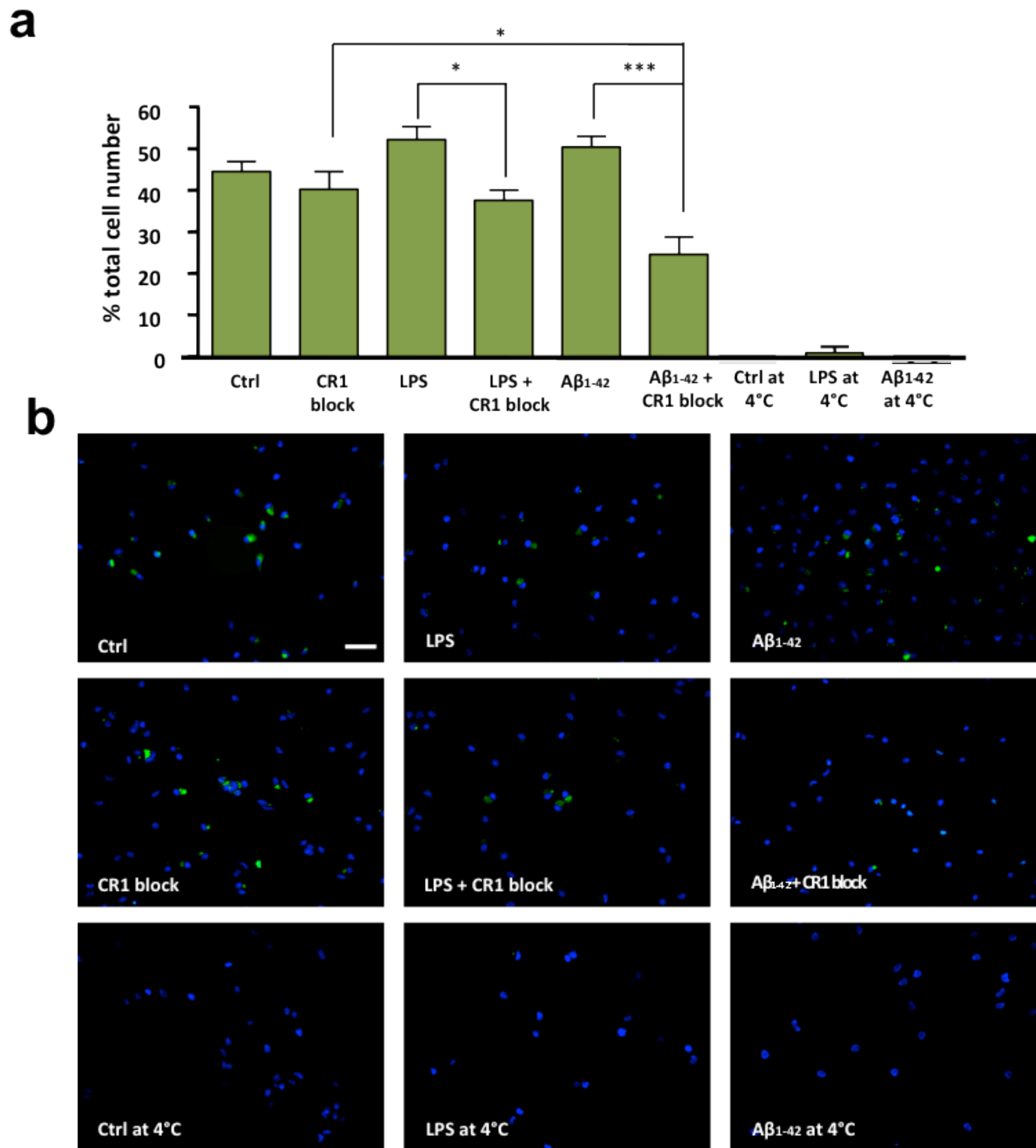


Figure 3.4: CR1 functional blocking antibody modulates microglial phagocytosis following treatment with Aβ or LPS.

(a) Quantification and (b) images of primary microglial phagocytosis of fluorescently tagged $A\beta_{1\text{-}42}$ after direct treatment of microglia with $A\beta_{1\text{-}42}$ (20 nM) or LPS (1 μg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL) for 24 h. Phagocytosis was measured as a percentage of the number of cells with a fluorescence signal to total microglial cell number/field of view from 3 fields per coverslip, 3 coverslips per condition, from 3 separate experiments. Data were analysed by one-way ANOVA ($\rho < 0.0001$) and Tukey post-hoc tests, ***$\rho < 0.001$ and *$\rho < 0.05$. Data are shown as mean ± SEM.

3.2.2 CR1 modulation has an effect on and TNFα and IL-1β secretion from microglia

Production of cytokines and complement activation are important microglial responses to CNS injury, particularly in AD (Smith et al. 2012; Di Bona et al. 2008; Di Bona et al. 2009). *In vitro* microglial activation with $A\beta_{1-42}$ leads to an increase in the production of a number pro-inflammatory cytokines including IL-1β, IL-6 and TNFα (Rogers & Lue 2001; Lue et al. 2001). Levels of the soluble neurotoxins, TNFα, IL-6 and IL-1β were measured in MGCM following 24 hr treatment with microglial activators and CR1 blocking antibody (Fig. 3.5). There was an increase in TNFα in MGCM from microglia treated with LPS (1 μg/mL) compared with control MGCM (Fig. 3.5a). TNFα levels were significantly reduced in MGCM from microglia co-treated with CR1 blocking antibody and LPS. No difference was seen in MGCM from microglia treated with $A\beta_{1-42}$ (20 nM) or with MGCM from microglia treated with $A\beta_{1-42}$ and CR1 blocking antibody compared with control. Although an increase in IL-6 production was observed in MGCM from primary microglia treated with LPS (1 μg/mL) there was no change detected in microglia-co-treated with CR1 blocking antibody (Fig. 3.5b). As in figure 3.5a, no difference was observed in MGCM from microglial co-treatment with $A\beta_{1-42}$ and CR1 blocking antibody and $A\beta_{1-42}$ alone (Fig. 3.5b). Fig. 3.5c shows that there was a decrease in IL-1β levels in MGCM from microglia co-treated with CR1 blocking antibody plus $A\beta_{1-42}$ compared with $A\beta_{1-42}$ activator alone. No significant release of IL-1β or regulation by CR1 block was seen following LPS stimulation (Fig. 3.5c). IL-10 release was also measured but significant modulation with CR1 block was not measured (data not shown).

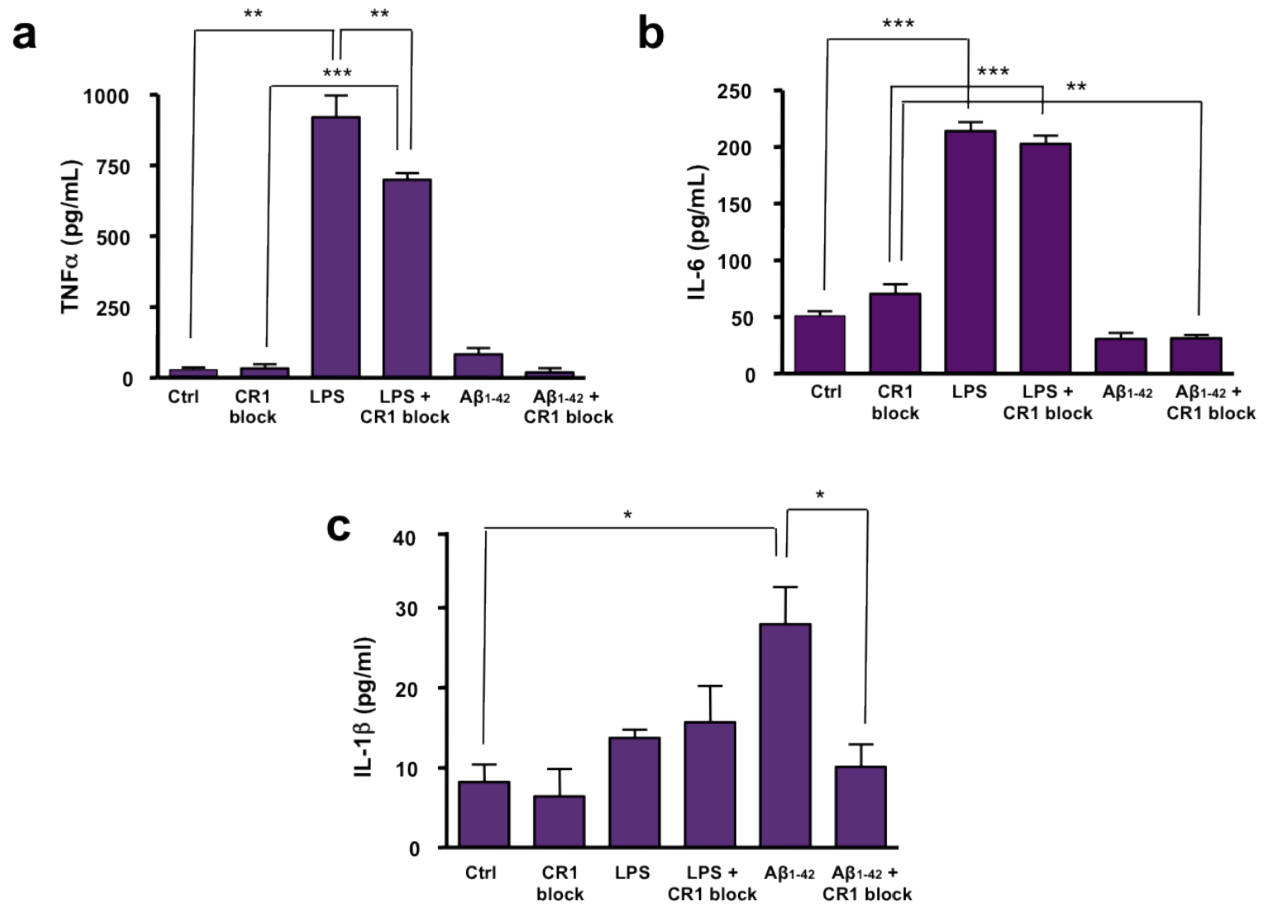


Figure 3.5: ELISA analysis of (a) TNFα, (b) IL-6 and (c) IL-1β secretion from primary cultured rat microglia following treatment with LPS (1 μg/mL), Aβ1–42 (20 nM) and CR1 blocking antibody (1 mg/mL), alone and in combination, for 24 h.

(a) Reduced TNFα secretion following LPS treatment in microglia pre-treated with CR1 blocking antibody. (b) A trend towards a decrease in IL-6 secretion and a significant decrease in IL-1β secretion (c) was observed in microglia pre-treated with CR1 blocking antibody compared with Aβ1–42 treatment alone. Data are the mean of supernatant values performed in duplicate from 3 separate coverslips per condition taken from 3 independent experiments and values were analysed by one-way ANOVA (TNFα $\rho < 0.0001$; IL-6 $\rho < 0.0001$; IL-1β $\rho < 0.0078$) and Tukey post-hoc tests, ***$\rho < 0.001$, **$\rho < 0.01$ and *$\rho < 0.05$. Data are shown as mean ± SEM.

3.2.3 CR1 block down-regulates iNOS expression in primary rodent microglia as measured by ICC

Microglial iNOS expression at high levels can mediate pathogenic effects with complement having a possible role (Montalto et al. 2003). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by microglia can react to form the highly toxic peroxynitrite in response to LPS or Aβ stimulation (Kim et al. 2009; Neher et al. 2011). The possibility of CR1 playing a role on iNOS expression was investigated. ICC data showed that LPS induced a significant increase in iNOS expression in microglia (Fig. 3.6a and 3.6b), which was attenuated by pre-treatment with CR1 functional blocking antibody. A similar trend was observed with the $A\beta_{1-42}$ –treated microglia (Fig 3.6a and 3.6b). Fig 3.6c shows the resultant Western blot data with a representative image of primary microglia treated with LPS (1µg/mL), $A\beta_{1-42}$ (20nM) and in some cultures co-incubation with CR1 blocking antibody (2µg/mL) and incubated with anti-iNOS antibody. A trend was observed showing a reduction in iNOS expression in microglia with co-treatment with CR1 blocking antibody compared with LPS treatment alone, however this result was not significant.

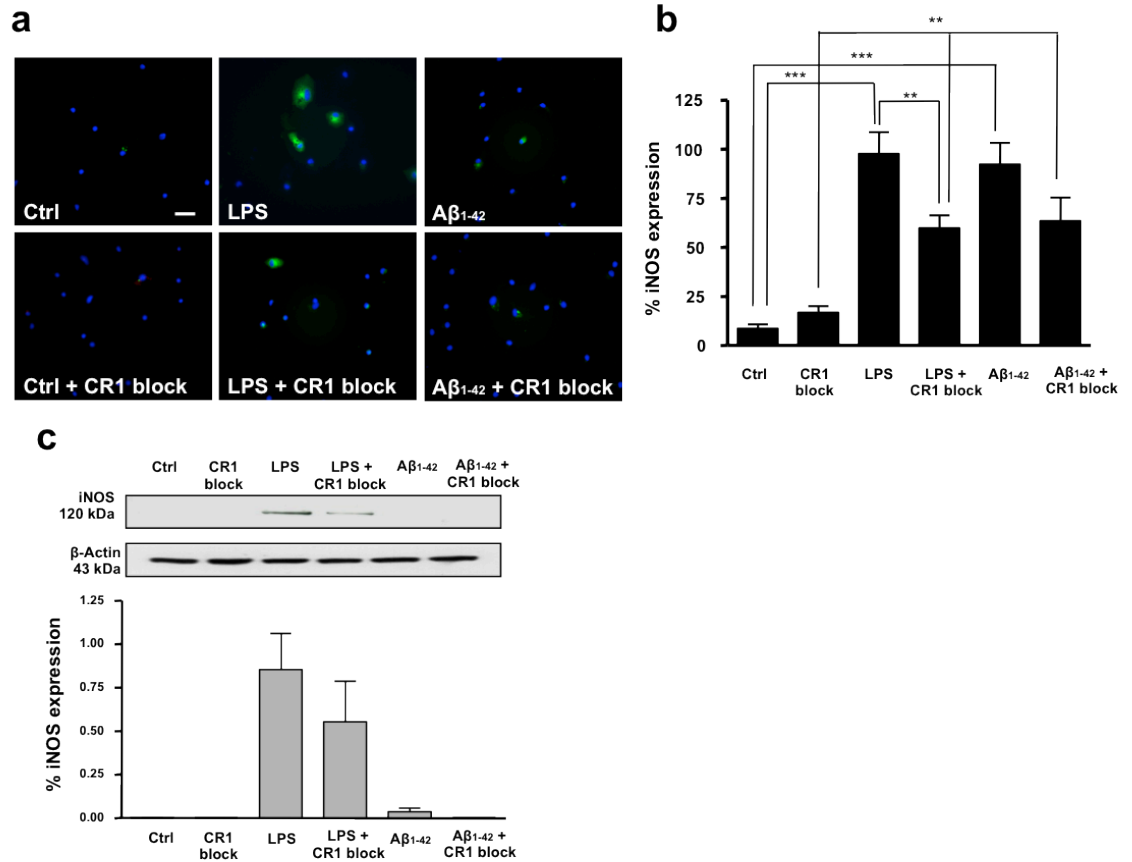


Figure 3.6 Immunocytochemistry, western blot and densitometric analysis of iNOS expression in primary rat microglia after treatment with Aβ1-42 (20 nM) or LPS (1 μg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL) for 24 h.

(b) Quantification of the presence of microglia staining positive for iNOS after treatments given in (a). (c) is a representative Western blot probing for the expression of iNOS from primary microglia following 24h treatment. ICC data analyses were carried out as a percentage of the number of cells with a fluorescence signal to total microglial cell number/field of view from 3 fields per coverslip, 3 coverslips per condition, from 3 separate experiments and western blot data were analysed from three separate blots from three independent experiments and compared by ANOVA ($\rho < 0.0001$) and Tukey post-hoc tests, ***$\rho < 0.001$, **$\rho < 0.01$ and *$\rho < 0.05$. Data are shown as mean ± SEM.

3.2.4 CR1 block inhibits $O_2^{\cdot-}$ production in microglia

The immune system can battle against invading pathogens through a respiratory burst which is a rapid release of ROS from, in this case, microglia. Previously, there has been a positive correlation between an increase in respiratory burst and expression of C3b, the binding of which to its corresponding receptor, CR1, promotes signalling to

NADPH oxidase to produce superoxide (Hoogerwerf et al. 1990; Gessler & Dahinden 2003). Superoxide production was measured by DHE staining, and here, primary microglia treated with LPS (1 μg/mL) for 24 h showed a significantly higher intracellular $O_2^{\cdot-}$ production compared with control, which was attenuated by functional antibody CR1 block (Fig. 3.7a and 3.7b). A similar significant reduction in $O_2^{\cdot-}$ production was observed in primary microglia treated for 24 h with $A\beta_{1-42}$ (20 nM) and CR1 block compared with $A\beta_{1-42}$ treatment alone. Primary microglia were also co-treated with apocynin (10μM), an inhibitor of NADPH oxidase which prevents superoxide production through inhibiting the translocation and assembly of the active NADPH oxidase complex (Stefanska & Pawliczak 2008; Mead et al. 2012b) and $O_2^{\cdot-}$ production was inhibited on these cells. Similar $O_2^{\cdot-}$ production was observed in BV2 microglia (Fig. 3.8a and 3.8b). Levels of $O_2^{\cdot-}$ were significantly reduced in BV2 microglia co-treated with CR1 blocking antibody compared with LPS alone and a trend towards a reduction compared to $A\beta_{1-42}$ treatment alone. $O_2^{\cdot-}$ production was also measured in BV2 microglia transfected with Crry siRNA, the murine ortholog of the human CR1 gene (Killick et al. 2012). LPS or $A\beta_{1-42}$ treated BV2 microglia displayed significantly increased $O_2^{\cdot-}$ production compared with control and this was reduced in cells transfected with Crry siRNA prior to LPS and $A\beta_{1-42}$ treatment (Fig. 3.9a and 3.9b). BV2 cells transfected with cyclophilin B control siRNA prior to treatment with LPS demonstrated similar $O_2^{\cdot-}$ production to control. Although a trend can be observed, there was no significant decrease in $O_2^{\cdot-}$ production between LPS treatment alone and LPS following control siRNA transfection. In addition, there was a significant decrease in $O_2^{\cdot-}$ production

between LPS and Crry siRNA treated/transfected cells versus LPS and control siRNA treatment/transfection suggesting that Crry may have a role in $O_2^{\cdot-}$ production. Additional controls would be needed to confirm this finding such as confirming knockdown of Crry by measuring Crry expression, therefore these data only show a preliminary look into the effect of Crry on $O_2^{\cdot-}$ production. $O_2^{\cdot-}$ positive fluorescence was also inhibited in BV2 microglia pre-treated with the NADPH oxidase inhibitor, apocynin, prior to LPS or $A\beta_{1-42}$ treatment.

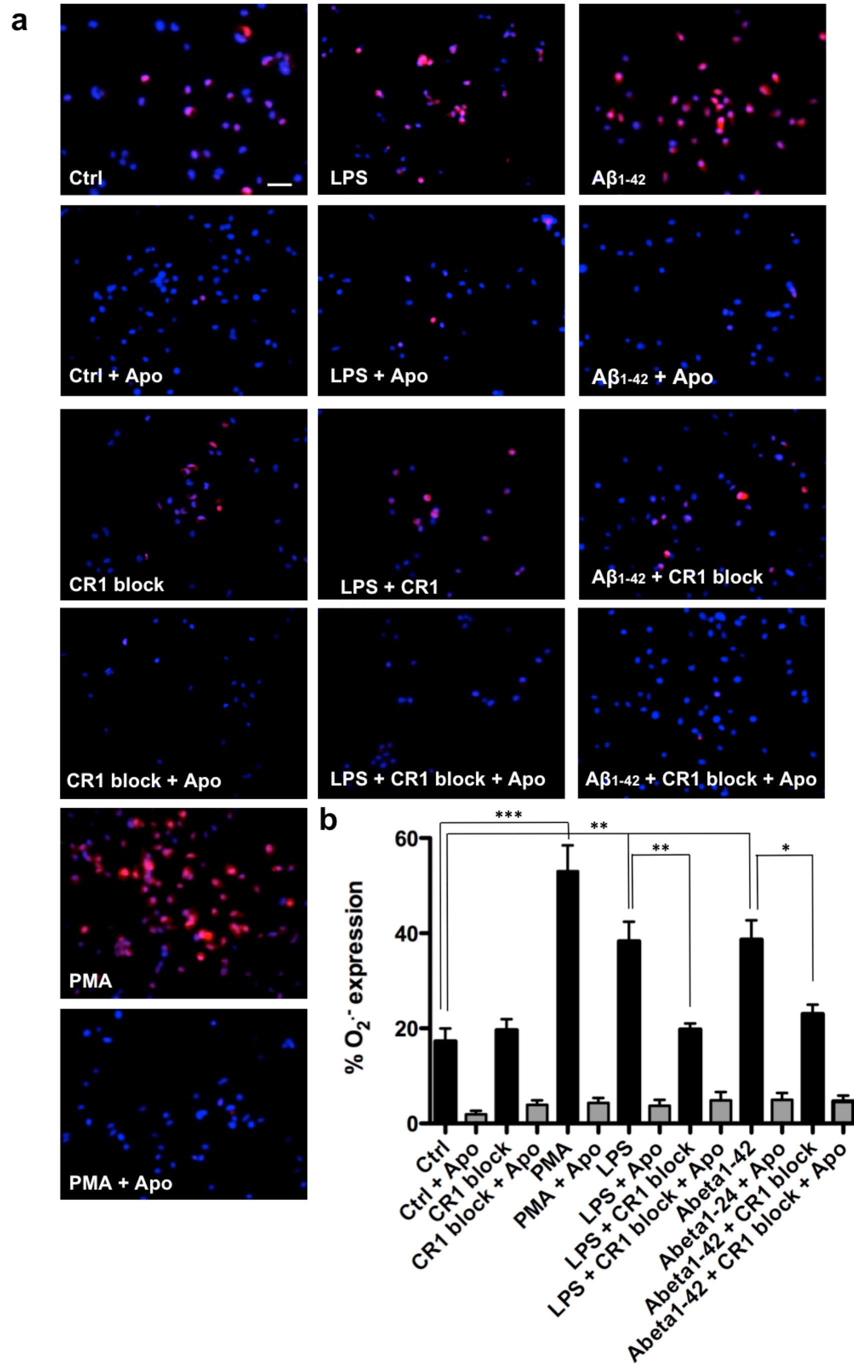


Figure 3.7: CR1 functional blocking antibody modulates intracellular superoxide (O2 •−) production in primary rat microglia.

(a) Live cell imaging of O2 •− generation monitored with dihydroethidium (DHE, pink fluorescence) in cells producing intracellular O2 •− in primary microglia, after treatment with $A\beta_{1-42}$ (20 nM), phorbol myristate acetate (PMA, 10 ng/mL) or LPS (1 µg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL) for 24 h or apocynin (Apo) (10 mM) during DHE loading, as a control for the specificity of DHE fluorescence. (b) Quantification of the percentage of microglia positive for O2 •− after treatments given in (a) (black bars). Grey bars represent treatments with apocynin and were not included in the statistical analysis. Data were analysed by one-way ANOVA (($\rho < 0.0001$) and Tukey post-hoc tests, ***$\rho < 0.001$, **$\rho < 0.01$ and *$\rho < 0.05$. Data are shown as mean ± SEM.

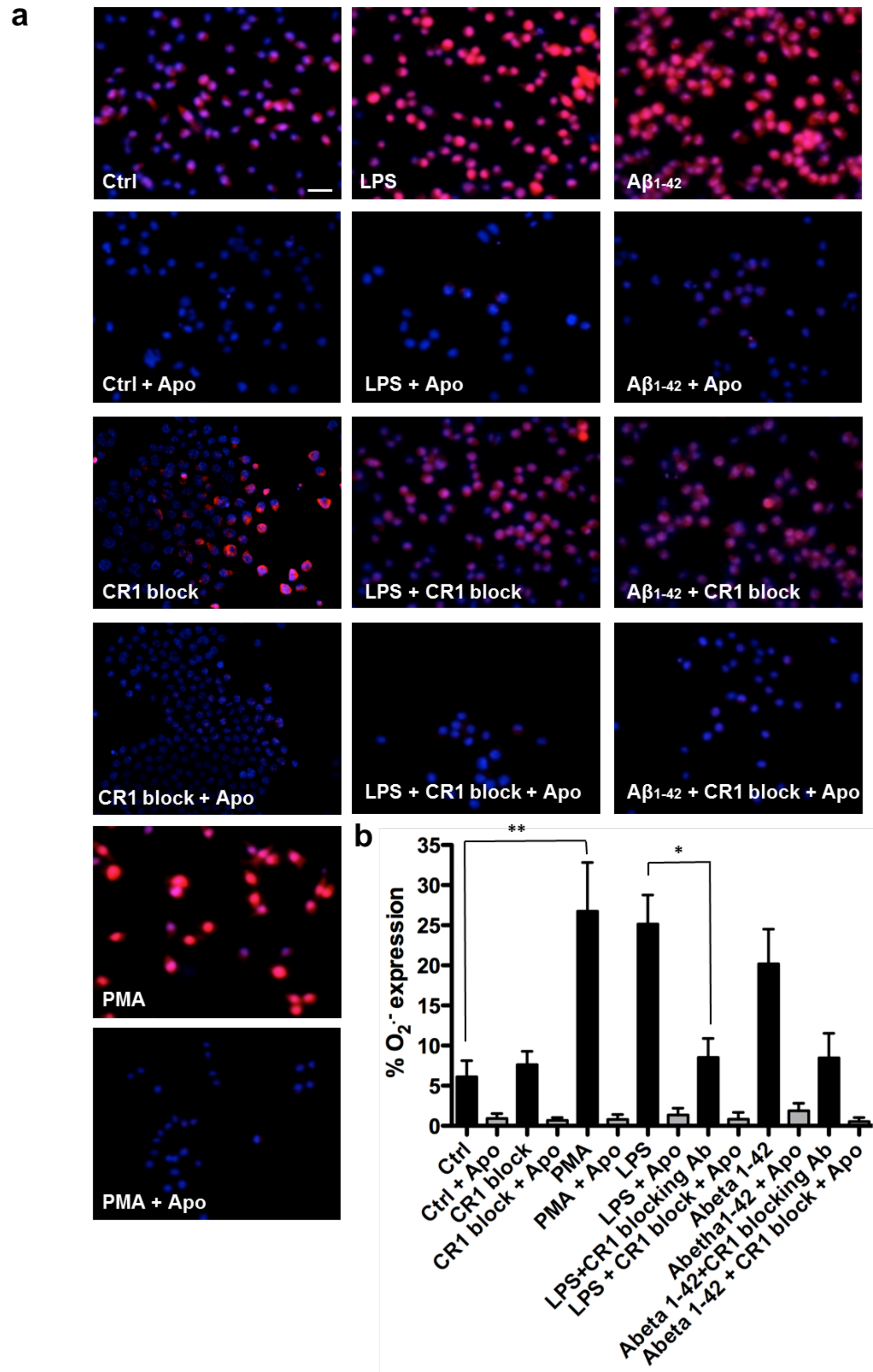


Figure 3.8: CR1 functional blocking antibody modulates intracellular superoxide (O2 •−) production in BV2 murine microglia.

(a) Live cell imaging of O2 •− generation monitored with dihydroethidium (DHE, pink fluorescence) in cells producing intracellular O2 •− in BV2 microglia, after treatment with $A\beta_{1\text{-}42}$ (20 nM), phorbol myristate acetate (PMA, 10 ng/mL) or LPS (1 µg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL) for 24 h or apocynin (Apo) (10 mM) during DHE loading, as a control for the specificity of DHE fluorescence. (b) Quantification of the percentage of microglia positive for O2 •− after treatments given in (a) (black bars). Grey bars represent treatments with apocynin and were not included in the statistical analysis. Data were analysed by one-way ANOVA ($\rho < 0.0001$) and Tukey post-hoc tests, **$\rho < 0.01$ and *$\rho < 0.05$. Data are shown as mean ± SEM.

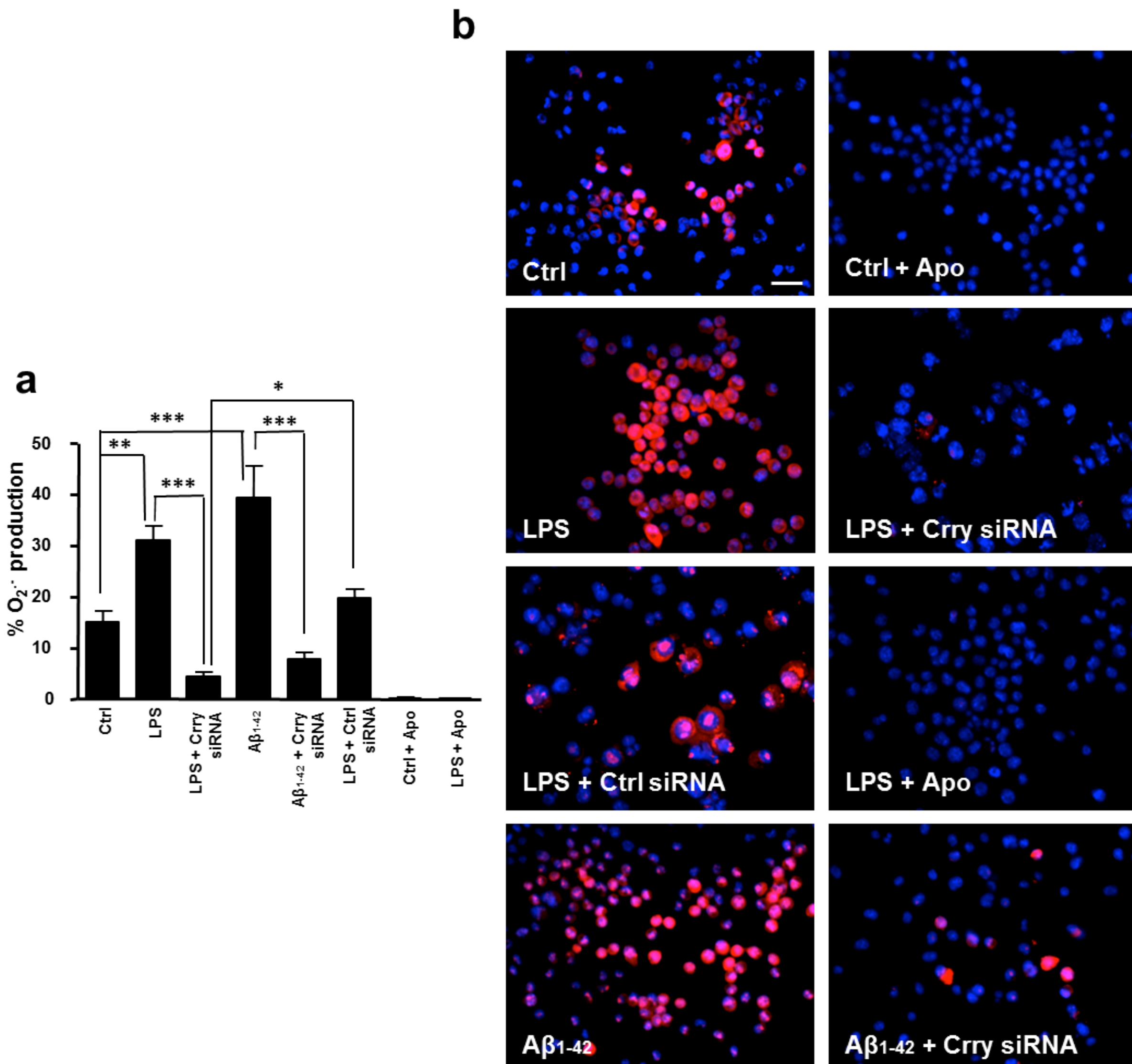


Figure 3.9: Quantification of the percentage of microglia positive for O2 •− after siRNA knockdown of Crry, the mouse homologue to CR1.

(a) and (b) Quantification was carried out following treatment with microglial activators, $A\beta_{1-42}$ (20 nM) and LPS (1 µg/mL) or apocynin (10 mM) during DHE loading, as a control for the specificity of DHE fluorescence. siRNA knockdown of Crry results in a modulation of O2 •− production after treatment with Aβ1–42 (20 nM) or LPS (1 µg/mL) for 24 h. Data were analysed by one-way ANOVA ($\rho < 0.0001$) and Tukey post-hoc tests, $^{**}\rho < 0.01$ and $^{*}\rho < 0.05$. Data are shown as mean ± SEM.

NBT analysis to measure superoxide production was performed to support the DHE fluorescence data (Fig. 3.10). This experiment was carried out at 4 h and 24 h and it appeared that NBT reduction had a similar trend at both time-points. A significant increase in NBT reduction compared to basal levels (NBT treatment alone) was seen following treatment with the positive control, PMA (10 ng/mL). This increase in reduction of NBT was also observed in BV2 cells treated with LPS (1 μg/mL) and $A\beta_{1-42}$ (20 nM) alone for 24 h. Co-treatment with CR1 blocking antibody (1 mg/mL) resulted in a significant decrease in NBT reduction. It was important to determine whether the LPS and $A\beta_{1-42}$ induced superoxide production, as shown by NBT production in BV2 microglia, was a consequence of NADPH oxidase activity. BV2 microglia were treated with LPS and $A\beta_{1-42}$ in the presence of the specific NADPH oxidase inhibitor apocynin (10 μM), and superoxide production was assessed by NBT reduction following 4 h and 24 h. Co-treatment with apocynin decreased NBT reduction and therefore superoxide production.

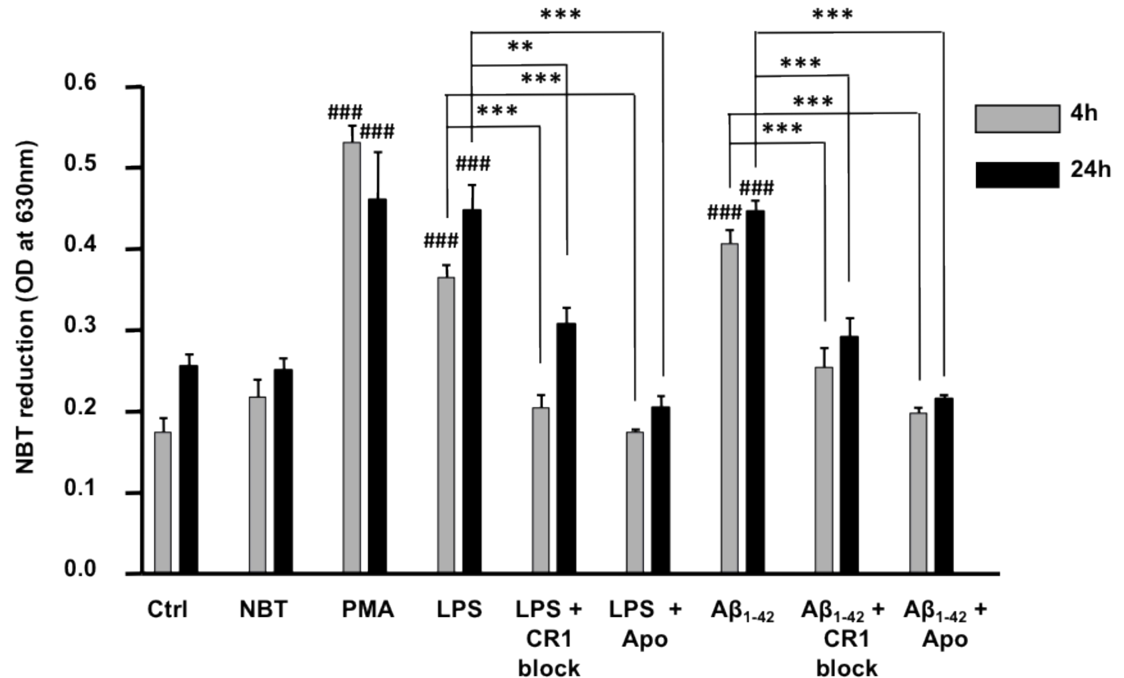


Figure 3.10: NBT assay showing reduction of superoxide production by CR1 blocking antibody. BV2 cells were treated with LPS (1 μg/mL), Aβ1-42 (20 nM) and CR1 blocking antibody (1 mg/mL) in the presence of NBT for 4 h and 24 h.

To determine whether NBT reduction following treatment with LPS and $A\beta_{1-42}$ was as a consequence of the NADPH oxidase, BV2 microglia were co-treated with LPS (1 μg/mL) and $A\beta_{1-42}$ (20 nM) in the presence of the NADPH oxidase inhibitor apocynin (Apo) (10 mM). Data were analysed by one-way ANOVA ($\rho < 0.0001$) and Tukey post-hoc tests, $^{***}\rho < 0.001$ and $^{**}\rho < 0.01$. Data compared with Ctrl and with NBT alone were marked with # and represent $^{\#\#\#}\rho < 0.001$. Data are shown as mean ± SEM.

3.2.5 CR1 block does not alter glutathione production in LPS and $A\beta_{1-42}$ treated microglia

As CR1 blockade was shown to attenuate LPS and $A\beta_{1-42}$ induced superoxide production in microglia, the mechanisms behind this were investigated further. Glutathione is an antioxidant produced by glial cells (Keelan et al. 2001), and as such plays a role in the defence system (Perricone et al. 2011). Microglial cells produce glutathione for defence against ROS produced by microglia or other cells such as astrocytes in the brain (Hirrlinger et al. 2000). Glutathione production was investigated

using the MCB assay following treatment of microglia with CR1 blocking antibody and microglial activators. There was no change in mean MCB fluorescence per cell following incubation with CR1 blocking antibody (1 mg/mL), LPS (1 µg/mL) and $A\beta_{1-42}$ (20 nM) for 24 h (Fig. 3.11). Grey bars represent microglia pre-incubated with ethacrynic acid (EA) (1 mM) a potent inhibitor of glutathione S-transferases for 10 min prior to additional treatment (Ploemen et al. 1993) to confirm fluorescence was due to glutathione binding to MCB.

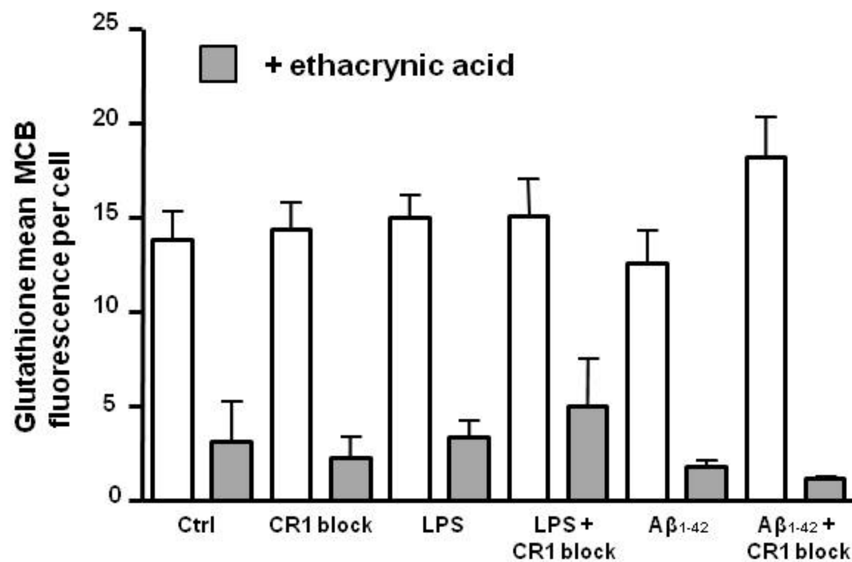


Figure 3.11: CR1 functional blocking antibody does not alter glutathione production in microglia. CR1 functional blocking antibody does not alter glutathione production in primary rat microglia investigated by live cell imaging of monochlorobimane (MCB) fluorescence.

Quantification of mean MCB fluorescence per microglial cell after treatment with $A\beta_{1-42}$ (20 nM) or LPS (1 µg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL) for 24 h (white bars) and co-treatment with ethacrynic acid (1 mM) as a control for MCB fluorescence (grey bars). Treatment with ethacrynic acid was not included in the statistical analysis. Data are the mean ± SEM of cellular fluorescence in approximately 50 cells per field, 3 fields per coverslip, 3 coverslips per condition from 3 independent experiments analysed with ImageJ software. Data were analysed by one-way ANOVA followed by Tukey post-hoc tests.

3.3 Discussion

It is hypothesized that in LOAD, it is not the rate at which Aβ is accumulated that is impaired but its clearance (Mawuenyega et al. 2010). CR1 is known to be involved in immune clearance of opsonised pathogens on erythrocytes (Kavai 2008; Li et al. 2010), and so it is thought that this receptor, shown here to be expressed in microglia, may provide a role in the clearance of Aβ or lack thereof in the AD brain. In healthy conditions, C3b, the ligand for CR1, is spontaneously produced and destroyed. However, in the presence of a pathogen, or in this instance a dextran bead, C3b binds to the bead and through the action of cofactors B and D develops into C3 convertase (Tohyama & Yamamura 2006). C3b is then cleaved to C3bi, which surrounds the bead and results in the capture of the bead by the phagocyte, which expresses CR3, the receptor for C3bi. When CR1 is blocked, it prevents the cleavage of C3b at two sites to produce C3bi, therefore preventing phagocytosis.

Microglia activated with LPS have been demonstrated to be a good cellular model to study neuroinflammation, and Toll-like receptors (TLRs) have been identified as important mediators of neuroinflammation (Le et al. 2001; Sheng et al. 2003; Arroyo et al. 2011). In order to examine the phagocytic capability of microglia in a proinflammatory environment, the cells were treated with LPS, of which the resulting activation is mediated by TLR-4 (Chow 1999; Lien et al. 2000). A significant reduction in phagocytosis of dextran beads was observed in microglia treated with LPS. This was not reversed by LPS and CR1 blocking antibody compared with control at 37°C (Fig. 3.3a and 3.3c), suggesting that CR1 does not play a role in a general phagocytosis

promoted by activation of microglia through the TLR-4 signalling pathway. Dextran bead phagocytosis of microglia previously exposed to A$\beta_{1\text{-}42}$ was also significantly lower than in control cells (Fig 3.3a). However, an increase in phagocytosis of dextran beads was observed in microglia treated with A$\beta_{1\text{-}42}$ and CR1 blocking antibody compared with A$\beta_{1\text{-}42}$ alone (Fig. 3.3a and 3.3c). The ability of Aβ binding to erythrocyte CR1 as previously mentioned, may extend to microglial CR1, and as such could contribute to a reduction in phagocytosis due to direct binding to the receptor. With the reduction of CR1 receptors available for binding this may contribute to an increased microglial activation response, through binding to components of the microglial cell surface receptor system resulting in increased phagocytosis. Microglial cells exposed to Aβ undergo phagocytosis through a different receptor-linked pathway to that induced by LPS. Microglia interact with fAβ through an Aβ cell surface receptor CD36, $\alpha_6\beta$ integrin and CD47 (Koenigsknecht & Landreth 2004) as well as to the Receptor for Advanced Glycation End-product (RAGE) (Yan et al. 1996; Fang et al. 2010). Microglial phagocytosis of fluorescently tagged A$\beta_{1\text{-}42}$ following LPS or cold A$\beta_{1\text{-}42}$ stimulation was prevented by CR1 block, suggesting that CR1 block may have distinct consequences for different phagocytotic pathways in microglia. This may reflect changes in ongoing phagocytosis versus pinocytosis (Pratten & Lloyd 1984). The decreased uptake of fluorescently tagged A$\beta_{1\text{-}42}$ in microglia treated with A$\beta_{1\text{-}42}$ and CR1 block may be associated with a reduction in IL-1β induced phagocytosis. An increase in IL-1β has been described to stimulate microglial motility (Ferreira et al. 2011), which correlates with the findings in Figure 3.5c showing a reduction in IL-1β

release in primary microglia treated with $A\beta_{1-42}$ and CR1 block compared to $A\beta_{1-42}$ alone. This reduction in IL-1β expression following treatment with CR1 block may consequently contribute to a reduced phagocytic effect. The exposure of 2 μM fluorescently-labelled $A\beta_{42}$ to microglia for 2 hrs prior to imaging may have also had an effect on microglial phagocytosis. Although it has been previously shown that oligomeric and fibrillar $A\beta_{42}$ do not lessen primary microglial cell viability until used at concentrations of over 5 μM and 10 μM respectively (Pan et al. 2011), addition of the 2 μM fluorescently-labelled $A\beta_{42}$ to the culture for 2 hrs may contribute to the microglial activation state, thus possibly amplifying the phagocytic response. If this additional 2 hr exposure to a higher concentration of $A\beta_{42}$ did have an effect on microglial phagocytosis, it may contribute to the differences in levels of phagocytosis observed between both the experiments measuring uptake of latex beads and uptake of fluorescently-labelled $A\beta_{42}$. A possible control for extracellular binding may be to image the uptake experiment by confocal microscopy and labelling the microglia with CD11b, thereby confirming that the fluorescently-labelled $A\beta_{42}$ was intracellular and not due to extracellular binding.

Cytokine production is an important microglial response to CNS injury and is associated with microglial activation. This cytokine production can lead to further activation of microglia by a positive feedback mechanism (Hanisch 2002). As previously noted, this proinflammatory cytokine production contributes to neuroinflammation implicated in AD pathogenesis. The proinflammatory cytokine, TNFα, is exorbitantly expressed coinciding with increased levels of Aβ plaque deposition (Fillit et al. 1991;

Paganelli et al. 2002). Additionally, three TNFα polymorphisms have been implicated with increased risk of LOAD, two of which are associated with higher TNFα secretion (Collins et al. 2000) further implicating this cytokine in the neuroinflammatory role in AD. A number of animal studies have shown an upregulation of TNFα production in AD mouse models which correlated with microgliosis (Janelsins et al. 2005; Janelsins et al. 2008) and further down-regulation of this cytokine in AD correlating with significant modulation of amyloid and tau related pathologies (Montgomery et al. 2013). Activation in a number of cell types have shown LPS to induce activation of extracellular-signal-regulated kinase-1 (ERK1) and ERK2, c-Jun N-terminal kinases (JNKs) and p38 of which all have been shown to be involved in either TNFα production or translation (Liu et al. 1994; Arditi et al. 1995; Hambleton et al. 1996; Sanghera et al. 1996; Nijenhuis et al. 1999). A significant increase in TNFα secretion was observed following treatment of primary rat microglia with LPS however this increase in secretion was not replicated following activation with $A\beta_{1-42}$ (Fig. 3.5a). This correlates with data from neuronal-glial mixed cultures showing that low doses of Aβ did not increase levels of TNFα secretion but the toxicity was thought to arise from ROS production (Qin et al. 2002). TNFα and IL-1β stimulate an upregulation of C3 and factor B biosynthesis, which are activator proteins of the alternative complement pathway (refer to fig. 1.5) in umbilical cord endothelial cells (Kawakami et al. 1997). This could suggest that the already continuously activated innate immune response pathway contributes to an increased C3b deposition. However, TNFα and IL-1 have been found on neutrophils to upregulate CR1 expression (Ogle et al. 1990), which could bind the

excess C3b. The reduction in TNFα secretion shown in figure 3.5a could be as a result of low expression of TNFα cleavage proteins such as TNFα converting enzyme (TACE or ADAM17). TACE is responsible for cleavage of the membrane-bound precursor TNFα (mTNFα) to its mature soluble TNFα (sTNFα) form which is secreted from the membrane into the extracellular compartment (Black et al. 1997; Mohan et al. 2002). TACE requires certain conditions for the cleavage of mTNFα and subsequent release of sTNFα, which may be affected by blocking CR1. Further investigation into a possible interaction would need to be addressed.

The proinflammatory cytokine, IL-6 is also thought to play a role in the pathogenesis of AD with studies reporting increased serum levels of this cytokine in AD patients compared with control (Cojocaru et al. 2011) and immunostaining detected around senile plaques and large cortical neurons of AD brains (Strauss et al. 1992). Furthermore a genetic polymorphism of the IL-6 gene has been associated with increased risk for the development of AD and modulation of time of onset (Papassotiropoulos et al. 1999; Bagli et al. 2000). Figure 3.5b shows an increase in IL-6 secretion from primary rodent microglia following 24 h hour stimulation with LPS which is consistent with known LPS activation data (Minogue et al. 2012). In human skin fibroblasts IL-6 has been shown to increase the rate of synthesis of C3 and factor B (Katz et al. 1989). This pathway is further accelerated by IL-6, contributing to the protection of the body by mediating an inflammatory response, pathogen opsonization and stimulating phagocytosis. Blocking CR1 may result in an accumulation of C3b due to reduced binding and subsequent removal from circulation. Excess C3b deposition

results in red cell deposition of which CR1 binding can have a protective effect (Odhiambo et al. 2008). Although there is an increase in rodent microglia IL-6 secretion observed following LPS activation, pre-treatment with CR1 blocking antibody did not appear to have any effect on IL-6 secretion (Fig. 3.5b). One of the components of the MAC complex, C5b, has also been hypothesized to contribute to IL-6 expression through the activation of transcription factors NF_kB and AP-1 in smooth muscle cells (Viedt et al. 2000), implicating a positive feedback mechanism is at play. This may attribute to the continuous secretion of IL-6 observed following increased C3b deposition.

IL-1β is another pro-inflammatory cytokine that has been indicated to be associated with the pathogenesis of AD. Both genetic data linking polymorphisms in IL-1β to increased AD risk (Licastro et al. 2004) and studies investigating IL-1β levels in post-mortem brain tissue and in CSF have found a significantly higher concentration of this cytokine in AD patients versus aged-matched controls (Cacabelos et al. 1991; Cacabelos et al. 1994). *In vitro* data demonstrate that microglia are the primary source of IL-1β in the brain (Chauvet et al. 2001). Figure 5.3c does not show an increased secretion of IL-1β following microglial activation with LPS as seen with the other cytokine ELISA measurements. It has been reported that IL-6 can act as an anti-inflammatory under certain conditions, and a study investigating the impact of IL-6 secretion on LPS release of IL-1β from glial cells found that co-treatment of LPS and recombinant IL-6 demonstrated a partially attenuated release of IL-1β (Minogue et al. 2012). Minogue and colleagues also postulated that it could be due to an increase in

the anti-inflammatory cytokine IL-10. However this study saw no change in IL-10 secretion between treatments (data not shown). Figure 3.5c does show an increase in IL-1β secretion following activation of primary rodent microglia with $A\beta_{1-42}$, a result shown to correlate with data investigating oligomeric and fibrillar $A\beta_{1-42}$ effects on glial cultures (White et al. 2005). Prior to IL-1β secretion from microglia, biologically inactive pro-IL-1β is cleaved by caspase-1 to generate the mature, biologically active IL-1β (Eder 2009). The first protein of the complement cascade, C1q, is able to suppress pro-caspase 1 and pro-IL-1β cleavage and therefore reducing IL-1β secretion (Benoit et al. 2012). Perhaps by blocking a complement regulatory protein such as CR1 could contribute to the mechanism by which C1q attenuates the cleavage of these factors. Increased binding of IL-1β to C3b following human serum activation (Borth et al. 1990), may suggest a possible reason for a decrease in IL-1β release from $A\beta_{1-42}$ and CR1 block treated cells compared to $A\beta_{1-42}$ alone. Binding to the increased C3b available through blocking CR1 could possibly regulate IL-1β release as observed in figure 3.5c.

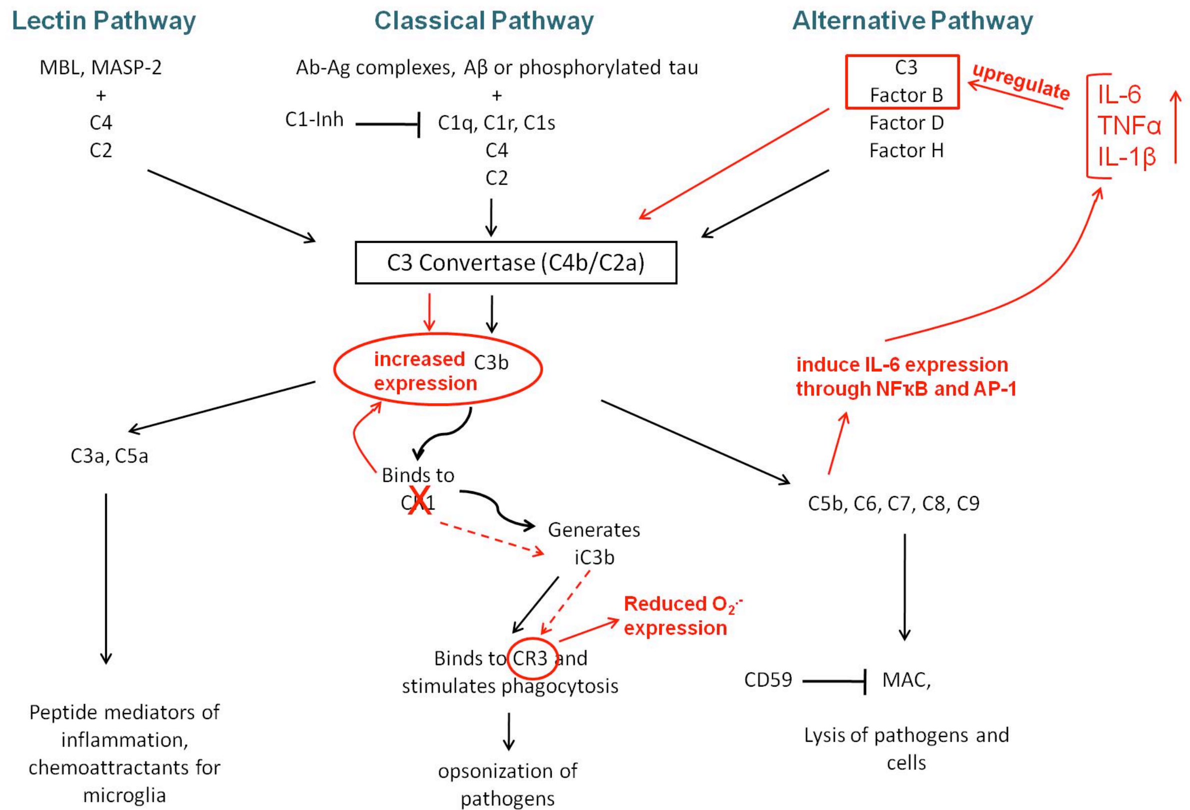


Figure 3.12: Possible interactions within the complement system following modulation of CR1 in neuroinflammation.
Microglial activation increases secretion of IL-6, TNFα and IL-1β which could upregulate C3 and Factor B of the alternative pathway. This may then contribute to increased C3b expression which could lead to increased cell lysis and increased $O_2^{\cdot-}$ through activation of C3. Blocking CR1 could lead to less production of iC3b which may reduce $O_2^{\cdot-}$ expression in addition to possibly reducing phagocytosis.

iNOS expression has been detected in the AD brain and associated with senile plaques and neurofibrillary tangles (Lee et al. 1999) and in figure 3.6a and 3.6b, there is an observed increase in expression following treatment with neuroinflammatory activator markers, LPS and $A\beta_{1-42}$. The increase observed through immunofluorescence was reduced in primary microglia co-treated with LPS and CR1 blocking antibody and a

trend towards a decrease in expression observed in $A\beta_{1-42}$ and CR1 blocking antibody co-treatment (Fig. 3.6a and 3.6b) proposing that CR1 may play a role in iNOS expression in microglia. The Western blotting data also showed a trend towards CR1 blockade decreasing LPS and $A\beta_{1-42}$ induced iNOS expression, however these data were not significant.

iNOS is involved in mediating the production of NO, which when it interacts with $O_2^{\cdot-}$ is known to have detrimental consequences to cells through the production of peroxynirtite. Superoxide production via NADPH oxidase has been previously shown to play a role in AD (Shimohama et al. 2000; Ansari & Scheff 2011). This study sought to investigate if CR1 was involved in this toxic role. TNFα, IL-6 and IL-1β upregulate C3 and Factor B leading to the increased expression of C3b, which has been previously mentioned to have a positive correlation with increased respiratory burst in neutrophils (Hoogerwerf et al. 1990). Figures 3.7 and 3.8 appear to complement this finding demonstrating an increase in $O_2^{\cdot-}$ expression in primary rat microglia and BV2 microglia respectively. An increase that can be attributed to NADPH oxidase production of $O_2^{\cdot-}$ due to the coinciding co-treatments with the NADPH oxidase inhibitor, apocynin, demonstrating an almost complete absence of $O_2^{\cdot-}$ expression. In leukocytes, CR3, the receptor for iC3b, mediates the activation of protein tyrosine kinases (PTKs). PTKs in turn stimulate the phos-phoinositide 3-kinase (PI-3K) activity and these function as src-homology 2 (SH2) docking sites. This leads to the activation of RAC2, which as part of the granulocyte NADPH oxidase complex ultimately leads to a respiratory burst (Wymann et al. 2000). Blocking CR1 would result in reduced

cleavage of C3b to C3bi, and as a consequence reduced binding to CR3 and subsequent reduced $O_2^{\cdot -}$ expression (Fig. 3.7 and 3.8). This result was replicated in BV2 microglia using Crry siRNA (Fig. 9), however further controls need to be done in this experiment to help reinforce the data indicating that blocking CR1 inhibits $O_2^{\cdot -}$ expression in microglia. Superoxide production was also measured by a NBT assay to confirm the findings. The data from this experiment replicates the decrease in superoxide production following blocking CR1 that was observed by DHE fluorescence measurement (Fig. 3.10). Figure 3.7, 3.8, 3.9 and 3.10 provide data that suggest CR1 may play a role in the superoxide production via NADPH oxidase activity.

Oxidative stress is an imbalance between ROS and its removal by antioxidant systems. Microglia produce glutathione for the defence of ROS (Hirrlinger et al. 2000) and a decline in glutathione concentration is seen in blood from male AD patients compared to controls (H Liu et al. 2004). Perhaps the oxidative stress observed following blocking CR1 is not only exclusively due to an increase in ROS such as $O_2^{\cdot -}$ but also through a change in glutathione to counteract this toxicity. However, following investigations as to whether CR1 played a role in microglial release of glutathione, the results demonstrated no change in production (Fig. 11) implying that CR1's contribution to the oxidative stress balance remains likely to be solely through ROS production. However, further investigation measuring other anti-oxidant systems could be carried out to confirm this.

3.4 Conclusions

These results possibly demonstrate that CR1 plays a role in microglial phagocytosis, particularly when triggered by Aβ. This modulation is interesting as it revealed CR1 to enhance dextran phagocytosis but reduce Aβ phagocytosis. Thus, taken together, GWAS studies mutations in CR1 may lead to loss of function and a decrease in Aβ clearance, which correlates with the data presented here. What is also clear here is the proinflammatory influence CR1 has on neurotoxicity, and our data suggest this is via actions of microglial $O_2^{\cdot -}$ and cytokine production. Mice express the Cr1-related protein Y (Crry) which is more similar to human Cr1 in its protein sequence and function than murine Cr1 and deletion of Crry has been recently shown to influence tau phosphorylation and complement production (Jacobson & Weis 2008; Killick et al. 2012). In this study, results for Crry knockdown in murine BV2 microglia are the same as those using the CR1 blocking antibody in rat microglia, suggesting similar downstream pathways. It should be noted that further experiments also using IgG control and a Fc receptor blocker would need to be carried out to control for the CR1 blocking antibody. Considering the recent data demonstrating a genetic link between CR1 and LOAD, it is crucial to determine what impact this has on the cellular and molecular ramifications of CR1 activation on neuronal survival and consequences of activity in AD.

Chapter 4

Ramifications of CR1 activation on neuronal survival

4. Ramifications of CR1 activation on neuronal survival

4.1 Introduction and summary of results

In the previous chapter, blocking microglial CR1 was shown to reduce a number of responses from microglia that are potentially neurotoxic such as iNOS expression, superoxide production and secretion of cytokines such as TNFα. It was important to investigate the ramifications of microglial CR1 modulation on neuronal survival, which may have important consequences for future AD therapies.

Survival of cerebellar granule neuronal cells (CGCs) was examined following direct treatment with LPS and $A\beta_{1\text{-}42}$ alone and also in the presence of CR1 blocking antibody. Neuronal death was also investigated following treatment with microglial conditioned medium (MGCM) from microglia incubated with LPS, $A\beta_{1\text{-}42}$ and CR1 blocking antibody. Modulating CR1 was found to have an effect on neuronal death.

Following on from the observed microglial TNFα secretion and iNOS expression, this study also aimed to determine if these microglial mediators of neurotoxicity, were causative factors in the observed neuronal death. It was observed that pharmacologically inhibiting microglial iNOS and TNFα did not have a protective effect on neurons, interestingly it appeared to increase the percentage of apoptosis.

4.2 Results

4.2.1 CR1 modulation has an effect on neuronal death

Evidence suggests that apoptosis can stimulate the complement system, and such activation contributes to the phagocytosis of subsequent apoptotic cells (Köhl 2006). Figure 4.1 shows the results following measuring percentage of cell death, as assessed by as assessed by fluorescence microscopy using PI, which is a marker for necrosis and late apoptosis and Hoechst 33342, a nuclear stain which stains smaller brighter apoptotic nuclei. Percentage of cell death was measured following either 48 h treatment of CGC cultures directly with LPS (1 μg/mL), $A\beta_{1\text{-}42}$ (20 nM) and CR1 blocking antibody (2 μg/mL) (Fig. 4.1a) or 48 h treatment with MGCM from microglial cultures treated for 24 h with LPS, $A\beta_{1\text{-}42}$ and CR1 blocking antibody (Fig. 4.1c).

Direct treatment of CGC cultures with LPS (1 μg/mL) or addition of MGCM from LPS-treated microglia for 24 h induced significant cell death (Fig. 4.1a and 4.1c), suggesting the neurotoxin(s) responsible was soluble and stable. No death was induced when CGC cultures were directly treated with $A\beta_{1\text{-}42}$ (20 nM) for 48 h (Fig. 4.1a) but significant death was observed when CGC cultures were treated with conditioned medium from microglia treated with $A\beta_{1\text{-}42}$ (Fig. 4.1c), suggesting that $A\beta_{1\text{-}42}$ -MGCM contains stable neurotoxins which may not be potent in mixed neuronal-glial cultures. It may, however, be as a consequence of a dampening of the response due to Aβ activated astrocytes in the CGC culture mediating extracellular signal-regulated kinase (ERK) dependent neuroprotection (Abe et al. 2003; Yamamuro et al. 2003). A significant reduction in death was observed in CGCs directly pre-treated with CR1

functional blocking antibody compared with CGCs treated with LPS alone (Fig. 4.1a). Also data presented in Fig. 4.1b indicate that the total cell number did not vary between treatments reducing the possibility of the decrease in apoptosis being due to an increase in phagocytosis of the apoptotic cells or floating off of dead cells. No difference in CGC levels of death were seen following direct treatment for 48 h with $A\beta_{1-42}$ with or without the presence of CR1 blocking antibody (Fig 4.1a). Data presented in Fig. 4.1c indicated that neuronal death was enhanced after treatment of CGC cultures with MGCM from microglia treated with LPS or $A\beta_{1-42}$. This neuronal death was reduced when the CGC cultures were treated with MGCM from primary microglia treated with $A\beta_{1-42}$ and CR1 blocking antibody.

In order to investigate the potential protective effect of blocking CR1 in one of the regions in which neurodegeneration is known to occur early on in AD, the hippocampus, a live cell assay was performed on hippocampal neurons. Similar findings were observed with these neurons compared with CGCs (Fig. 4.1e). Calculation of NeuN positively stained neurons was carried out to determine neuronal viability (Fig. 4.1e).

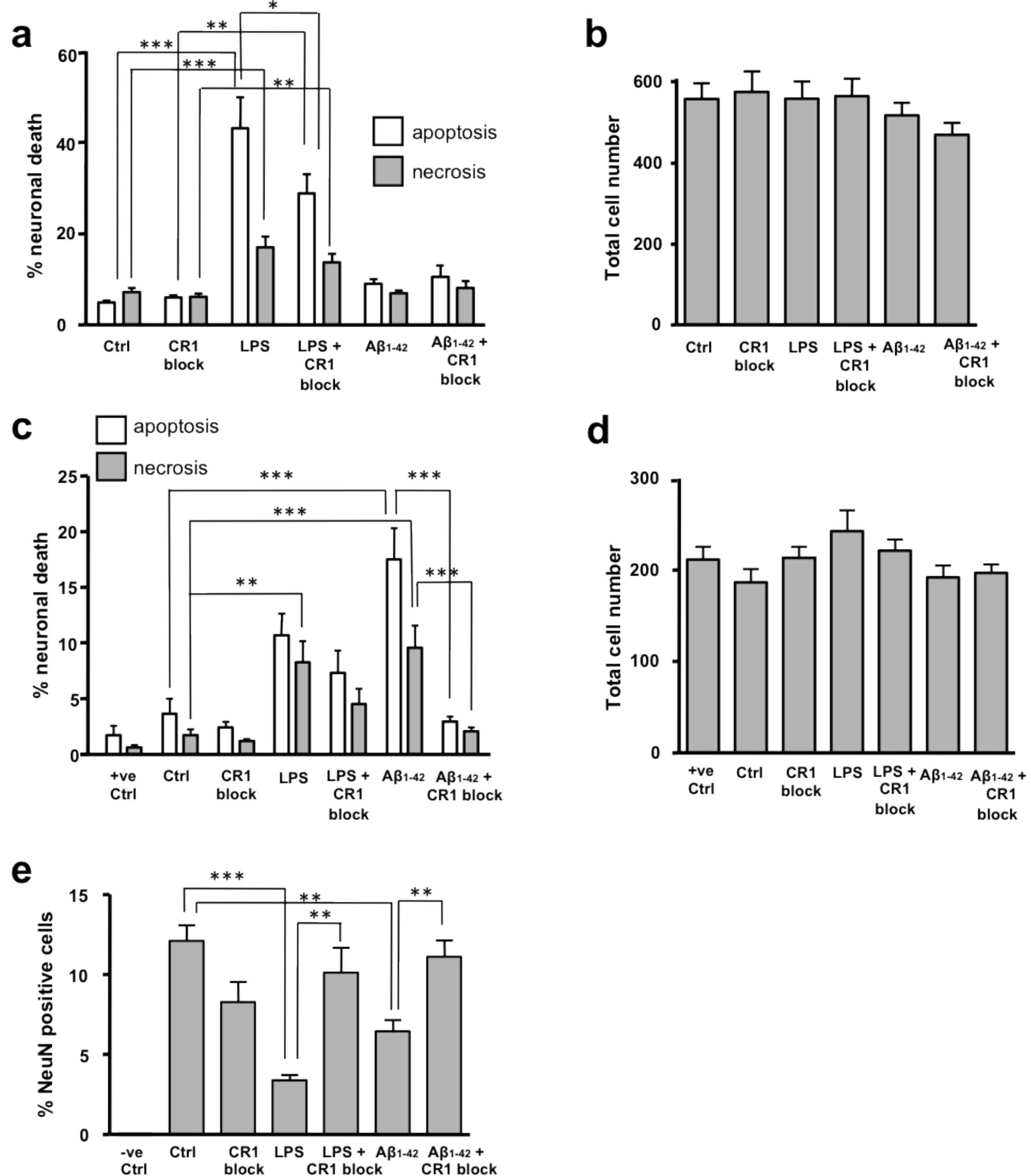
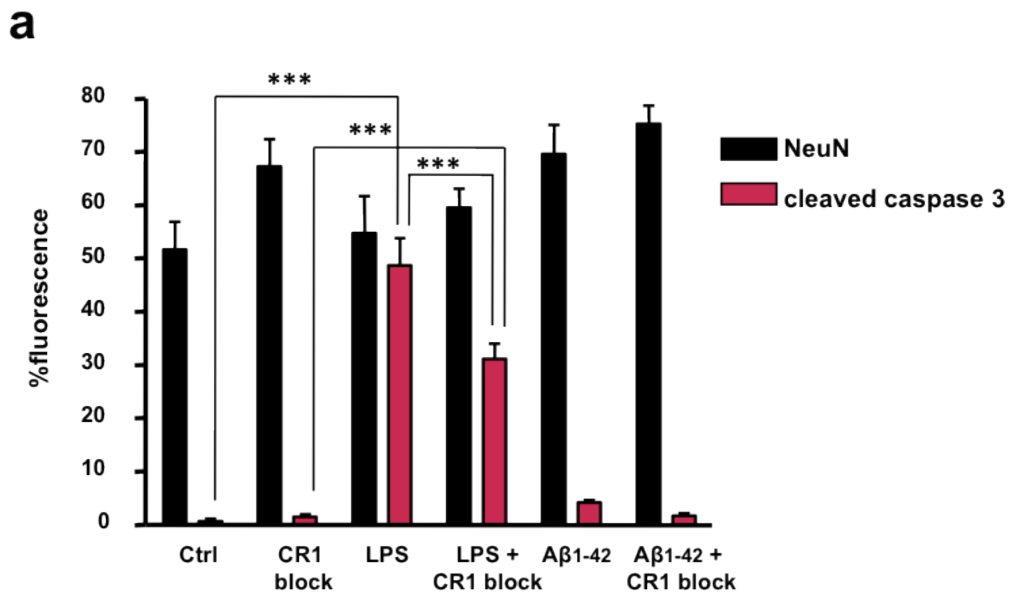


Figure 4.1: CR1 functional blocking antibody protects against microglial-evoked neurotoxicity following direct and indirect treatment with Aβ1-42 (20 nM) or LPS (1 μg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL).

(a) Live–dead cell staining of CGC neuronal cultures with Hoechst 33342 (white bars) and propidium iodide (grey bars) after 48 h direct treatment. (b) Total cell number per condition. (c) Live–dead cell staining of CGC cultures treated with MGCM from microglia treated with the conditions in (a) for 48 h with +ve Ctrl representing cells with no treatment of MGCM. (d) Total cell number per condition after MGCM treatment of CGCs. (e) Hippocampal neuronal protection under the same conditions monitored by NeuN staining. In all of the experiments data are the mean of 3 fields per coverslip, 3 coverslips per condition from 3 separate experiments. Data were analysed by one-way ANOVA ($\rho < 0.0001$ for a,c and e) and Tukey post-hoc tests, ***$\rho < 0.001$, **$\rho < 0.01$ and *$\rho < 0.05$. Data are shown as mean ± SEM.

Following the findings that CR1 block conferred neuronal protection against LPS and $A\beta_{1-42}$ treatment, the mechanisms of this protection were investigated further. Activation of the caspase-3 pathway, which is a hallmark of apoptosis (Thornberry & Lazebnik 1998), was investigated to determine whether blocking CR1 down-regulated this cell death cascade (Fig. 4.2). Treated and PFA fixed CGCs showed a significant increase in cleaved caspase-3 immunoreactivity following treatment with LPS compared to control. This in turn was significantly reduced in CGCs co-treated with LPS and CR1 blocking antibody (Fig. 4.2b). There was no change in cleaved caspase-3 immunoreactivity following CGC treatment with $A\beta_{1-42}$ and also with co-treatment with CR1 blocking antibody. These results are in line with the CGC live fluorescence assay following direct treatment (Fig. 4.1a).



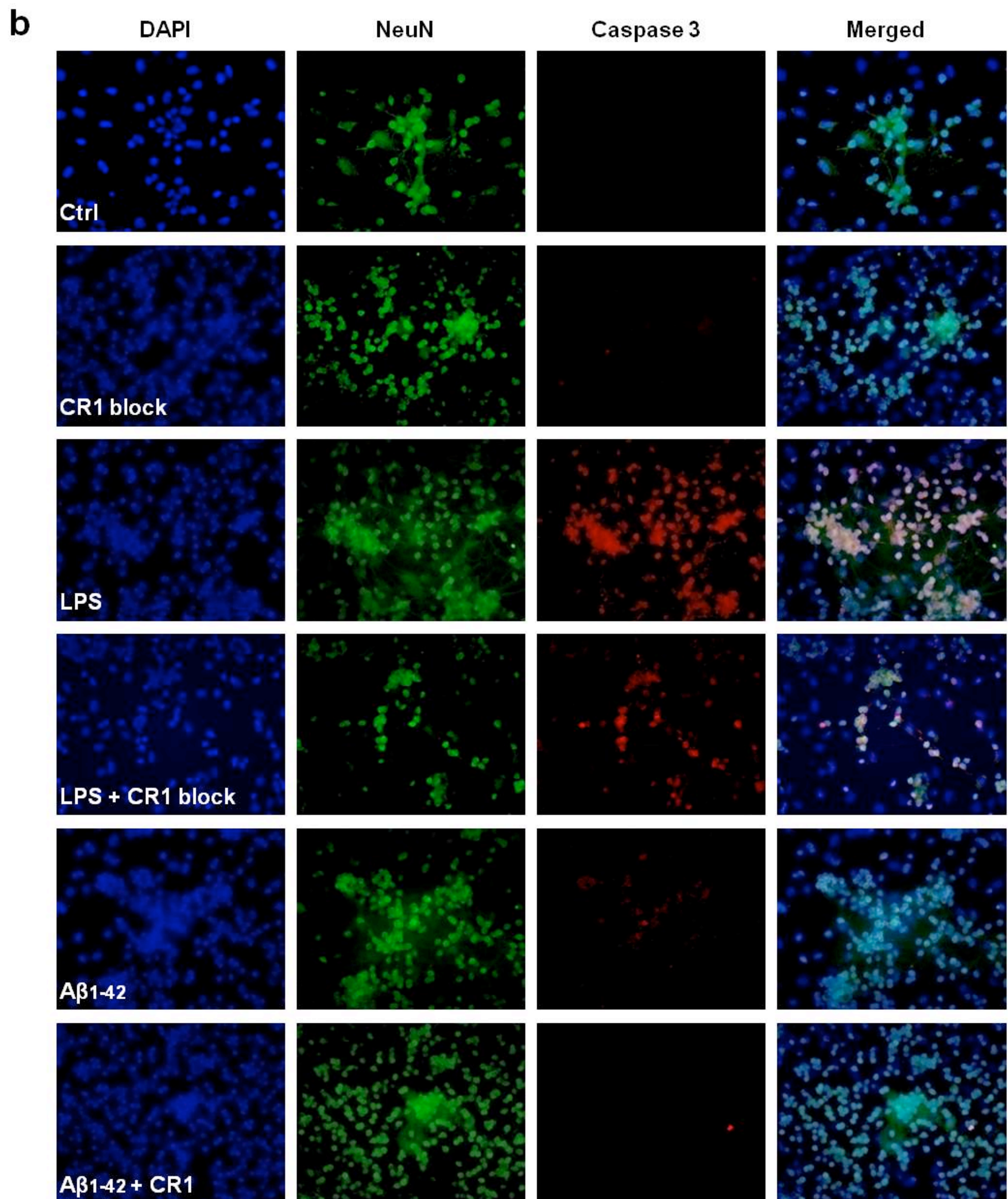


Figure 4.2: Cleaved caspase-3 cleavage can be attenuated by CR1 functional blocking antibody following activation of CGCs.

(a) Quantification and (b) images of localization of caspase-3 expression in CGC cultures performed using ICC after treatment with $A\beta_{1-42}$ (20 nM) or LPS (1 µg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL). After treatment for 48h, cultures were fixed and incubated with anti-NeuN (green) for neuronal identification and anti-cleaved caspase-3 (red). Data were analysed by one-way ANOVA ($\rho < 0.0001$) and Tukey post-hoc tests, ***$\rho < 0.001$, **$\rho < 0.01$ and *$\rho < 0.05$. Data are shown as mean ± SEM.

To ascertain whether the cell-death was due to microglia present in the CGC culture, microglia were selectively depleted using LME (25 mM) and CGC live fluorescence assay was carried out to measure cell death. Firstly, to confirm the optimum concentration to deplete microglia without increasing neuronal death, 25 mM LME for 1 h which was previously optimized in this lab, was tested in CGCs and found to have satisfactory results (Fig.4.3).

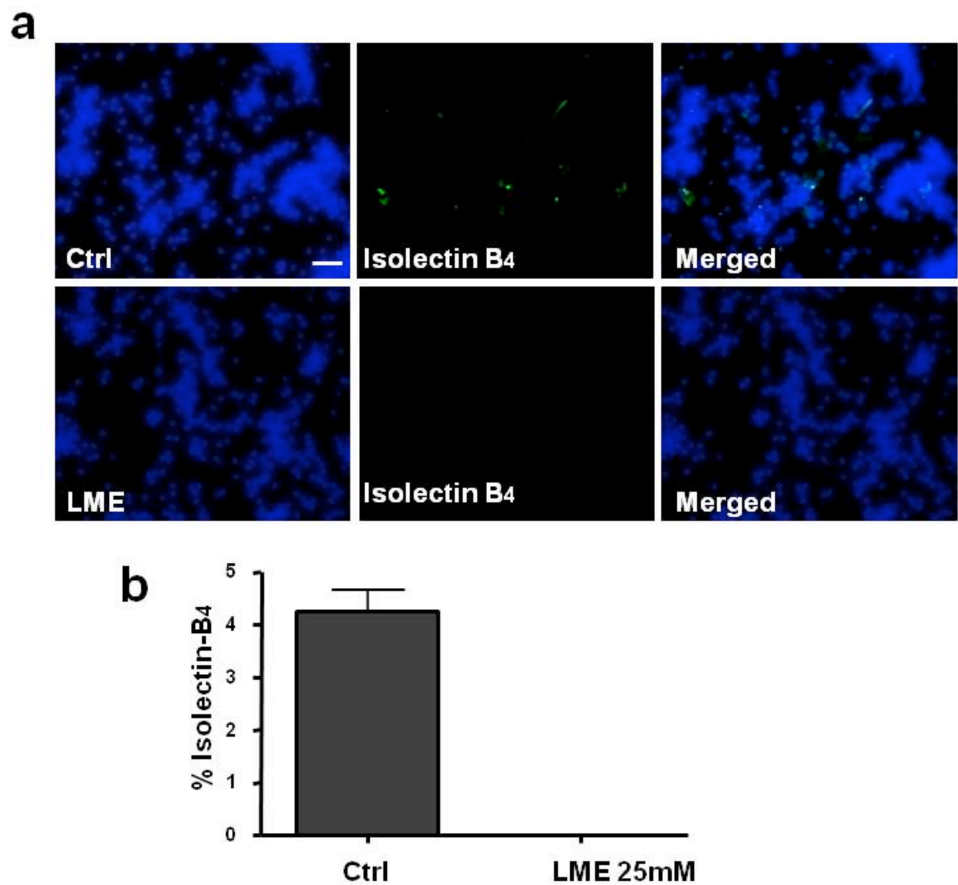


Figure 4.3: Initial characterization of LME treatment of CGCs.
(a) Representative images of ICC experiments performed on CGC cultures after treatment with 25 mM LME for 1 h. ICC was performed with DAPI (blue) for quantification of total cell number and those displaying apoptotic morphology and Isolectin B_4 (green) for microglial identification and quantification. (b) Quantification of microglial number, as percentage of control levels. Data were analysed by student's t-test. Data are shown as mean ± SEM.

Following experimental confirmation that 25 mM successfully depleted microglial cell number in CGC cultures, live fluorescence assays were carried out to investigate whether the neuronal death observed in figures 4.1 and 4.2 were due to the microglia present in the CGC cultures. Figure 4.4a displays the results from this experiment which have been separated into graphs separately showing apoptosis (Fig. 4.4b) and necrosis (Fig. 4.4c) for more a straightforward observation. Apoptosis levels were comparable between CGC cultures directly treated with control, CR1 blocking antibody (1 mg/mL), LPS and CR1 blocking antibody and $A\beta_{1-42}$ (20 nM) and $A\beta_{1-42}$ and CR1 blocking antibody and those that were also pre-treated with LME (Fig. 4.4b). However a similar trend was not observed in CGC cultures treated with LPS compared to the same treatment in LME pre-treated cultures. A higher percentage of apoptosis was observed in LPS treated CGCs minus LME (i.e. containing microglia). Measurement of necrosis by PI also demonstrated a similar result (Fig. 4.4c), showing a trend towards a decrease in cell death in LME pre-treated CGC cultures compared with non-LME treated cells following activation. This result showed a trend towards a higher percentage of cell death in non-microglia depleted cultures treated with $A\beta_{1-42}$ thereby suggesting that microglia contribute to neuronal death under these conditions.

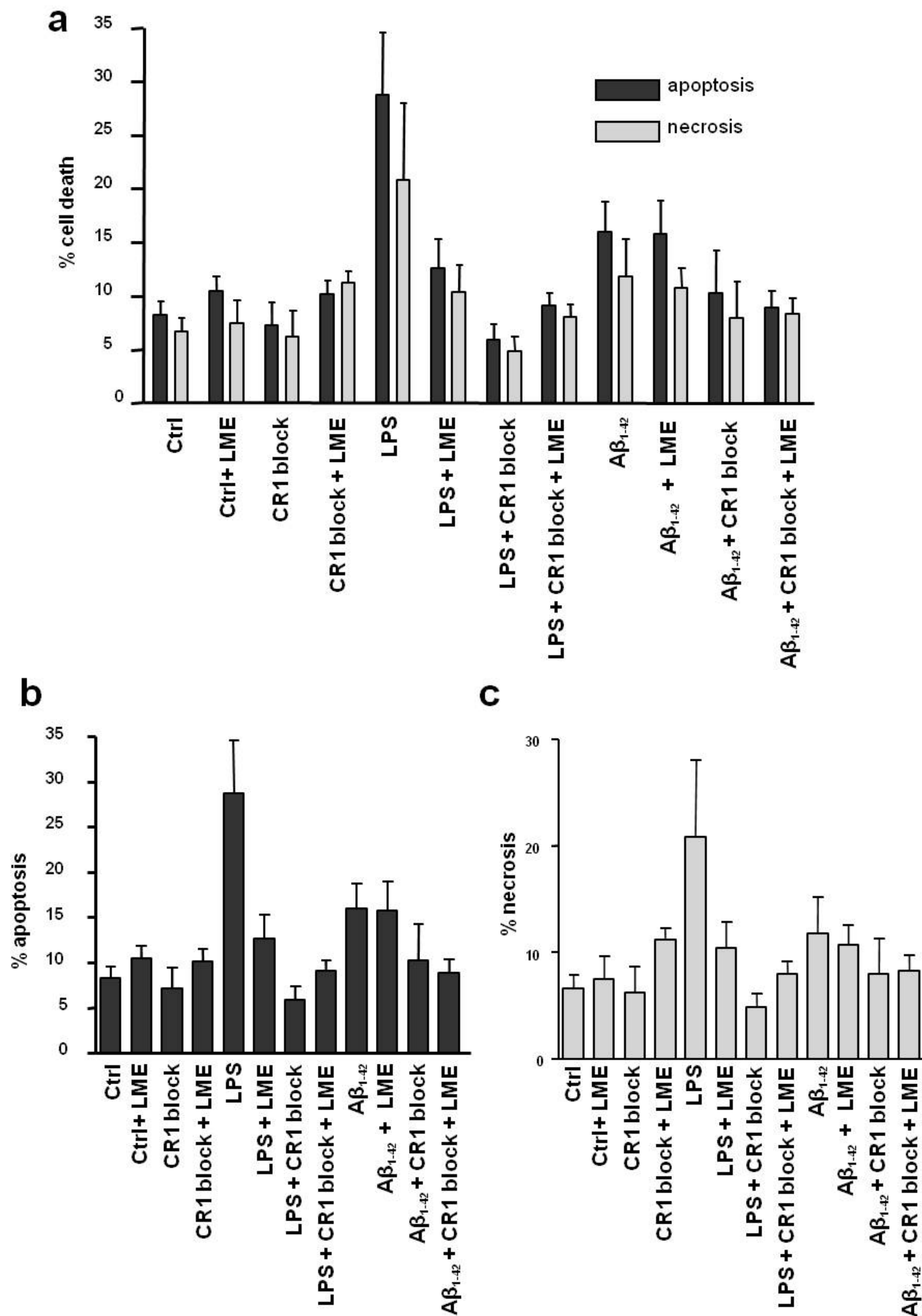


Figure 4.4: CGC neurotoxicity can be down-regulated by CR1 in the presence of microglia. Hoechst 33342 and PI staining of live CGC cultures for the quantification of apoptotic nuclear morphology and necrosis. Some cultures were depleted of microglia prior to treatment with LME. Cultures were treated with $A\beta_{1-42}$ (20 nM) or LPS (1 µg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL). (a) Graph showing all treatments and apoptosis and necrosis measurements. (a) Apoptosis measurement of CGC treatments. (b) Necrosis measurement of CGC treatments. Experiment carried out in duplicate.

4.2.2 Inhibition of microglial iNOS and TNFα result in a different neuronal response which may not mediated by CR1

Following on from the findings that microglial depletion was protective in LPS activated CGCs, and from findings in the previous chapter that microglial treatment induced the release of iNOS and TNFα in a CR1 dependent manner, it was important to investigate the contribution of these released factors to neuronal cell death. As previously shown by ICC, CR1 block alters iNOS expression by LPS activated microglia (Fig. 3.6), and this chapter has shown that blocking CR1 in LPS activated CGC cultures confers protection by reducing neuronal death. It was therefore important to evaluate if it was iNOS that was responsible for having the effect on the neuronal death observed. CGCs were incubated with 1400W dihydrochloride (1400W) at 100 μM, as used in previously published work (Yuan et al. 2012) to inhibit iNOS activity 30 min prior to treatment with LPS (1 μg/mL), $A\beta_{1\text{-}42}$ (20 nM) and CR1 blocking antibody (1 mg/mL) for 48 h (Fig. 4.6a). The data in this study also demonstrated that this concentration was not neurotoxic at this concentration (Fig. 4.5).

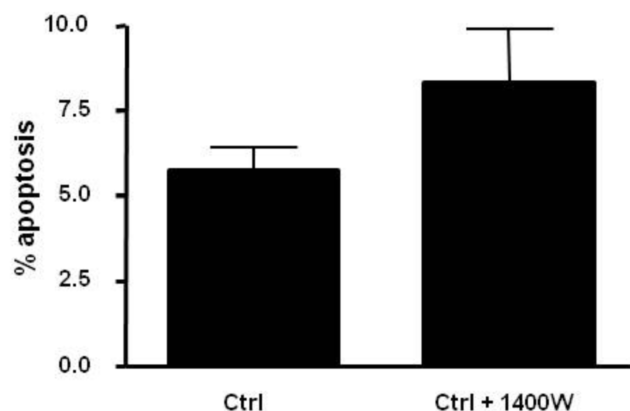


Figure 4.5: Percentage apoptosis of total cell number in control CGCs in the presence and absence of 1400W dihydrochloride (1400W) (100 μM) for 48 h demonstrates concentration is not neurotoxic.
In all of the experiments data are the mean of 3 fields per coverslip, 3 coverslips per condition from 3 separate experiments. Data were analysed by student's t-test. Data are shown as mean ± SEM.

Statistical analysis of this data was carried out comparing the percentage of apoptotic nuclei in CGCs from each treatment to their respective controls (i.e. LPS v Ctrl or LPS + CR1 block v CR1 block) and also comparing the percentage of apoptosis in each treatment in the presence and absence of 1400W pre-incubation. Direct treatment of CGCs with LPS (1 µg/mL), $A\beta_{1-42}$ (20 nM) and CR1 blocking antibody (1 mg/mL) for 48 h showed a significant decrease in apoptosis in LPS activated cells pre-incubated with 1400W. A similar significant result of decreased apoptosis was also observed in LPS and CR1 blocking antibody treated CGCs. There was however, no change in the $A\beta_{1-42}$ treated cells following pre-treatment with 1400W. The experiment was also repeated treating CGC cultures for 48h with MGCM from microglia previously treated with LPS, $A\beta_{1-42}$ and CR1 blocking antibody for 24 h (Fig. 4.6b).

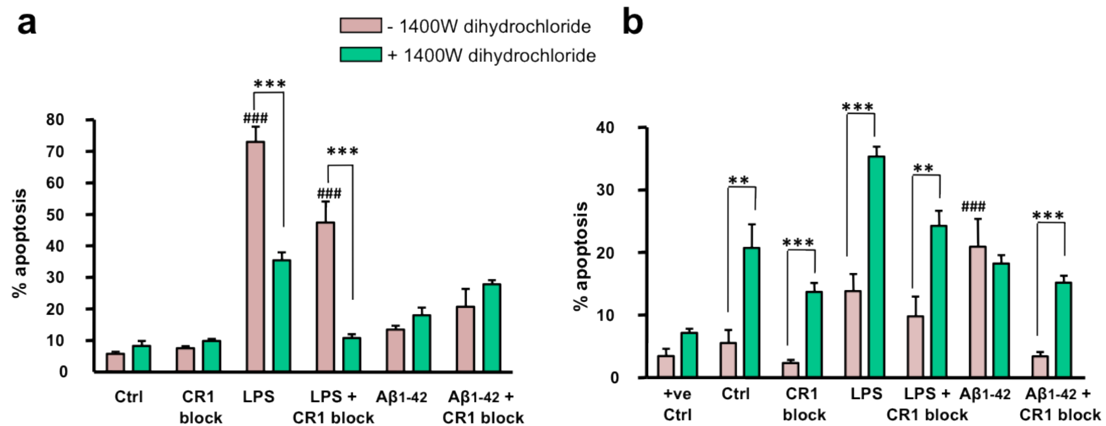


Figure 4.6: Direct 1400W dihydrochloride treatment and indirect 1400W dihydrochloride (1400W) treatment through MGCM of CGCs shows modulation of apoptosis.

Hoechst 33342 staining of live CGC cultures for the quantification of apoptotic nuclear morphology. Some CGC cultures were (a) directly pre-treated with 1400W 30 min prior to activation with $A\beta_{1-42}$ (20 nM) or LPS (1 µg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL). CGC cultures were also (b) treated with MGCM from microglia pre-treated with 1400W prior to activation with conditions as in (a). +ve Ctrl represents absence of MGCM. In all of the experiments data are the mean of 3 fields per coverslip, 3 coverslips per condition from 3 separate experiments. Data were analysed by one-way ANOVA ($\rho < 0.0001$ for a and b) and Tukey post-hoc tests, $^{***}\rho < 0.001$, $^{**}\rho < 0.01$ and $^{*}\rho < 0.05$. Data compared with respective controls (Ctrl or CR1 block) were marked with # and represent $^{###}\rho < 0.001$. Data are shown as mean ± SEM.

The results from this experiment showed an increase in apoptosis from the CGCs treated with the MGCM from the microglia pre-incubated with 1400W in all conditions except $A\beta_{1\text{-}42}$ (Fig. 4.6b).

TNFα secretion was also previously shown by ELISA to be increased in LPS treated microglia and further decreased with CR1 blocking antibody co-treatment (Fig. 3.5) which coincided with data from this chapter showing a reduction in LPS activated neuronal death (Fig. 4.1a) and a trend towards a decrease in neuronal death following MGCM exposure (Fig. 4.1c). Investigation into whether TNFα played a role in this reduction of neuronal death was carried out through pharmacological inhibition of TNFα synthesis. To confirm that Thalidomide was sufficiently inhibiting TNFα synthesis at the concentration used, an ELISA to measure TNFα secretion in MGCM from microglia pre-incubated with Thalidomide was carried out. Thalidomide (10 μg/mL) appeared to sufficiently inhibit TNFα secretion in primary microglia (Fig. 4.7).

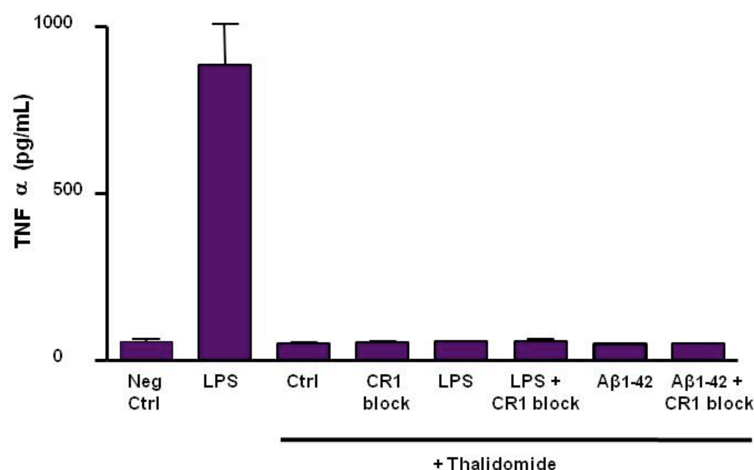


Figure 4.7: ELISA analysis of TNFα secretion from primary cultured rat microglia.
Analysis after treatment with LPS (1 μg/mL), Aβ1–42 (20 nM) and CR1 blocking antibody (1 μg/mL) for 24 h, following 1 h pre-treatment with Thalidomide (10 μg/mL). Reduced TNFα secretion following 1 h pre-treatment with Thalidomide.

CGC cultures were pre-incubated with Thalidomide at 10 µg/mL, previously recognised as optimum concentration to inhibit TNFα (Mohty et al. 2002), for 1 h before treatment with LPS (1 µg/mL), $A\beta_{1-42}$ (20 nM) and CR1 blocking antibody (1 mg/mL) for 48 h (Fig. 4.8a). Statistical analysis of this data was carried out similar to the previous experiment with 1400W, comparing the percentage of apoptotic nuclei in CGCs from each treatment to their respective controls (i.e. LPS v Ctrl or LPS + CR1 block v CR1 block). Comparison of the percentage of apoptosis in each treatment in the presence and absence of thalidomide pre-incubation was also carried out. Significant reduction in apoptosis was only observed in CGCs treated with LPS and LPS with CR1 blocking antibody. When the neuronal apoptotic effect was investigated following microglial TNFα inhibition, an increase in apoptosis was measured in CGCs treated with $A\beta_{1-42}$ treated MGCM and also with co-treatment with CR1 blocking antibody (Fig. 4.8b).

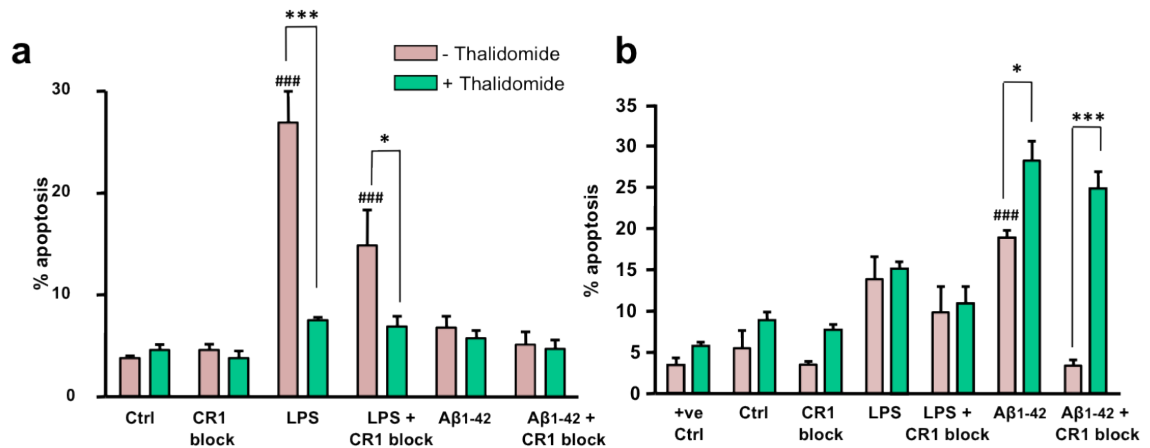


Figure 4.8: Direct thalidomide treatment and indirect thalidomide treatment through MGCM of CGCs shows modulation of apoptosis.

Hoechst 33342 staining of live CGC cultures for the quantification of apoptotic nuclear morphology. Some cultures were (a) directly pre-treated with Thalidomide 1 h prior to activation with $A\beta_{1-42}$ (20 nM) or LPS (1 µg/mL) in the presence or absence of CR1 blocking antibody (1 mg/mL). CGC cultures were also (b) treated with MGCM from microglia pre-treated with Thalidomide prior to activation with conditions as in (a). In all of the experiments data are the mean of 3 fields per coverslip, 3 coverslips per condition from 3 separate experiments. Data were analysed by one-way ANOVA ($\rho < 0.0001$) and Tukey post-hoc tests, ***$\rho < 0.001$, **$\rho < 0.01$ and *$\rho < 0.05$. Data compared with respective controls (Ctrl or CR1 block) were marked with # and represent $^{###}\rho < 0.001$. Data are shown as mean ± SEM.

4.3 Discussion

Exploring the consequences of blocking CR1 on microglial-neuronal interactions by 48 h treatment with MGCM from microglia treated with CR1 blocking antibody, LPS or $A\beta_{1-42}$, and after direct treatment with these activators demonstrated that modulating CR1 has an effect on neuronal survival. Direct treatment with LPS resulted in increased neuronal death (Fig. 4.1a) and this death was decreased in cells co-treated with CR1 blocking antibody (Fig. 4.1a). LPS is capable of activating the complement system in macrophages as measured by iC3b ELISA (Sladowski et al. 2001) ultimately leading to cell lysis. Blocking CR1, of which binding to C3b is necessary for the opsonization of pathogens, may therefore lead to a reduction in neuronal apoptosis and necrosis. This result is also in line with data from the previous chapter showing an increase in TNFα secretion following LPS activation, together with a decline in secretion observed upon co-treatment with CR1 blocking antibody (Fig. 3.5a). This link was further investigated and will be discussed in greater detail later.

A similar result to direct LPS treatment of CGCs was found with microglial-neuronal interactions, showing an increase in neuronal death following LPS MGCM incubation (Fig. 4.1c), however this was not modulated by the co-treatment with CR1 blocking antibody as it was with direct neuronal treatment. Microglial release of TNFα is very fast and a time-course analysis after the addition of 1 μg/mL LPS has shown 6 h to be the peak of TNFα release after which it gradually decreases (Nakamura et al. 1999; Pinteaux-Jones et al. 2008). MGCM for this experiment was collected following 24 h LPS treatment and as such the levels of TNFα present in the MGCM would have

been lower, possibly contributing to the lower amount of neuronal death measured compared with direct treatment with LPS. However TNFα is not the only inflammatory factor important for determining the outcome of neuronal survival. A significant amount of NO, matching the peak production of TNFα, was released at 24 h following LPS treatment (Nakamura et al. 1999) which may also reflect the increase in neuronal death (Fig. 4.1c).

The rescuing effect observed with direct treatment of CGCs with CR1 blocking antibody together with LPS was not replicated in neurons directly treated with $A\beta_{1-42}$ (Fig.4.1a). This may be due to the low nanomolar concentration of $A\beta_{1-42}$ used (20 nM) which may not produce a direct toxic effect to neurons, but can have damaging effects on neurons through the inflammatory role of glia (Neniskyte et al. 2011). The increased apoptosis of CGCs treated with $A\beta_{1-42}$ MGCM for 48 h (Fig. 4.1c) was significantly decreased when the neurons were exposed to MGCM from microglia treated with CR1 blocking antibody together with $A\beta_{1-42}$. Binding of $A\beta_{1-42}$ to the receptor for advanced glycation end products (RAGE) can induce a microglial mediated inflammatory and neurotoxic response (Yan et al. 1996) and Aβ oligomers, fibrils and aggregates can all bind to this receptor (Sturchler et al. 2008). This behavior creates a harmful cellular environment, in which the persistent activation of microglia causes them to adopt a predominantly neurotoxic phenotype, resulting in neuronal cell death (Fang et al. 2010). The decrease in apoptosis seen in CGCs treated with MGCM from microglia treated with $A\beta_{1-42}$ and CR1 blocking antibody suggests that manipulating CR1 has a positive effect on neuronal survival (Fig. 4.1c.). Total cell number was also measured to

eliminate the possibility that a reduction in neuronal death was a consequence of changes in cell number (Fig. 4.1b and 4.1d). These results appeared to correlate with hippocampal neuronal viability data (Fig. 4.1e).

Caspase-3 is one of the proteases that serves as a primary mediator for apoptosis (Thornberry & Lazebnik 1998) therefore to further support the live cell assay with direct treatment of CGCs from figure 4.1a, ICC probing for cleaved caspase-3 and NeuN was carried out (Fig. 4.2). This experiment demonstrated an increase in cleaved caspase-3 expression after CGC treatment with LPS, which was then shown to be significantly decreased in the presence of CR1 blocking antibody (Fig. 4.2). These data show the same result and are in line with the live assay, thus strengthening the finding that inhibiting CR1 may have a protective role on neurons.

A small number of microglia, ~5%, are also present in CGC cultures (despite Ara-C treatment) (Fig. 4.3) and so it is was important to investigate whether the microglia present in the culture contribute to neuronal death. After successfully confirming that 25 mM LME was sufficient to deplete a CGC culture of microglia (Fig. 4.3), all conditions previously used in the live assay were added to the LME treated CGCs. Depletion of all microglia from the CGC culture resulted in a trend towards a reduction in apoptosis (Fig.4.4b) and necrosis (Fig. 4.4c) in LPS treated cells, suggesting that microglia influenced the neuronal death observed. Microglia release neurotoxic factors upon activation with pro-inflammatory stimuli such as LPS resulting in neuronal

death (Block et al. 2007) explaining the reduction in neuronal death in CGC cultures in absence of microglia.

iNOS expression as measured by ICC in chapter 3 demonstrated the ability of blocking CR1 on reducing the expression of iNOS from LPS and $A\beta_{1-42}$ treated microglia (Fig. 3.6a and 3.6b), which is a somewhat similar trend to the neuronal loss data measured in this study (Fig. 4.1 and 4.2). To confirm the involvement of increased microglial iNOS with neuronal death, the enzyme was inhibited before activation of microglia and the MGCM was incubated with the CGCs (Fig.4.6b). The contrasting results of the MGCM treated CGCs to the directly treated CGCs (Fig. 4.6a) pose a question of whether iNOS works alone to bring about neurotoxicity and if so, whether disrupting this combination will cause an imbalance and subsequent increased neurtoxicity. However, control and CR1 block MGCM had a significantly increased amount of neuronal death when iNOS was inhibited indicating that this is not an activation specific response. Further investigation into the cause of this increase death would need to be carried out, perhaps by evaluating the soluble factors present in the MGCM.

As previously shown in chapter 3, LPS increases the secretion of the pro-inflammatory cytokine TNFα (Fig. 3.5a), which is able to activate the extrinsic pathway of caspase activation through binding to cell surface receptor, TNFR1, followed by oligomerization and cleavage of pro-caspase-8 and -10 ultimately resulting in apoptosis (Micheau & Tschopp 2003). This was in line with results in this study that were able to

demonstrate an increase in neuronal apoptosis following direct LPS treatment and incubation with MGCM from LPS treated microglia (Fig. 4.1a and 4.1c).

A similar correlation between TNFα secretion and neuronal death was also observed between reduced TNFα secretion from microglia by ELISA analysis following co-treatment with CR1 blocking antibody (Fig. 3.5a) and a trend towards a reduction in neuronal death in CR1 co-treated MGCM (Fig. 4.1c). To ascertain the involvement of TNFα secretion as a contributing element to the neuronal apoptosis, pharmacological inhibition of TNFα was performed (Fig. 4.7). Data from direct CGC treatment, shows that apoptosis was significantly reduced in LPS treated cells where TNFα was inhibited prior to incubation suggesting that this pro-inflammatory cytokine was in fact a contributor to the neuronal death. Therefore, the reduced TNFα secretion measured following co-treatment with CR1 blocking antibody may then be a tenable mechanism for reduced neuronal death following CR1 inhibition. However this measurement was in the neuronal culture and does not reflect the microglial TNFα measurements reported in chapter 3. Inhibition of microglial TNFα secretion did not appear to reduce apoptosis in the $A\beta_{1-42}$ MGCM treated CGCs (Fig. 4.8b) perhaps suggesting that TNFα may work in concert with another toxic mediator to contribute to neuronal death. This could be examined further by a more comprehensive cytokine array or by mass spectrometry. TNFα was decreased in the MGCM added to the CGCs as shown by ELISA (Fig. 4.7) therefore it is unlikely that this increase was due to unsuccessful TNFα inhibition. It is important to note that TNFα does not solely play a neurotoxic role. It's pleiotropic characteristic means it can also exert protective effects by binding to

different TNFRs (Sriram & O'Callaghan 2007). Microglia are have been shown to express both TNFR1 and TNFR2 and it is thought that the patterns of these receptors on microglia influence the outcome of TNFα binding (Dopp et al. 1997; Dziewulska & Mossakowski 2003). It may be possible that $A\beta_{1-42}$ has an effect on the distribution of TNFRs whereby increasing the expression of TNFR1 which could lead to an increase in neuronal apoptosis following $A\beta_{1-42}$ MGCM treatment (Fig. 4.1c & 4.8b) despite low levels of microglial TNFα secretion by microglia (Fig. 3.5a). Therefore it may have been interesting, to carry out Western blot experiments to investigate TNFR1 and TNFR2 protein level differences between treated cells. It is important to note that thalidomide has also been thought to inhibit other cytokines such as IL-6, IL-12 and IL-10 and also have an stimulatory effect on the production of IL-2, IL-4 and IL-5 (Singhal & Mehta 2002), therefore possibly effecting the cell survival of the neurons.

4.4 Conclusions

The aim of the research presented in this chapter was to investigate the effect of CR1 activation on neuronal survival and the results provide indications of how CR1 may play a role in neuronal cell death in neurodegeneration. Taken together with the results from chapter 3, it was interesting to find that CR1 modulation of microglial TNFα and iNOS may not be the main contributors to the neuronal death observed, suggesting that other contributors deserve attention.

Chapter 5

Preliminary investigation into CR1 expression in Alzheimer’s disease post-mortem brain tissue and control red blood cell CR1 levels

5. Preliminary investigation into CR1 expression in Alzheimer's disease post-mortem brain tissue and control red blood cell CR1 levels

5.1 Introduction and summary of results

The studies performed in the previous chapters have demonstrated that CR1 plays an important role in modulating microglial activation, phagocytosis and subsequent neuronal survival *in vitro*, and here, human post-mortem tissue was used to determine whether CR1 is up-regulated in the AD brain. It was hoped that this may provide evidence that CR1 may play an important role in *in vivo* as well as *in vitro*. The Aβ protein is transported in both directions across the blood brain barrier (Deane et al. 2009) which thereby maintains an equilibrium between soluble Aβ levels in the brain and blood. CR1 is expressed on human red blood cells (Dobson et al. 1981). Peripheral $A\beta_{42}$ clearance in the blood has been shown to occur through a C3b-dependent adherence to CR1 on red blood cells (RBCs) (Rogers et al. 2006). Therefore individuals who express low levels of CR1 on RBCs might be less efficient at immune-adherence mechanisms of Aβ removal, resulting in possible gradual accumulation of Aβ protein in the brain with aging and increased risk of AD.

To date conflicting reports exist as to whether and where CR1 is expressed in the brain (Singhrao et al. 1999; Singhrao et al. 2000; van Beek et al. 2005), therefore in order to investigate CR1 expression in the brain, paraffin sections from different regions of the human brain were immunostained. Aβ plaque burden, CR1 expression and cellular localization in frontal, temporal, hippocampal and parietal tissue of AD

patients and age-matched controls (AMC) was determined. A number of CR1 antibodies generated by collaborators, Dr Anne Nicholson-Weller and Dr Ionita Ghiran, targeting different epitopes, were tested and optimized for this use by IHC. Flow cytometry to quantify the levels of CR1 on RBCs isolated from fresh blood drawn from control individuals was also carried out to determine differences in RBC CR1 quantity among individuals and to investigate any inverse correlation with circulating Aβ levels in the blood.

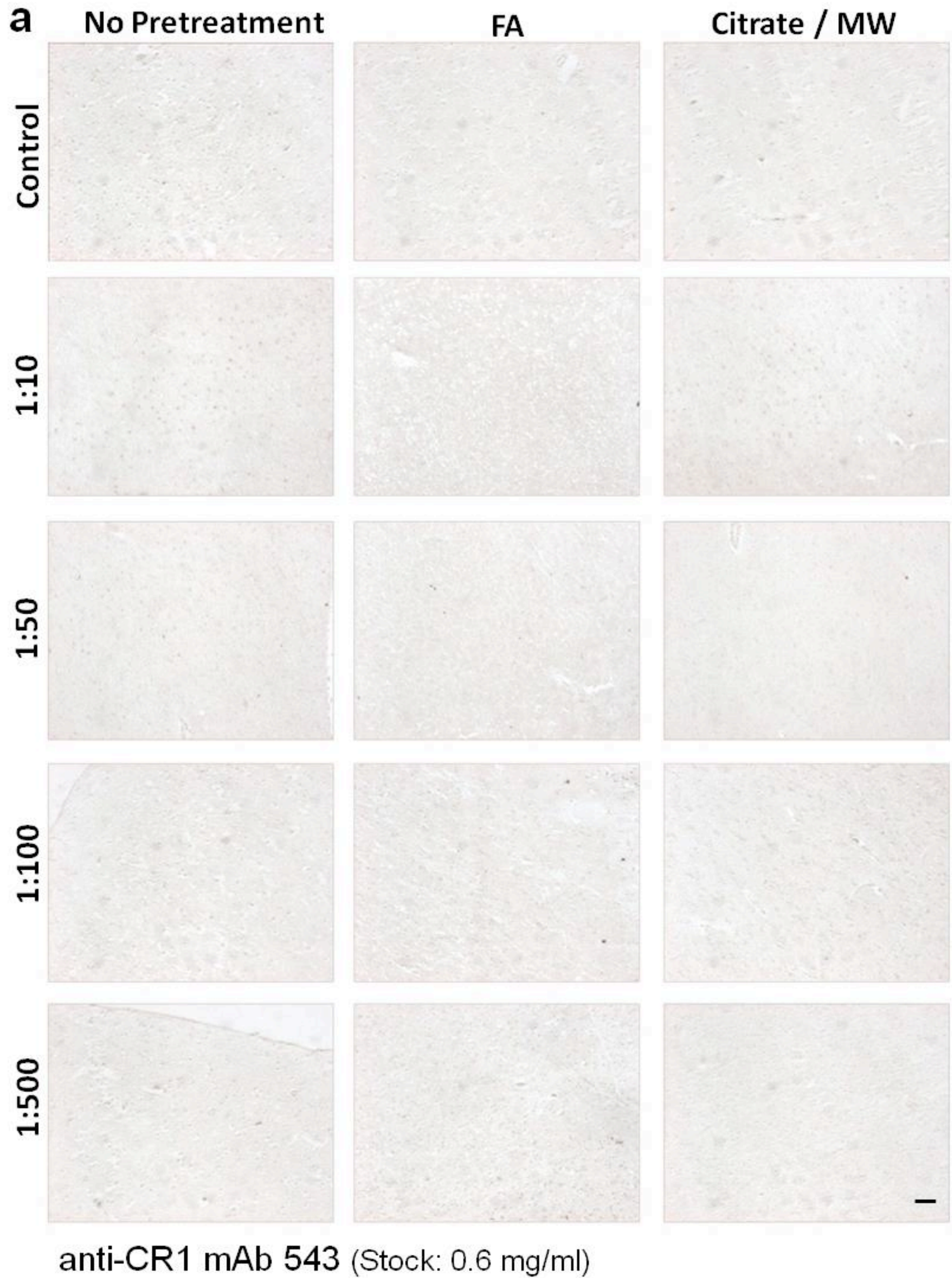
Data presented in this chapter provide evidence for the presence of CR1 in human AD and control brain and indicate differences in CR1 coverage between four brain regions in areas with high and low plaque burden. The findings of this study also indicate differences in RBC CR1 levels between individuals.

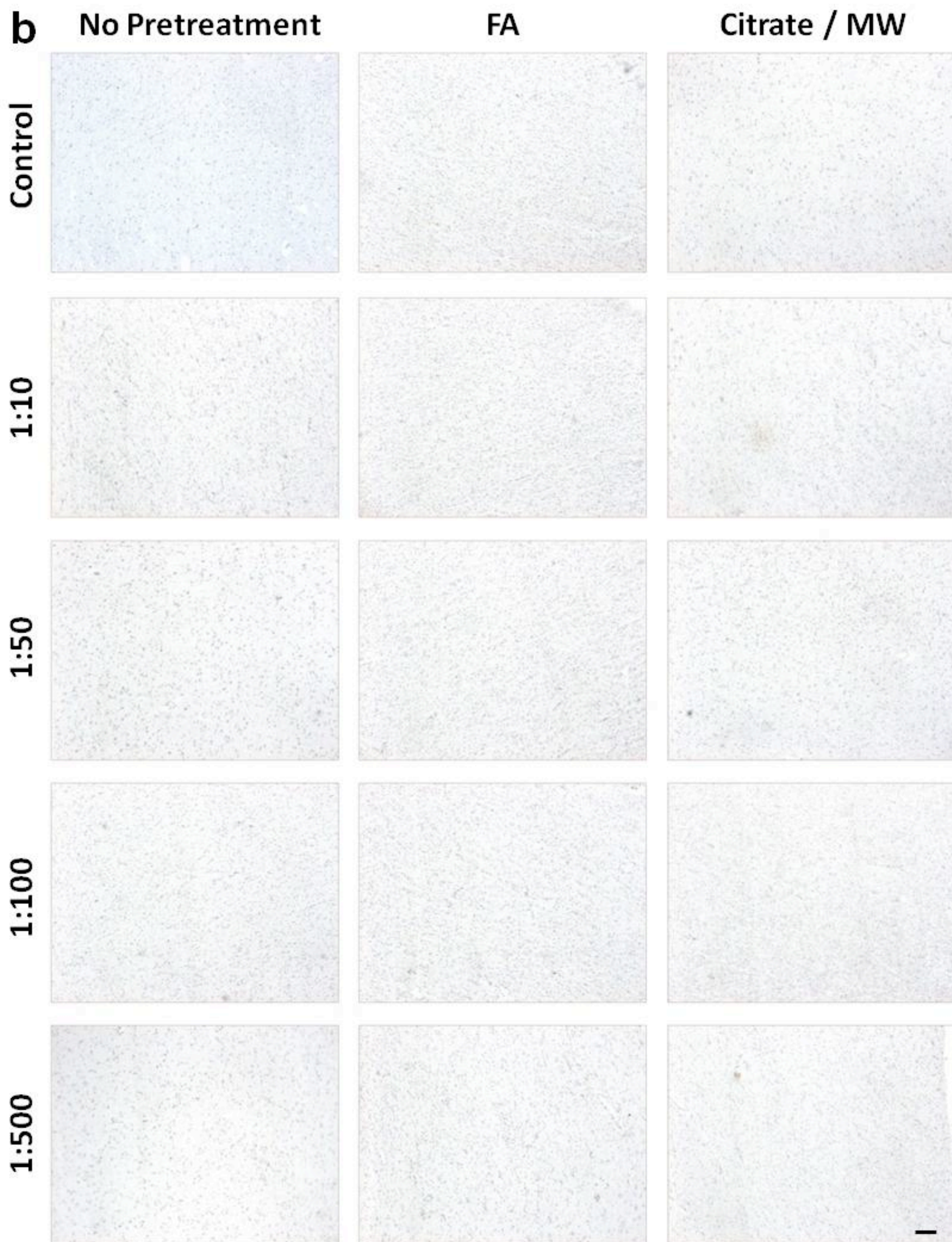
5.2 Results

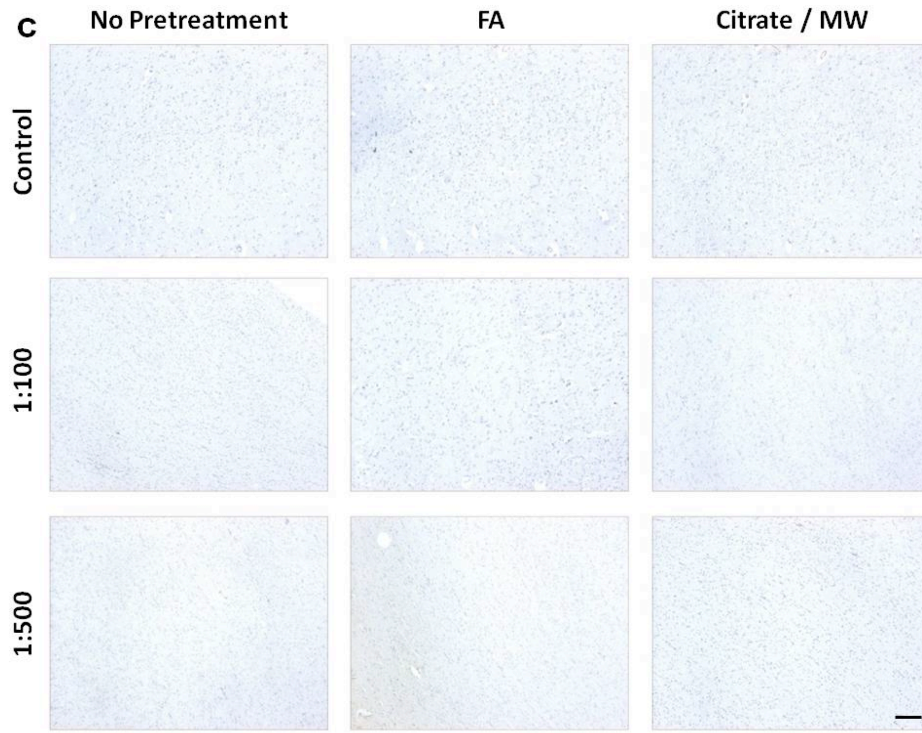
5.2.1 Optimization of CR1 antibodies indicates anti-CR1 6B1 is appropriate for investigation into CR1 expression in *post-mortem* brain tissue

There is very little published literature describing immunoreactivity of any commercially available CR1 antibody in human brain tissue. Therefore a great deal of optimization was required to begin investigation of CR1 expression in brain tissue through IHC using the range of antibodies available for this project. This study began by determining the optimal concentration and IHC pre-treatment procedures. Formalin or other aldehyde fixation treatments form protein cross-links that mask the antigenic sites in the tissue which can lead to weak staining or even false negative staining. There are a number of treatments that can be carried out to break these protein cross-links, thus unmasking the antigens and epiptopes in formalin-fixed and paraffin embedded tissue sections leading to enhanced staining intensity. Formic acid is a solution that is capable of breaking these protein cross-links and successfully works as a pre-treatment for IHC working especially well with Aβ antibody staining (Kitamoto et al. 1987). Heat-mediated antigen retrieval with citrate buffer in conjunction with a microwave treatment (citrate/mw) is another method of breaking the protein cross-links to enhance staining intensity and is suitable for most antibody applications (Shi et al. 1991). Immunoreactivity detected by 6 different CR1 antibodies was investigated in parietal tissue: anti-CR1 mAb 543, anti-CR1 pAb, anti-CR1 mAb 3C10, anti-CR1 mAb IC7, anti-CR1 mAb YZ1 and anti-CR1 mAb 6B1 (Fig. 5.1). In this study, immunoreactivity is only shown in parietal tissue with the anti-CR1 mAb 6B1 which targets LHR-D. Heat-mediated antigen retrieval was considered the optimum pre-treatment as it produced

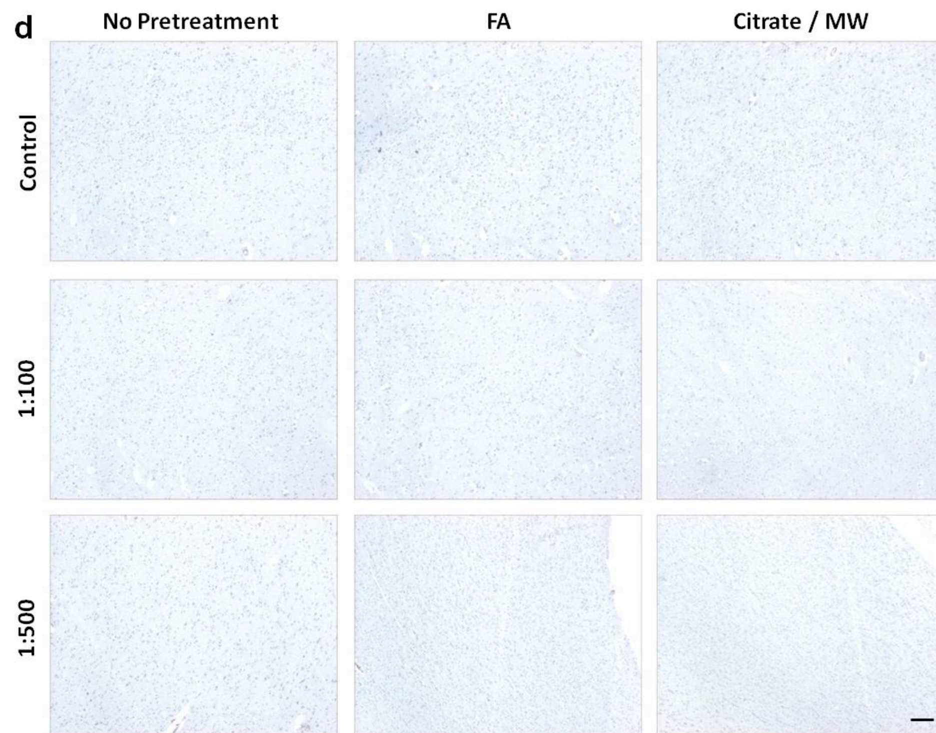
less damage to the tissue and 4.6 µg/mL was chosen as the optimum concentration of anti-CR1 6B1 to use (Fig. 5.1f).



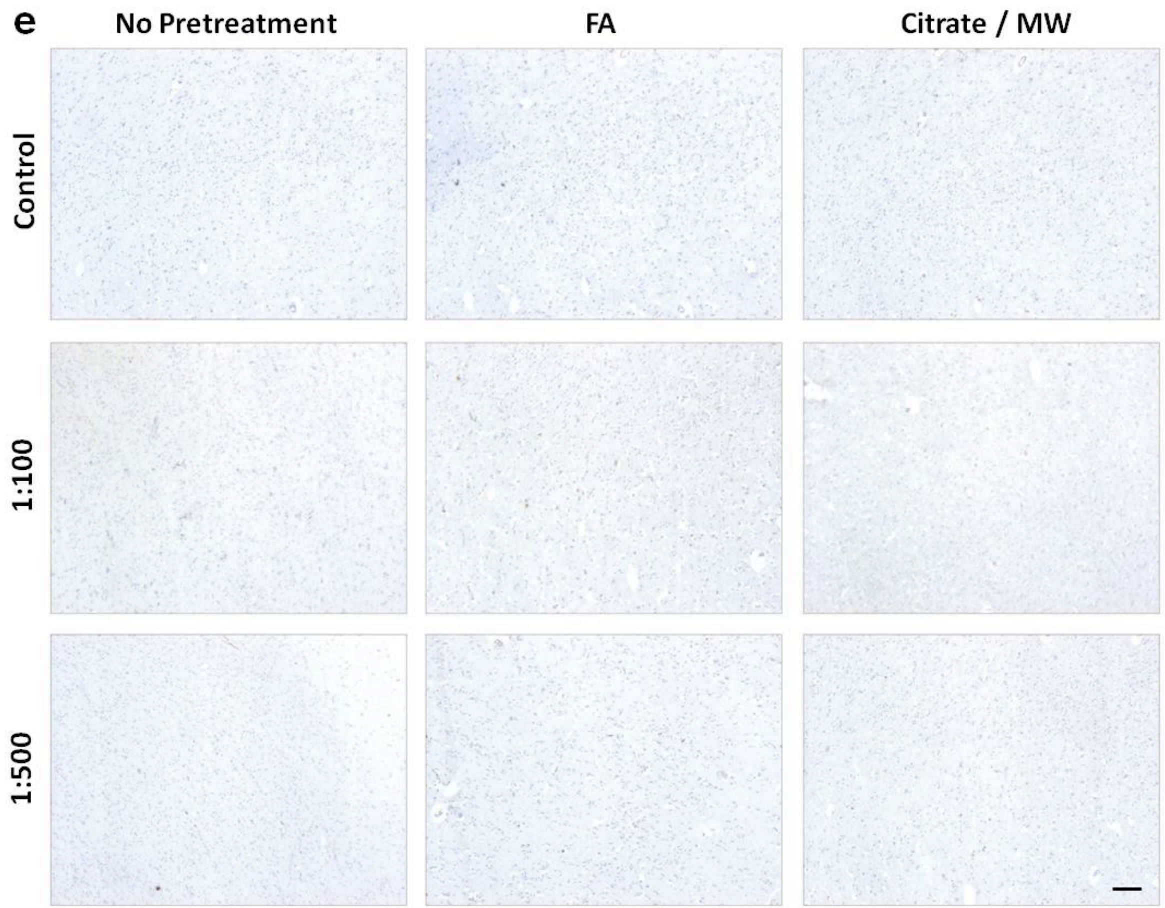

b
No Pretreatment
FA
Citrate / MW
Control
1:10
1:50
1:100
1:500
anti-CR1 pAb (Stock: 5 mg/ml)



anti-CR1 mAb 3C10 (Stock 0.2 mg/ml)



anti-CR1 mAb IC7 (Stock 0.8 mg/ml)


e
No Pretreatment
FA
Citrate / MW
Control
1:100
1:500
anti-CR1 mAb YZ1 (Stock 3 mg/ml)

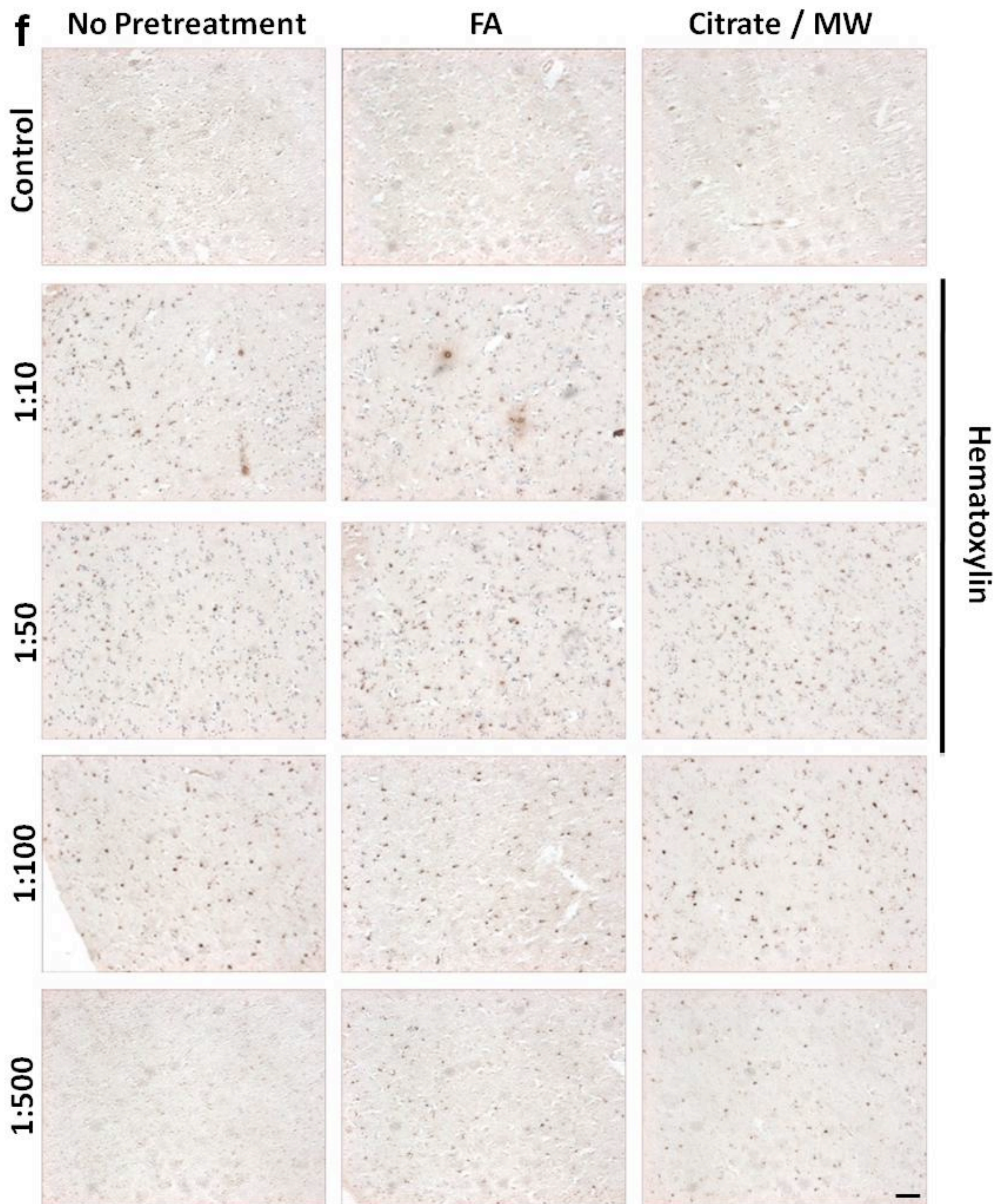


Figure 5.1: Optimization of immunohistochemistry in human post-mortem brain tissue using anti-CR1 antibodies at varying pretreatments and concentrations.

(a) anti-CR1 mAb 543 (stock: 0.6mg/ml), (b) anti-CR1 pAb (stock: 3mg/ml), (c) anti-CR1 mAb 3C10 (stock: 0.2mg/ml), (d) anti-CR1 mAb IC7 (stock: 0.8mg/ml), (e) anti-CR1 mAb YZ1 (stock: 3mg/ml) and (f) anti-CR1 mAb 6B1 (stock: 0.465mg/ml) were tested on paraffin-embedded parietal cortex tissue from an 84yr old male AD brain. Fixation length was 2 h in 10% formalin. Scale bar represents 100µm

Anti-CR1 mAb 6B1 was further investigated at a higher magnification using a 40x objective on an Olympus BX50 microscope (Fig.5.2). The tissue was also stained with the nuclear stain, hematoxylin, and it was found that CR1 immunoreactivity was colocalized to cells positively stained with hematoxylin.

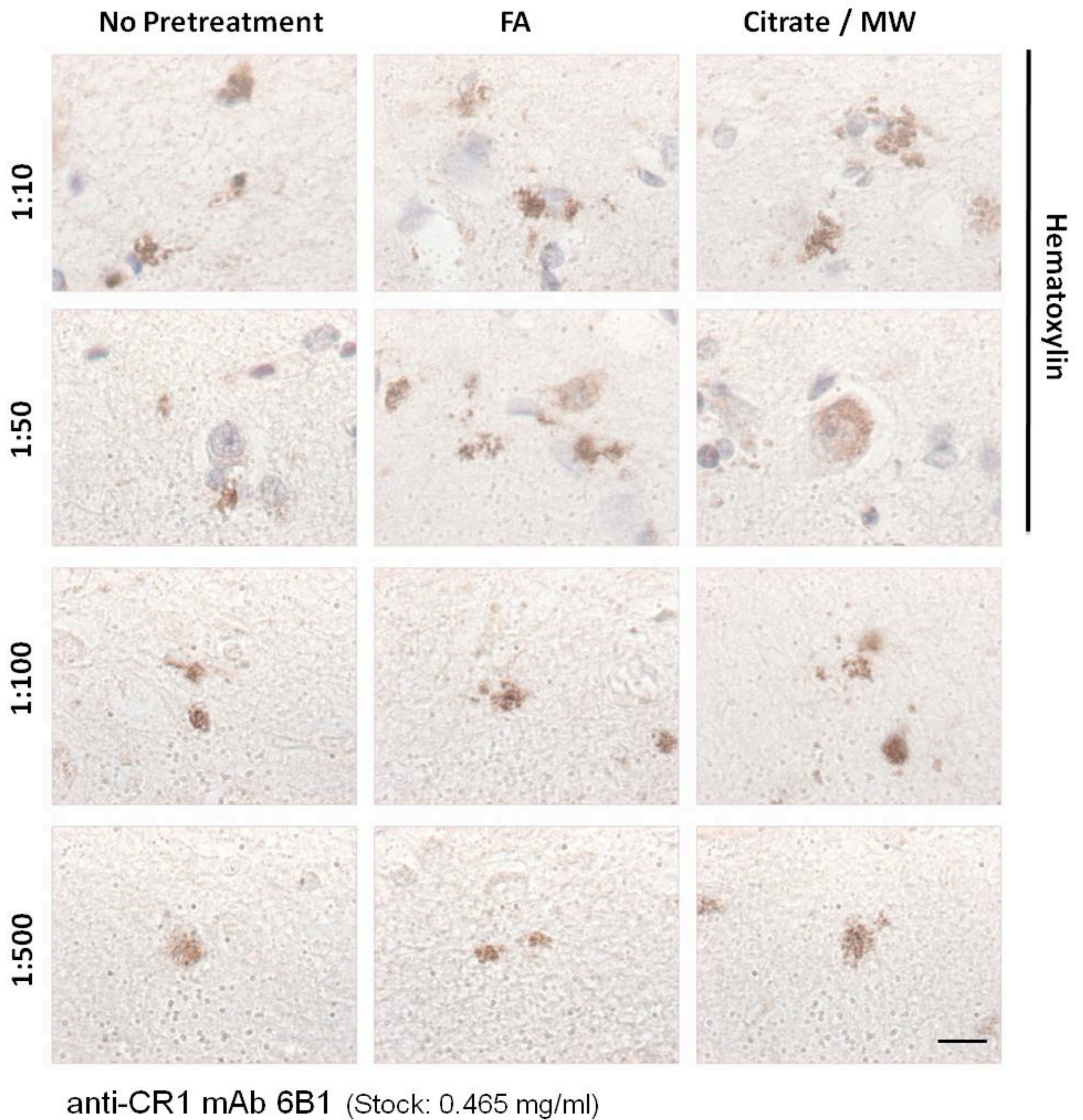


Figure 5.2: Immunohistochemistry in paraffin-embedded parietal cortex tissue from 84yr old male AD patient using anti-CR1 6B1 antibody.
Fixation length was 2 h in 10% formalin. Scale bar represents 50µm.

Another CR1 antibody that was commercially available and described to work well on paraffin-embedded tissue, anti-CR1 E11, was used for CR1 detection through IHC in the parietal cortex of an AD brain. (Fig.5.3). This antibody did not show any immunoreactivity.

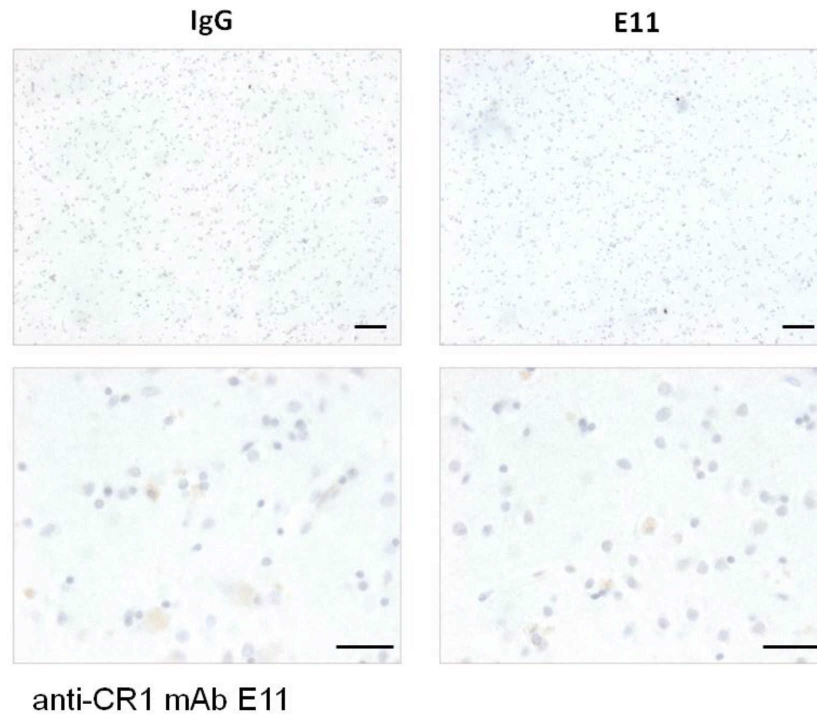


Figure 5.3: Immunohistochemistry in paraffin-embedded parietal cortex tissue from 81yr old female AD patient using anti-CR1 E11 antibody (5 μg/ml) compared with IgG control. Pretreatment for antigen retrieval was heat mediated with citrate buffer (citrate/MV). Fixation length was 2 h in 10% formalin. Scale bar represents 100um

In the periphery, CR1 aids in the removal of complement-opsonized particles by their transfer to macrophages in the liver and spleen (Ghiran et al. 2008b), and so immunoreactivity of these antibodies was tested in spleen tissue as a positive control (Fig. 5.4).

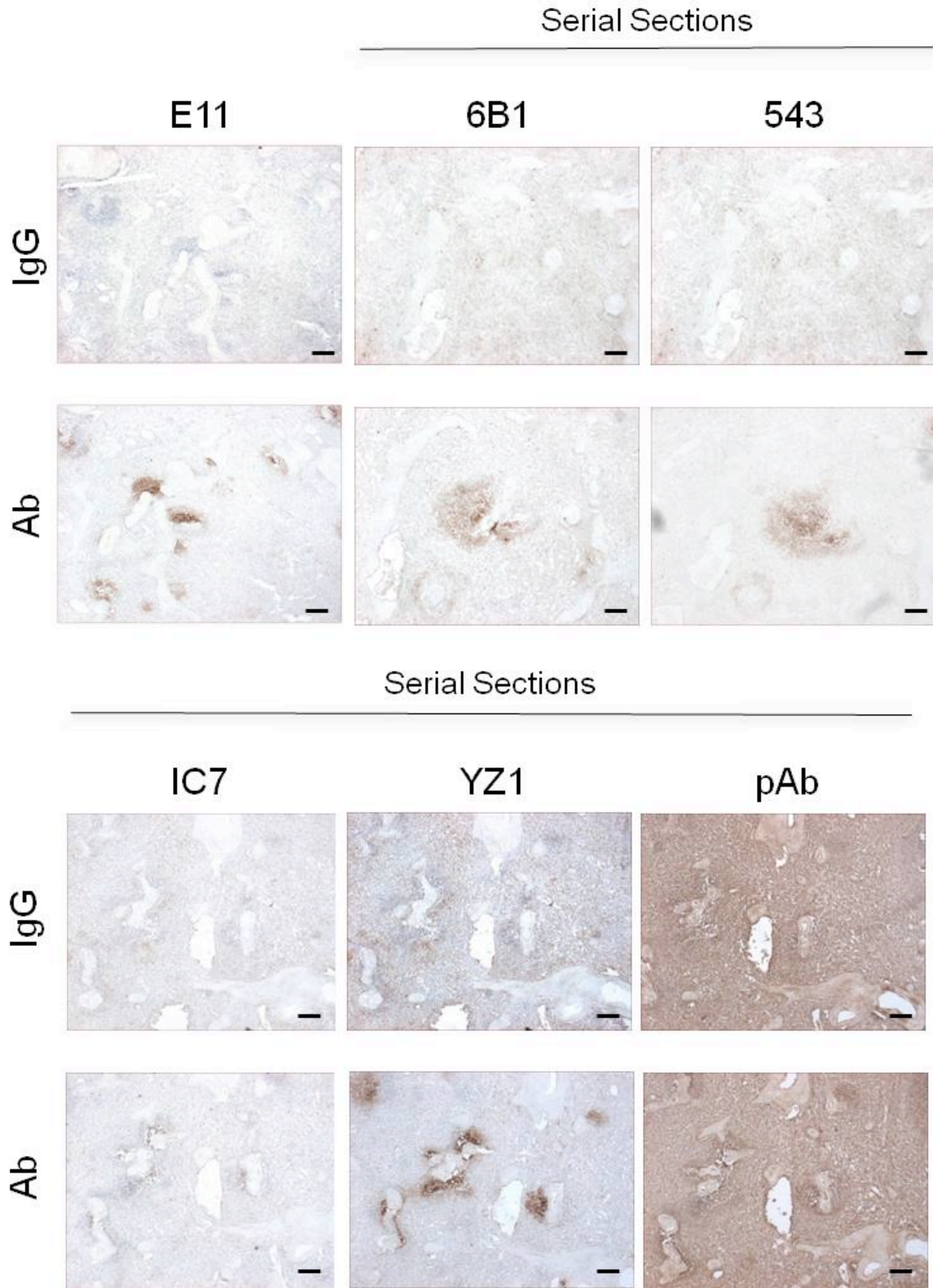


Figure 5.4: CR1 antibody optimization on spleen tissue.

Immunohistochemistry in paraffin-embedded, briefly fixed spleen tissue from 77yr old female AD patient using the range of CR1 antibodies targeting different CR1 LHR domains (see Table 2.3) and IgG control. Scale bar corresponds to 200µm

The results in Fig. 5.4 were able to show that there was immunoreactivity with anti-CR1 mAb- E11 (5 µg/ml), -6B1 (4.6 µg/ml), -543 (6 µg/ml) and YZ1 (30 µg/ml). Anti-CR1 pAb (50 µg/ml) appeared to have a high background which may be due to tissue quality and anti-CR1 mAb IC7 (8 µg/ml) did not appear to show any immunoreactivity in the control spleen tissue. Immunofluorescence (IF) staining using the range of anti-CR1 antibodies was performed however the experiment was unsuccessful after a number of optimizations with most antibodies. Anti-CR1 mAb E11 was the only antibody which was able to provide IF in control spleen tissue (Fig. 5.5).

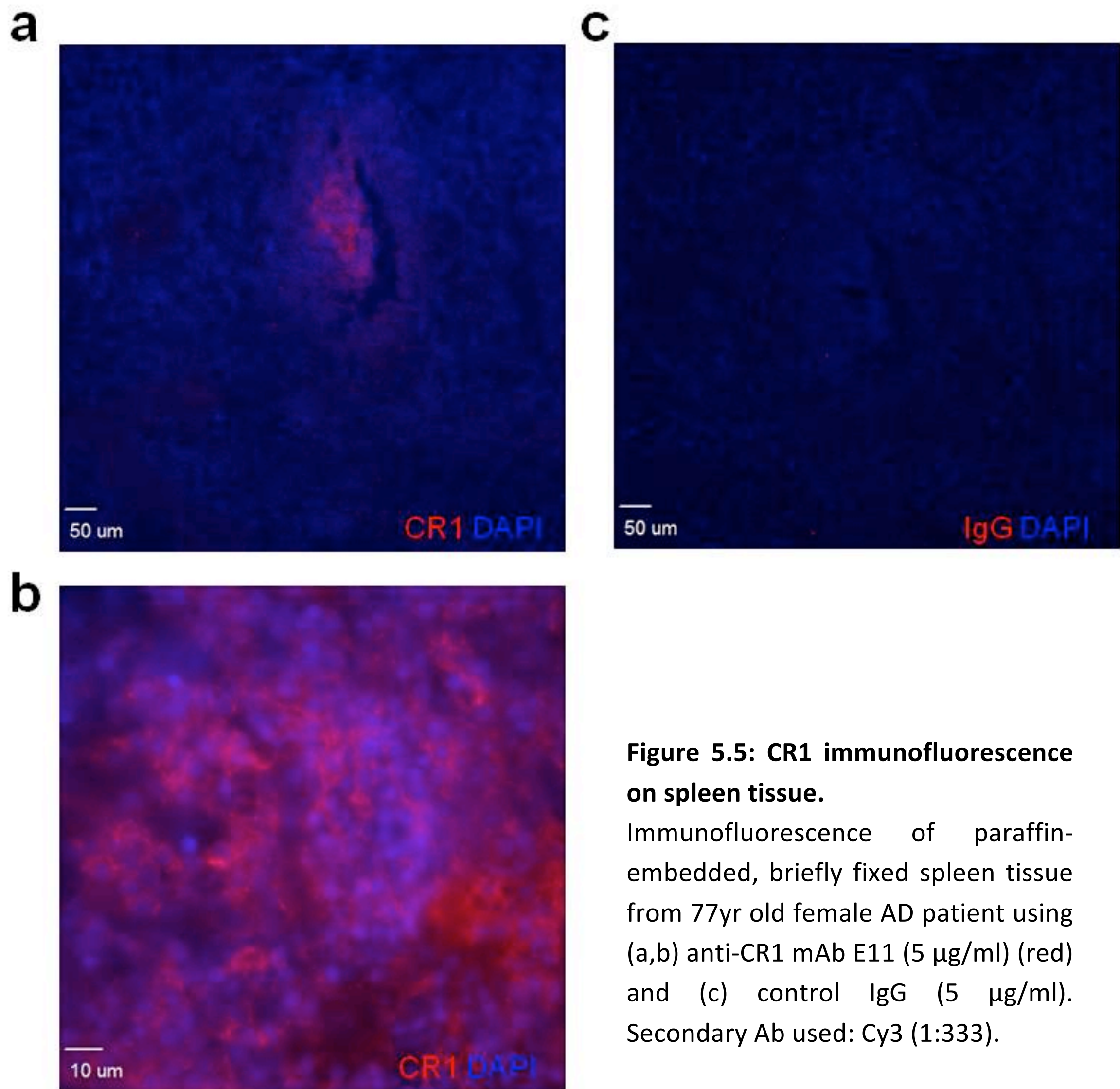


Figure 5.5: CR1 immunofluorescence on spleen tissue.
Immunofluorescence of paraffin-embedded, briefly fixed spleen tissue from 77yr old female AD patient using (a,b) anti-CR1 mAb E11 (5 µg/ml) (red) and (c) control IgG (5 µg/ml). Secondary Ab used: Cy3 (1:333).

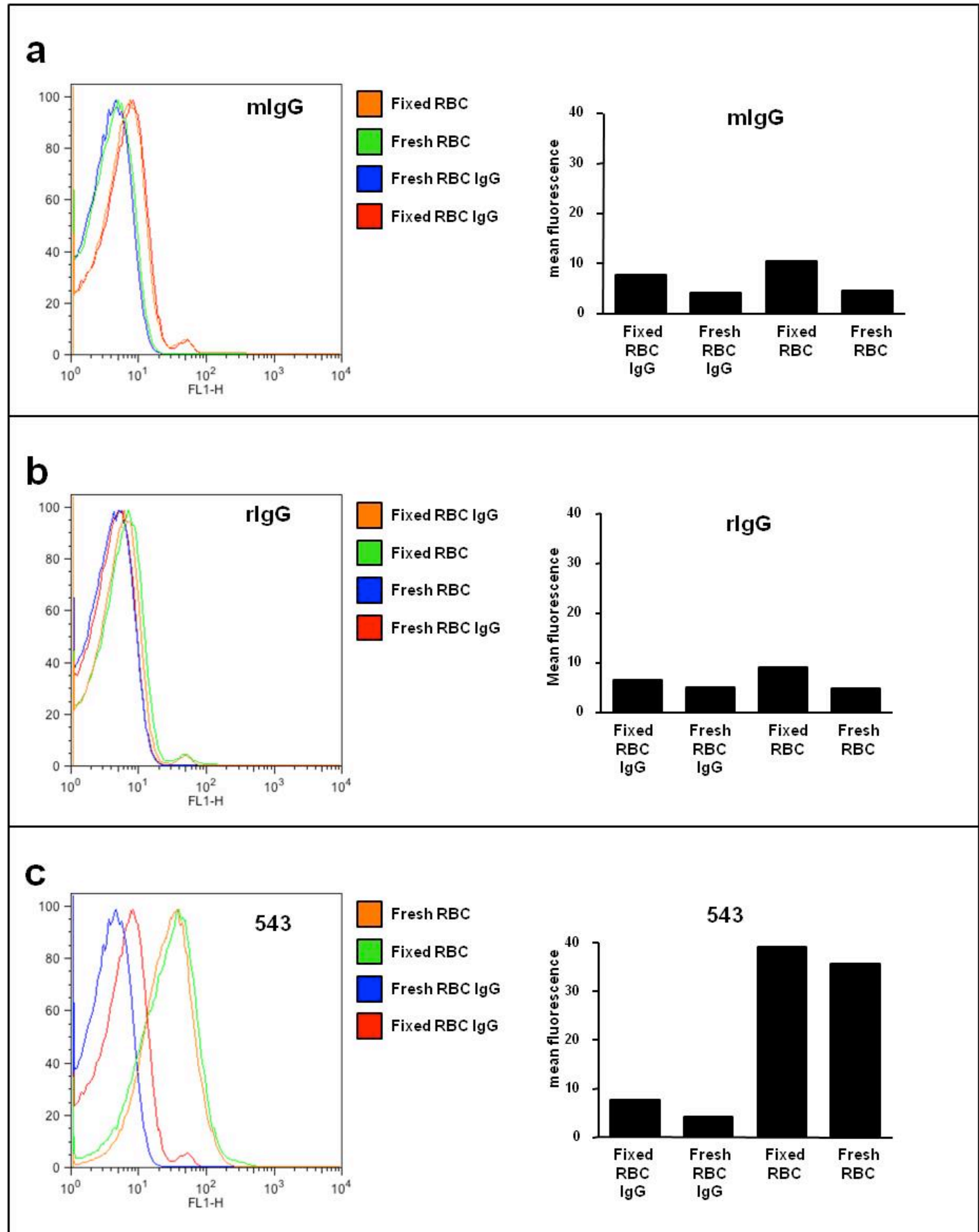
The washing steps in IHC commonly use TBS as a rinse buffer for the removal of antibodies unbound and weakly bound to non-specific sites. It is also used as a buffer for antibody incubation. The addition of the detergent Tween 20 to TBS (TBS-T) is capable of aiding in more effective rinsing and assisting in uniform spreading of the antibody. Therefore IHC was also carried out replacing TBS with TBS-T in the washing and incubation steps and probing with the range of anti-CR1 543, 3C10, YZ1, pAb and

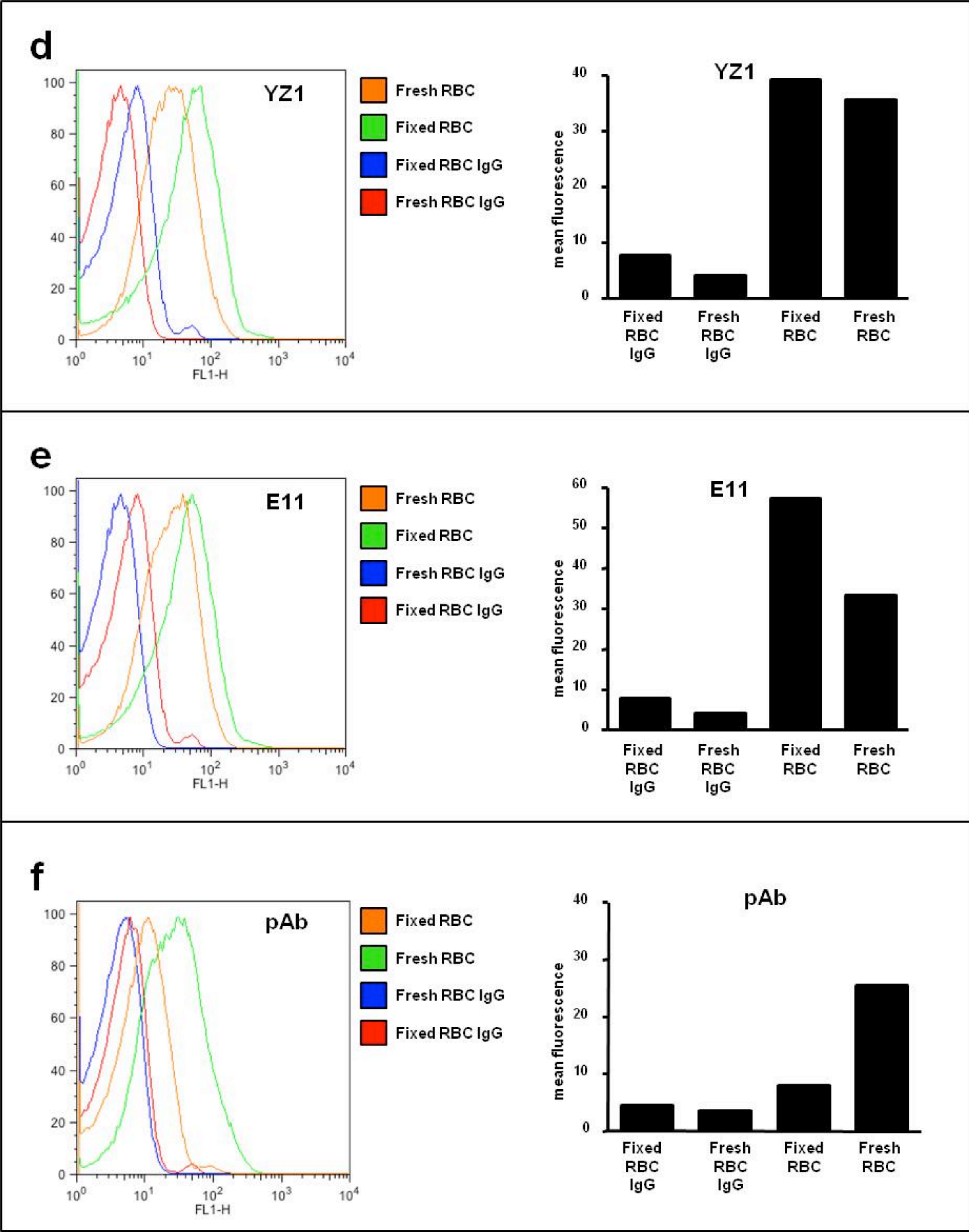
IC7. The results from this proved unsuccessful as there no immunoreactivity observed (data not shown).

Tissue fixation chemically cross-links proteins and can reduce protein solubility which can mask target antigens thus hindering IHC immunoreactivity (Otali et al. 2009). To test if the 2 h formalin fixation of the post-mortem brain tissue was responsible for the absence of immunoreactivity of a number of the CR1 antibodies, RBCs were fixed with 10% formalin and the sensitivity of the anti-CR1 antibodies was measured by flow cytometry.

Flow cytometry works on the basis of the movement of cells in suspension past a detector, which for the measurement of fluorescence requires fluorescence markers to be used. The prepared sample in suspension is taken up from a round bottom FACS tube under pressure and transported to the flow cell, where the sample combines with a faster flowing sheath fluid which transports the sample to a light source for excitation, where fluorescence is captured and detected by photodetectors. The flow cytometer is able to count single cell events by passing the cell suspension through a narrow tube at high pressure, which forces the cell suspension to pass the laser and detector in a stream of particles. To count the events, the flow cytometer relied in this experiment on the ability of cells to emit light when tagged with a fluorescent probe. Flow cytometry was used to fluorescently measure CR1 on both fixed and fresh RBCs to determine whether the fixation method affected the immunoreactivity with the range of CR1 antibodies (Fig. 5.6). The flow cytometry data demonstrated that anti-CR1

3C10 and anti-CR1 pAb were both fixation sensitive (Fig. 5.6f and 5.6h) as measured by mean fluorescence comparable to IgG isotype control. The remaining antibodies did however demonstrate a shift in mean fluorescence compared with IgG control.




d
YZ1
Fresh RBC
Fixed RBC
Fixed RBC IgG
Fresh RBC IgG
FL1-H
mean fluorescence
Fixed RBC IgG
Fresh RBC IgG
Fixed RBC
Fresh RBC
e
E11
Fresh RBC
Fixed RBC
Fresh RBC IgG
Fixed RBC IgG
FL1-H
mean fluorescence
Fixed RBC IgG
Fresh RBC IgG
Fixed RBC
Fresh RBC
f
pAb
Fixed RBC
Fresh RBC
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FL1-H
mean fluorescence
Fixed RBC IgG
Fresh RBC IgG
Fixed RBC
Fresh RBC

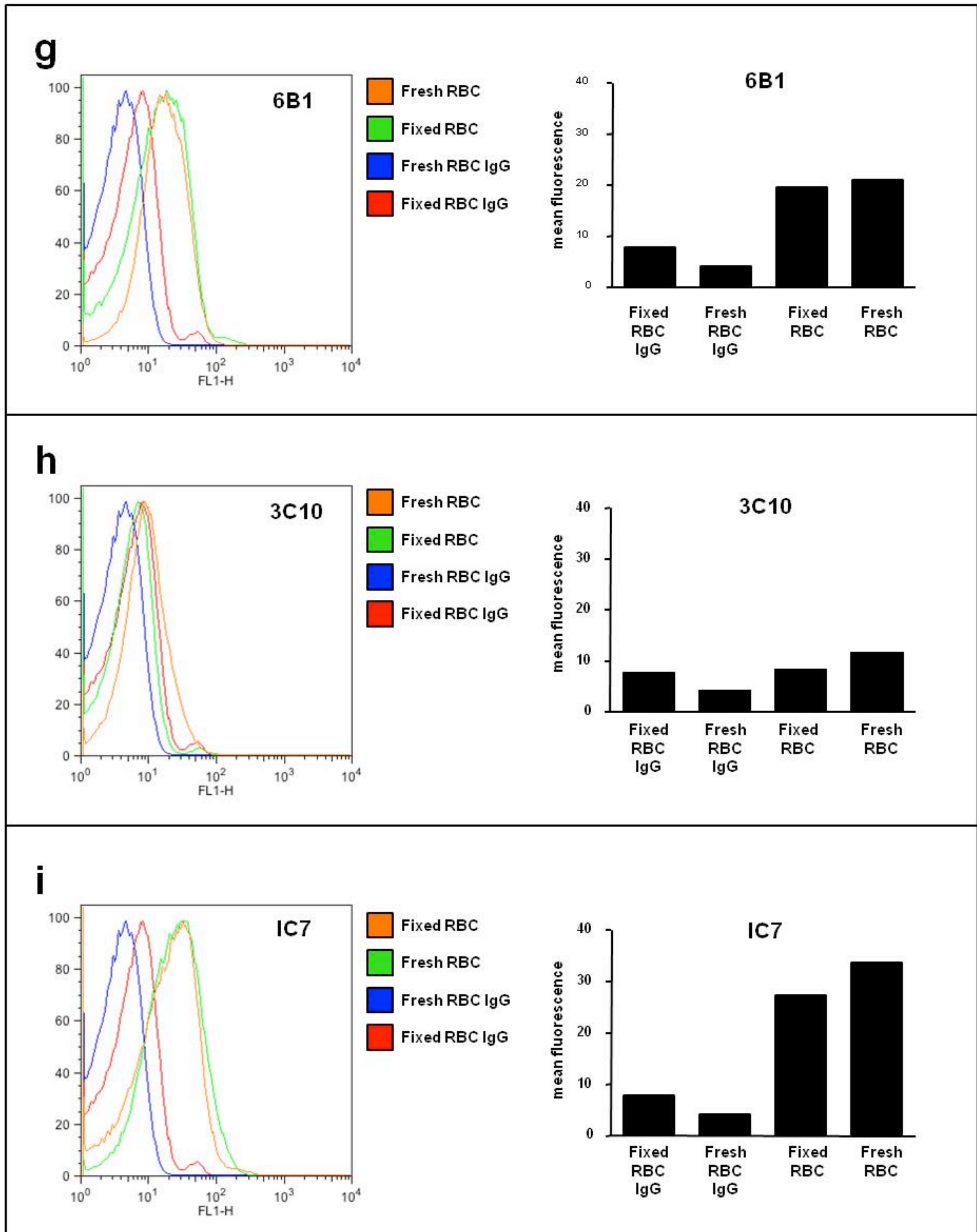
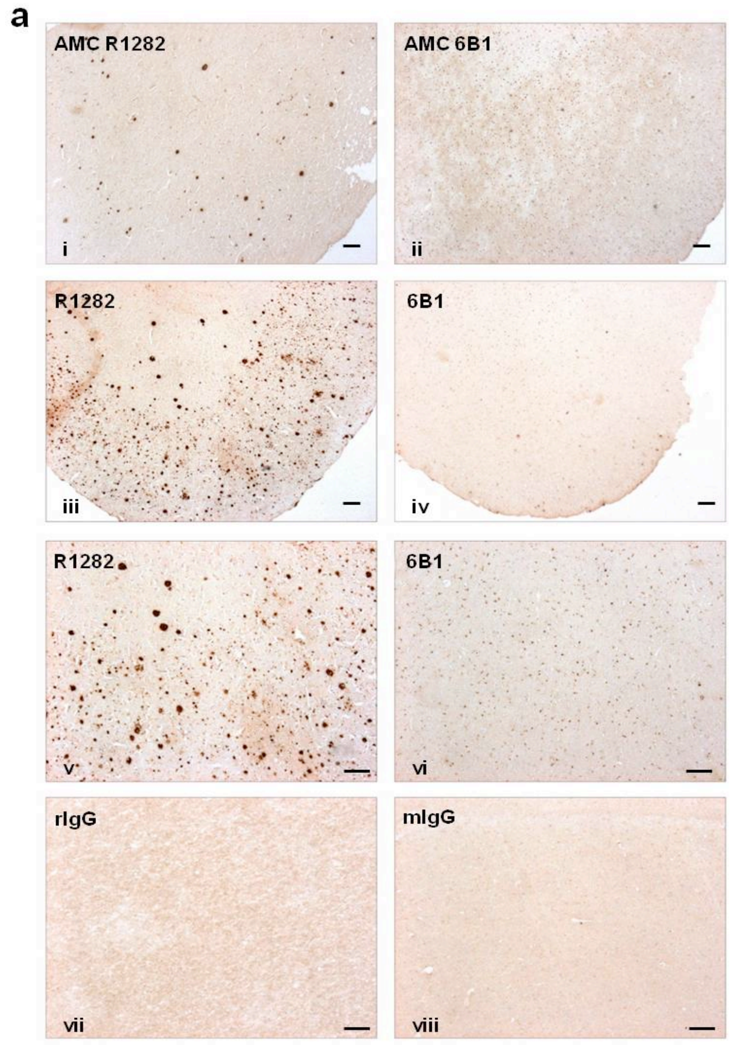


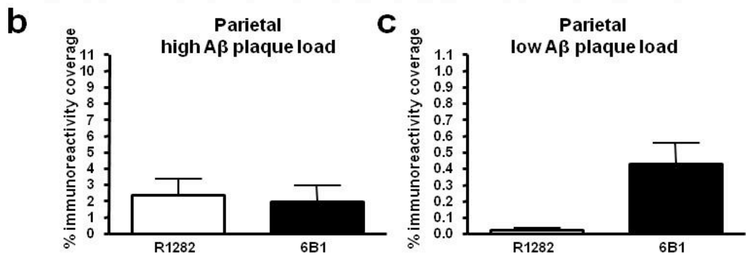
Figure 5.6: Flow cytometry analysis of fixed and fresh red blood cells using CR1 antibodies. RBCs were fixed in 10% formalin for 2 h prior to analysis. The histograms show a shift in fluorescence compared to IgG isotype control with (c) anti-CR1 mAb 543, (d) anti-CR1 mAb YZ1, (e) anti-CR1 mAb E11, (g) anti-CR1 6B1 and (i) anti-CR1 mAb IC7, which is also represented on the corresponding graphs. (f) anti-CR1 pAb and (h) anti-CR1 mAb 3C10 demonstrate little to no shift in fluorescence.

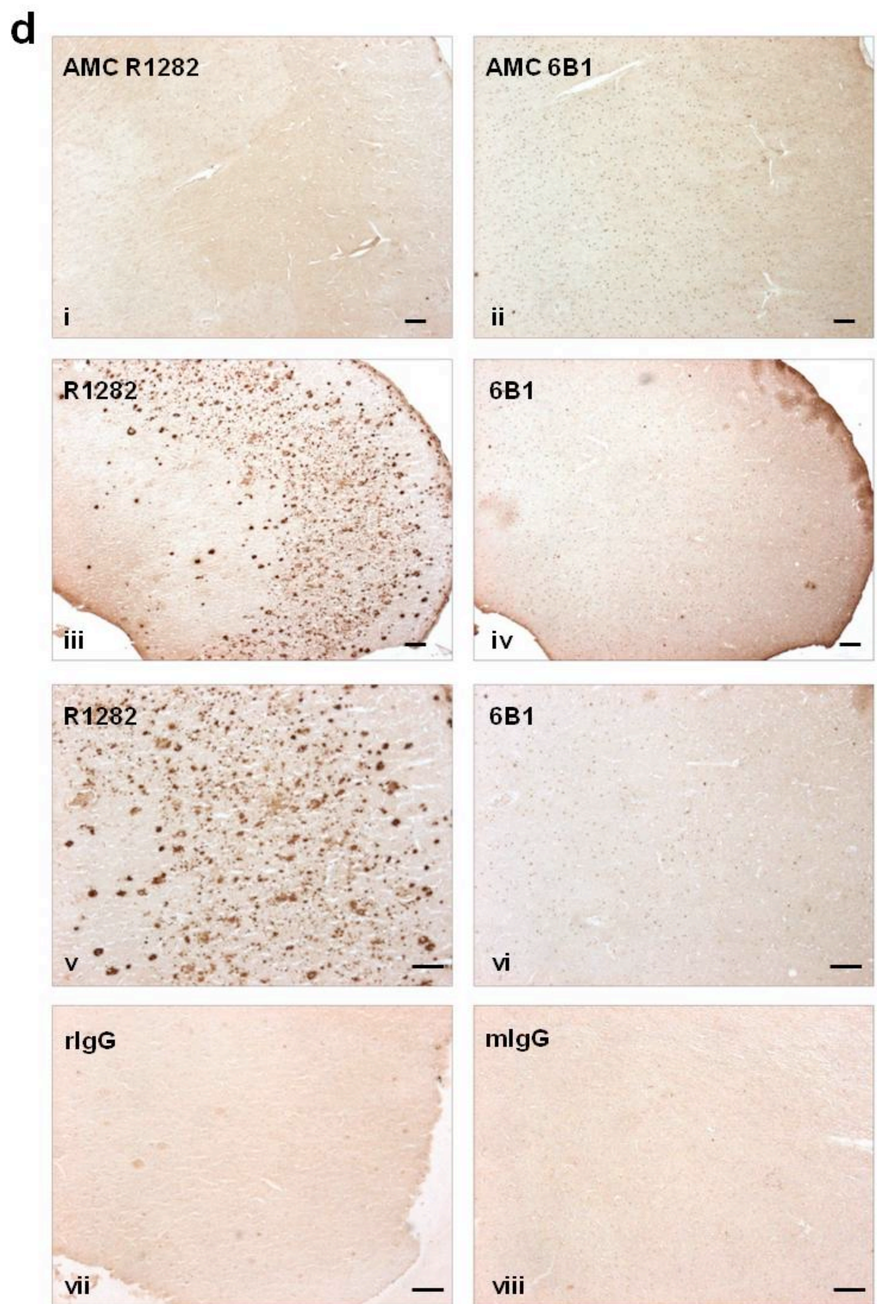
5.2.2 Higher CR1 expression is regionally present in low Aβ plaque areas of AD tissue, however increased CR1 presence overall is higher compared to low plaque areas

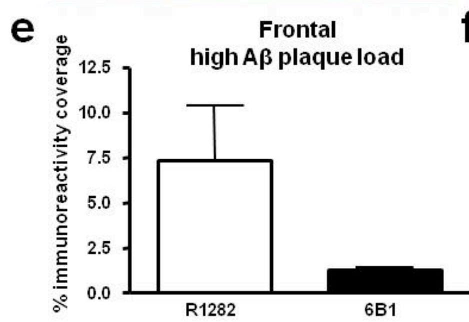
Following the optimization experiments, it was decided that the anti-CR1 6B1 antibody should be used for all further experiments. As such, CR1 immunoreactivity using 6B1 antibody was measured in areas of high and low Aβ plaque density within four regions of human AD and control brains (Fig. 5.7). The age-matched control (AMC) tissue were from donors who were not reported to have dementia, and were of similar age to the AD tissue donors at time of death. Aβ deposition was measured using R1282 antibody at dilution 1:1000. R1282 is a general Aβ rabbit polyclonal antibody, (a gift from Dr. Dennis Selkoe, Center for Neurologic Diseases, Boston, MA), was raised against synthetic $A\beta_{1\text{-}40}$ peptide and recognizes multiple epitopes and conformations. Figure 5.7a(iii-vi) and 5.7b showing this investigation in the parietal cortex demonstrated that in areas of high plaque load there was similar level of CR1 immunoreactivity. In areas with low volume of Aβ plaques, there was a comparatively higher ratio of CR1 present (Fig. 5.7c), however this still remained lower than the CR1 present in higher plaque areas. When this was investigated in the frontal cortex it was found that there was a trend towards lower CR1 present in areas of high Aβ plaque load (Fig. 5.7d(iii-vi) and 5.7e), which appeared to show an opposite trend in areas of low plaque load (Fig. 5.7c). Again, this percentage of CR1 in low plaque areas is still lower compared to that present in high plaque density. The Aβ plaque and CR1 ratio in the hippocampus was similar to that observed in the parietal cortex with similar immunoreactivity of Aβ plaques and CR1 in areas of high plaque density (Fig. 5.7g(iii-vi)

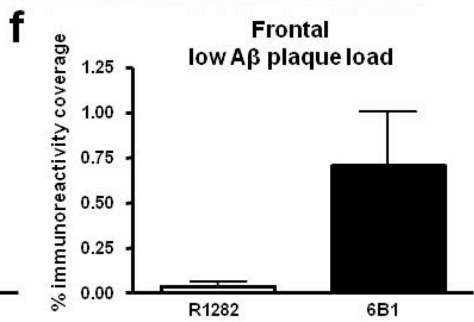
and 5.7h). Also, although lower than measured in high density plaque areas, there was a trend towards an increase in higher CR1 in low Aβ plaque areas (Fig.5.7i). Finally, data from the temporal cortex demonstrate a similar trend observed in the frontal cortex AD tissue (Fig. 5.7d and 5.7e and 5.7f). A lower density of Aβ plaques was measured in AMC compared to the corresponding areas in the AD brain. Representative images of these areas corresponding to high plaque load fields in AD brains are presented in Figure 5.6 with CR1 immunoreactivity also observed.

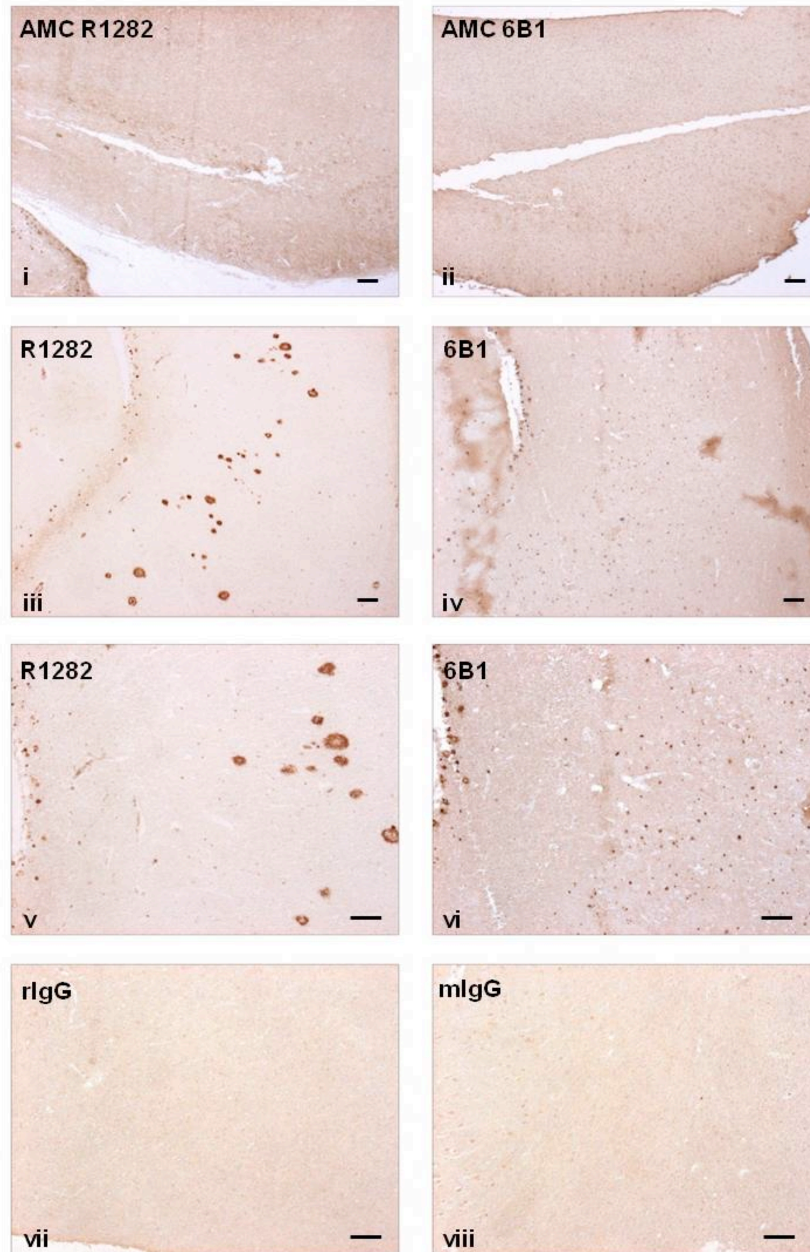

a
AMC R1282
AMC 6B1
R1282
6B1
R1282
6B1
rIgG
mIgG
i
ii
iii
iv
v
vi
vii
viii

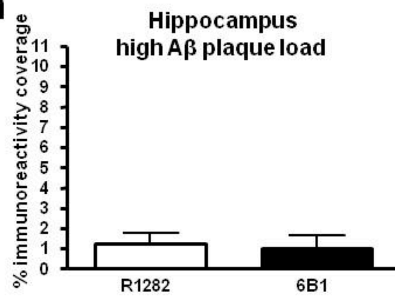

b
Parietal
high Aβ plaque load
% immunoreactivity coverage
R1282
6B1
c
Parietal
low Aβ plaque load
% immunoreactivity coverage
R1282
6B1

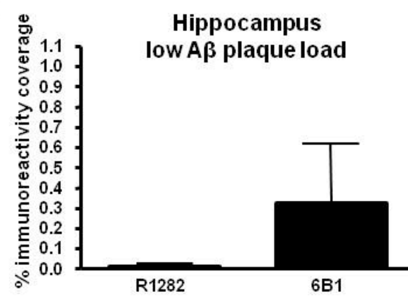

d
AMC R1282
AMC 6B1
i
ii
R1282
6B1
iii
iv
R1282
6B1
v
vi
rIgG
mIgG
vii
viii


e
Frontal
high Aβ plaque load
% immunoreactivity coverage
12.5
10.0
7.5
5.0
2.5
0.0
R1282
6B1


f
Frontal
low Aβ plaque load
% immunoreactivity coverage
1.25
1.00
0.75
0.50
0.25
0.00
R1282
6B1


g
AMC R1282
i
AMC 6B1
ii
R1282
iii
6B1
iv
R1282
v
6B1
vi
rIgG
vii
mIgG
viii


h
Hippocampus
high Aβ plaque load
% immunoreactivity coverage
11
10
9
8
7
6
5
4
3
2
1
0
R1282
6B1


i
Hippocampus
low Aβ plaque load
% immunoreactivity coverage
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
R1282
6B1

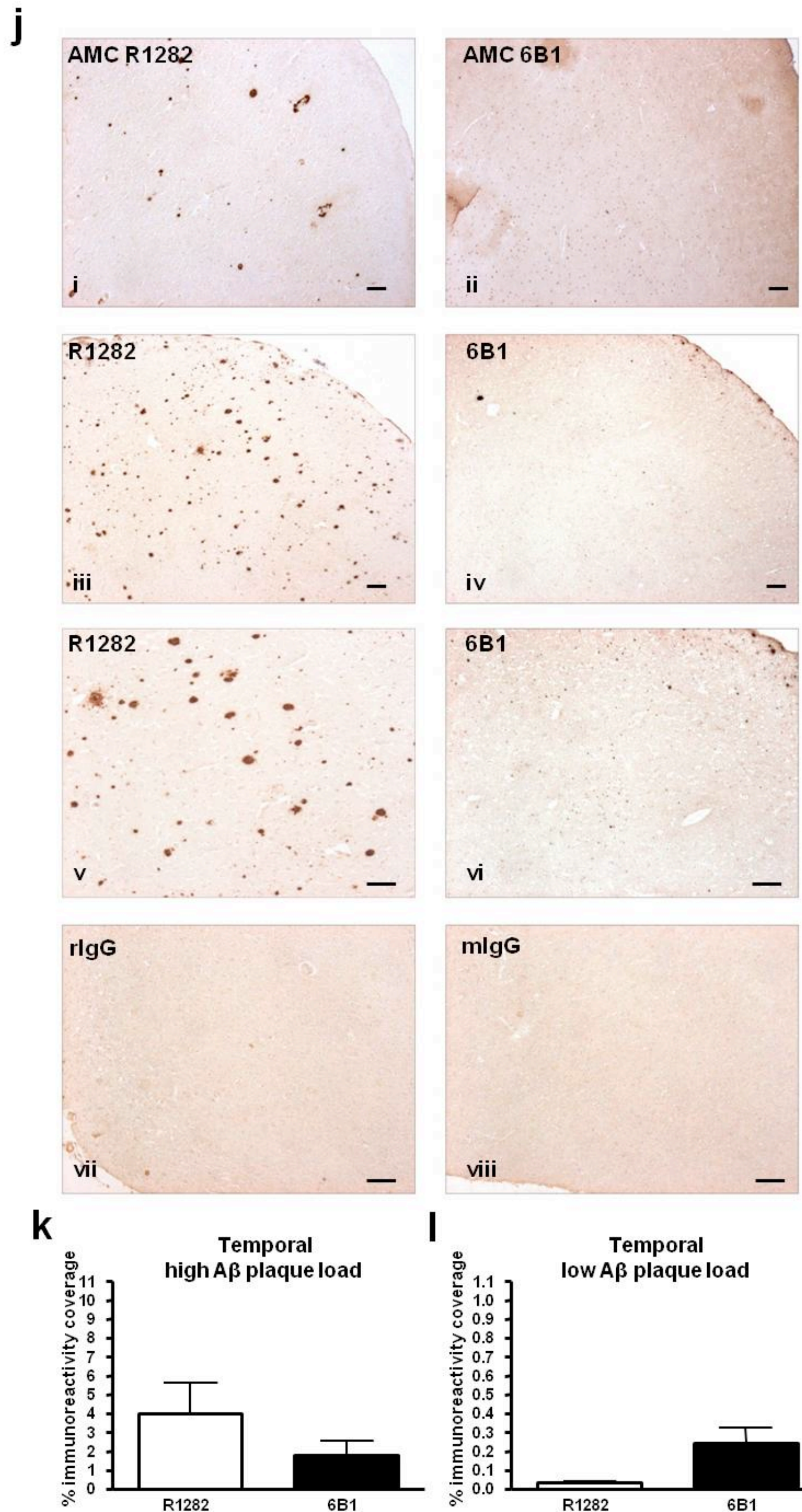


Figure 5.7: Immunohistochemical analysis of parietal, fronal, hippocampal and temporal cortex tissue in AD and aged-matched control (AMC) brains probing for Aβ plaques and CR1 immunoreactivity.
The representative images show R1282 (Aβ) and 6B1 (CR1) immunoreactivity in (i) and (ii) AMC, (iii) and (iv) area of high plaque load, (v) and (vi) same area of high plaque load at higher magnification and (vii) and (viii) probing with IgG isotype controls. Experiment carried out in 3 AD and AMC brains. Scale bar represents 100µm.

When percentage CR1 coverage was compared between the regions of the AD brain, a trend was observed for higher CR1 immunoreactivity with higher plaque load (Fig. 5.8b). This trend is not observed when the same regions are compared in the AMC brain (Fig. 5.8a). Parietal, frontal, cortex and hippocampal tissue from AD brain showed a trend towards a reduction in CR1 coverage in low Aβ plaque areas compared with AMC brains with CR1 coverage in hippocampal tissue demonstrating a similar trend in the high Aβ volume area also. However the opposite was observed in the temporal cortex, which showed a trend towards a higher percentage of CR1 present in high Aβ plaque regions in AD tissue compared to AMC

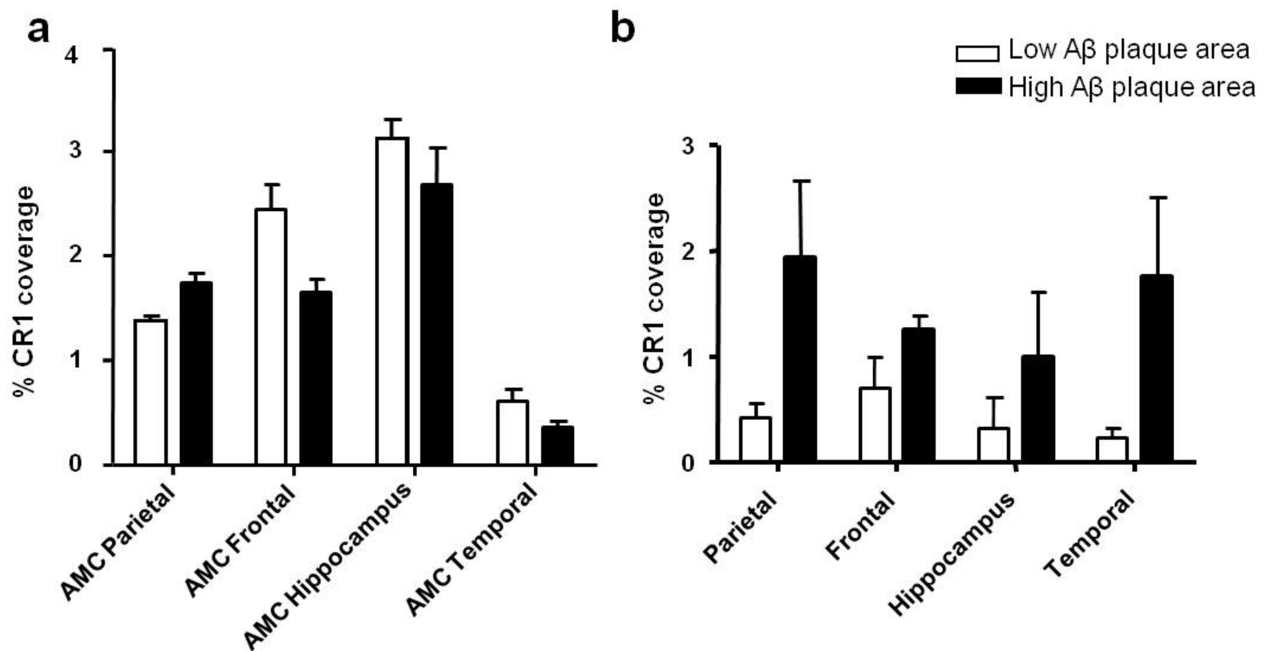


Figure 5.8: Comparison of CR1 coverage in regions of AD and AMC brain from Fig. 5.7.

CR1 was measured using anti-CR1 mAb 6B1 (4.65 μg/mL) and Aβ deposition was measured using general Aβ antibody R1282 (dilution 1:1000). (a) Graph comparing percentage of CR1 coverage in four regions of the AMC brain in both high and low plaque areas. (b) Graph comparing CR1 percentage coverage between similar four regions in AD brain. Data represents 3 human AD and AMC brains.

5.2.3 Quantification of CR1 on RBCs and comparison to circulating Aβ levels

The presence of CR1 on RBCs in blood vessels in paraffin-embedded brain tissue was investigated using a range of CR1 antibodies: anti-CR1 mAb 543, anti-CR1 pAb, anti-CR1 mAb 3C10, anti-CR1 mAb IC7, anti-CR1 mAb YZ1 and anti-CR1 mAb 6B1 (Fig. 5.9). CR1 staining (DAB) was not found on the RBCs in this tissue (Fig. 5.9b) however CR1 staining (DAB) was observed in blood smears that were fixed in 10% formalin for 2 hr (Fig. 5.9a).

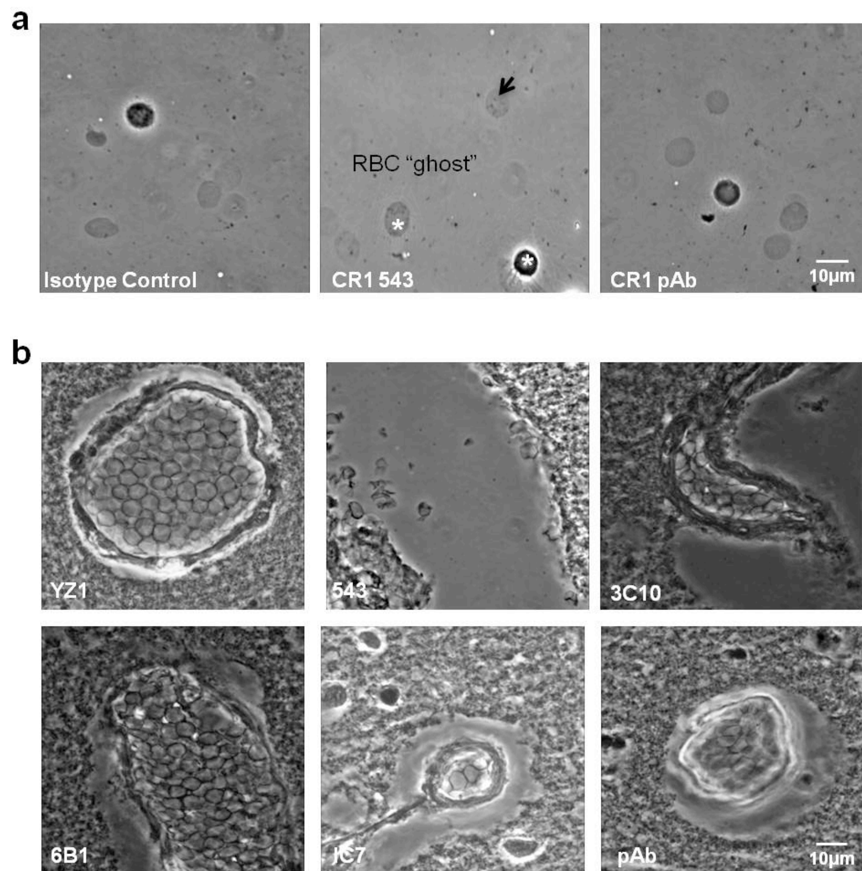
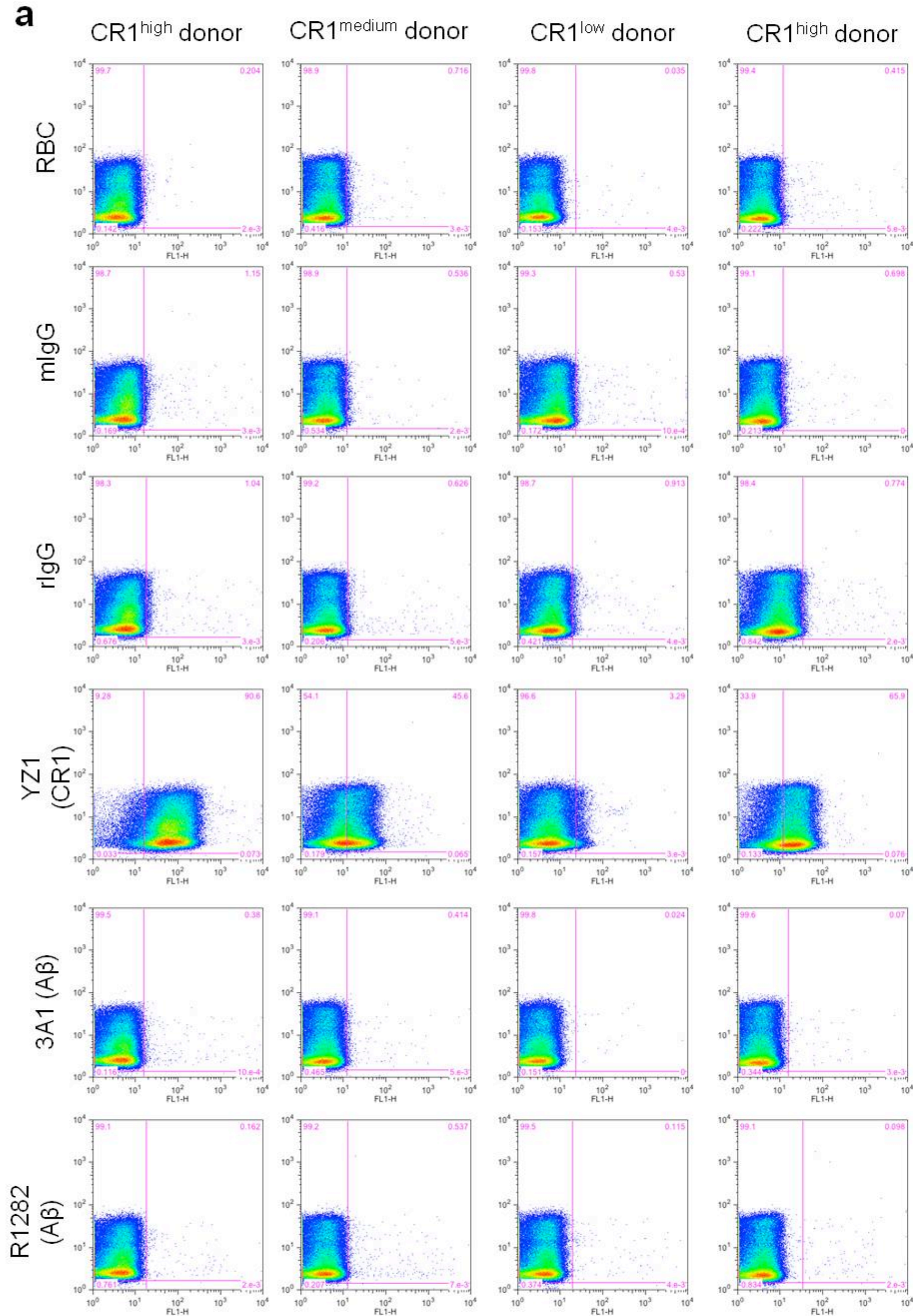


Figure 5.9: CR1 staining (DAB) of RBC from blood smears and brain tissue.
(a) CR1 is seen as black dots were visible on RBC ghosts as indicated by the arrow by anti-CR1 mAb 543. (b) CR1 was not observed on the RBCs in the parraffin-embedded brain tissue after investigation with ani-CR1 mAb- YZ1, 543, 3C10, 6B1, IC7 and pAb.

Levels of CR1 present on RBCs can vary between individuals and this may lead to differences in Aβ clearance. Therefore flow cytometry was used to measure these circulating levels of CR1 and Aβ in control blood samples to investigate any inverse correlation on RBCs (Fig. 5.10).



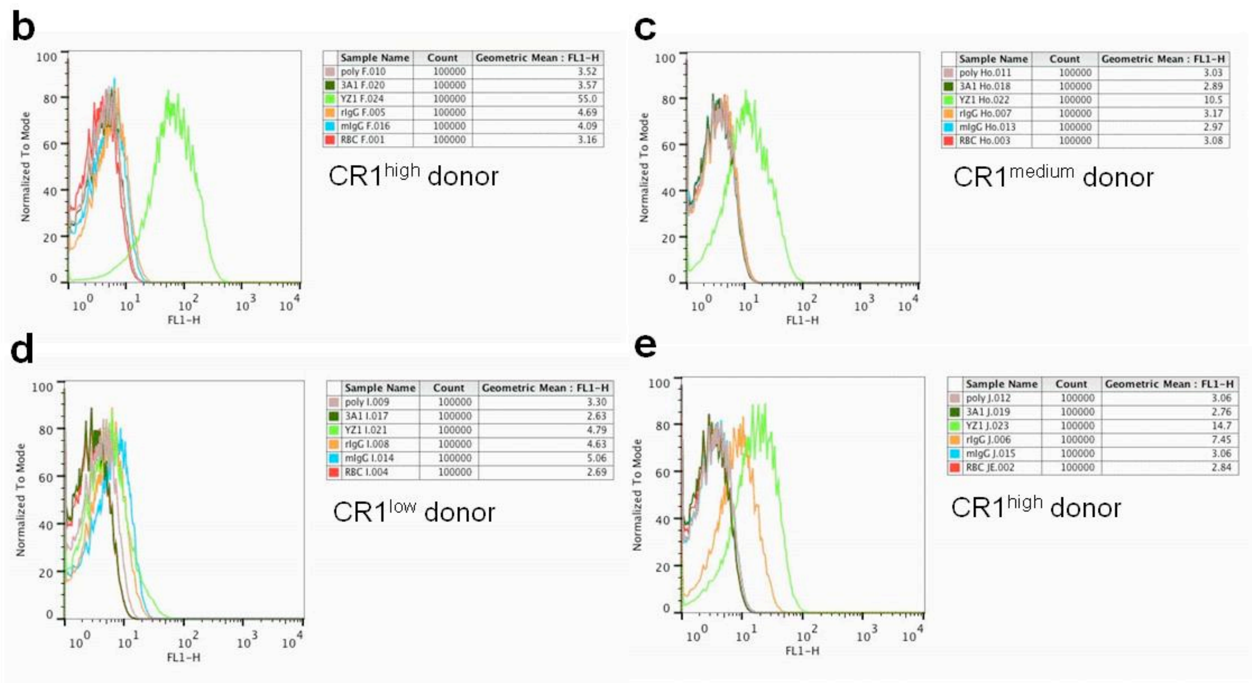


Figure 5.10: Flow Cytometry analysis of four blood donors measuring CR1 and Aβ expression. (a) Gating analysis demonstrating CR1 levels measured by fluorescence of YZ1 (1 µg/mL) and Aβ levels measured by fluorescence of R1282 (1 µg/mL) and 3A1 (1 µg/mL). Fluorescence measurement of IgG isotype controls mouse IgG (1 µg/mL) and rabbit (1 µg/mL) and RBC alone also carried out. (b) Histogram depicting the mean fluorescence from the CR1 high donor in the first column in (a), (c) showing all the mean fluorescence of the CR1 medium donor from the second column in (a), (d) showing the mean fluorescence of the CR1 low donor from the third column in (a) and (e) showing the mean fluorescence of the CR1 high donor from the fourth column in (a). 10,000 events counted.

The gating analysis shows clearly that the four donors express CR1 at different levels, as measured by anti-CR1 YZ1 antibody: high, medium and low (Fig. 5.10a). There was no obvious correlation with Aβ levels observed which was measured by two Aβ antibodies, R1282 and 3A1. This data is also presented by mean fluorescence on histograms, which can also clearly see the differences of CR1 expression between individuals (Fig. 5.10b-e). Fig. 5.9a demonstrates a high level of mean fluorescence of YZ1 compared to that of the donor measurements recorded in Fig. 5.10b and 5.10c. This high CR1 level did not appear to have an impact on the Aβ levels as measured by

R1282 and 3A1. Fig. 5.10c and Fig. 5.10d show individuals who would be considered medium and low expressors of CR1 respectively and both these donors did not show any correlation between the CR1 and Aβ levels also. Fig. 5.10e shows an additional individual that would be considered a high CR1 expressor with no differences in Aβ levels either.

It was possible to visualise the increased presence of CR1 on RBCs by IF on fresh blood smears using a number of CR1 antibodies. This study investigated CR1 using E11 (5 μg/mL), 543 (1 μg/mL) and 6B1 (1 μg/mL) along with control mIgG (5 μg/mL) on blood smears from individuals already shown to be high and low expressors of CR1 through flow cytometry. All three CR1 antibodies were able to show CR1 presence on RBCs through IF. Figure 5.11 shows representative images of RBC CR1 IF using anti-CR1 E11. The graph shows the average volume of CR1 'dots' per donor. The experiment was carried out by measuring CR1 on a set number of RBCs from 9 fields of view from 3 cover-slipped blood smears. The results were in line with the flow cytometry data showing there are differences in CR1 levels between individuals.

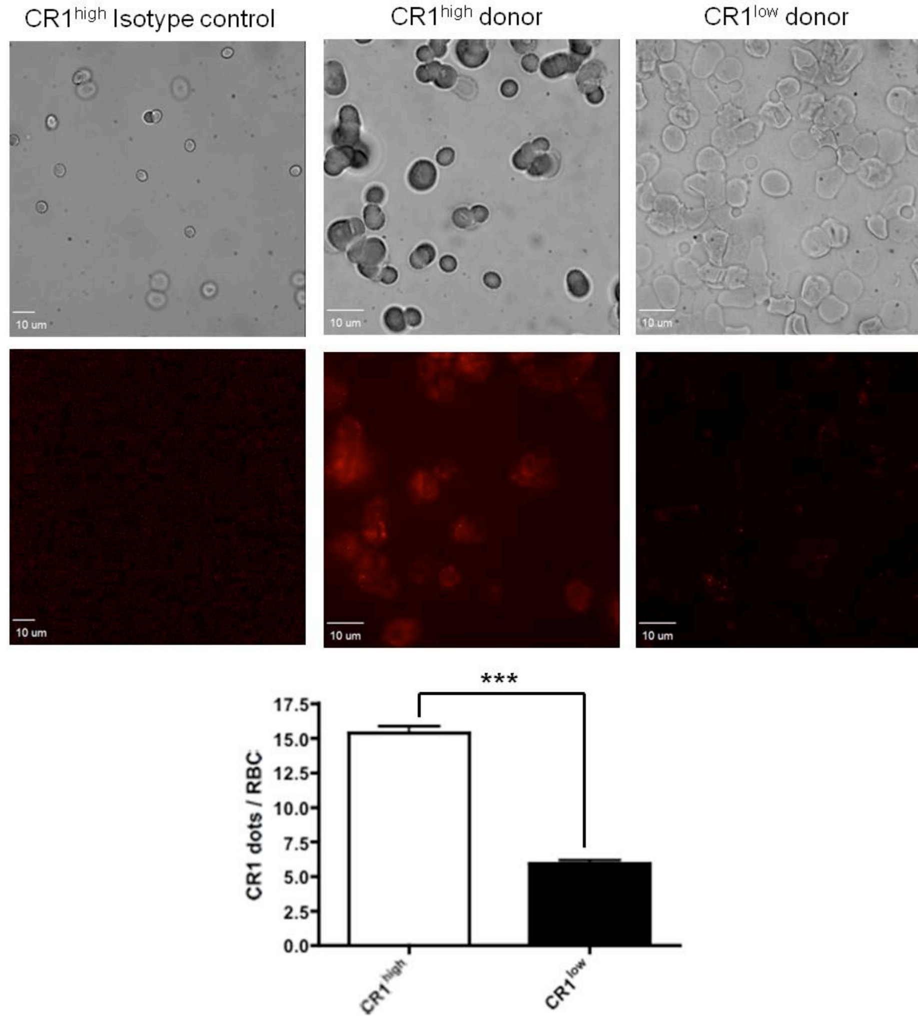


Figure 5.11: Immunofluorescence of RBCs with anti-CR1 mAb E11 shows a difference in CR1 volume between control blood donors.

Representative images following probing with E11 (5µg/mL) and mAb IgG isotype control (5 µg/mL). Graph shows average volume of RBC CR1 'dots' per donor. Experiment measured on a set number of RBCs from 9 fields per coverslip and 3 coverslips per donor. Data were analysed by Student's t-test with pair-wise analysis as indicated, ***$\rho < 0.001$, **$\rho < 0.01$ and *$\rho < 0.05$.

5.3 Discussion

The association of complement fragments with AD lesions was first reported over 3 decades ago (Eikelenboom & F. C. Stam 1982; Ishii & Haga 1984). This has been further strengthened over the years with the development of monoclonal antibodies for the specific detection of complement, and complement component knock-out mice to validate these (Yasojima et al. 1999; Afagh et al. 1996; Fonseca et al. 2004). The recent reports of CR1 polymorphisms being associated with AD, suggest new roles for complement (Lambert et al. 2009; Hazrati et al. 2012), which may provide a novel approach in augmenting Aβ clearance. Although there is escalating evidence of blood-brain barrier disruption in AD (Bowman & Quinn 2008) this is not observed in all cases and there is also evidence of complement gene expression in the AD brain (Shen, Li, et al. 1997; Walker et al. 1995). As previously mentioned a number of complement components have been identified in the AD brain however there have been few reports of the presence of CR1 in the AD brain. This does not necessarily mean an absence of CR1 in the brain, as it may be due to lack of specific reagents used in the investigation, such as high affinity antibodies that are highly specific for different LHR domains of CR1 or possibly due to tissue preservation and staining methods. The present study used a range of highly specific CR1 antibodies that target LHR-A, -B, -C and –D to investigate the presence of CR1 in the human AD brain. Formalin fixation is one of the most widely used fixation methods used in histology and although it can provide excellent preservation of tissue and cellular morphology it has been implicated in the masking or even loss of epitopes in immunohistochemistry. Fixation with 10%

formalin can reduce immunohistochemical activity through a number of ways, such as blocking access of antibodies to the epitopes by protein cross-linking, directly binding to the epitope and modifying it or causing a change in the conformation of the proteins (Dapson 2007; Bogen et al. 2009; O'Leary et al. 2009). The additional steps in tissue processing; dehydration in alcohols, establishing a hydrophobic environment such as infiltration with xylene and the embedding of the tissue in the hydrophobic environment, paraffin, may also contribute to altering of epitopes (Dapson 2007). Performing the optimum antigen retrieval method is important for the re-exposure of the epitope, which ultimately restores immunoreactivity. Heat-mediated antigen retrieval is able to reverse the formaldehyde-protein adduct formation through reducing the number of ribonuclease A molecules participating in the cross-linking (O'Leary et al. 2009), giving rise to increased immunoreactivity. The protein denaturing agent formic acid also functions to successfully retrieve antigens and improve immunoreactivity (Shi et al. 1991). Both these retrieval methods were performed with the paraffin-embedded tissue to determine an optimum antigen retrieval method for CR1 immunoreactivity. Immunoreactivity was only observed with anti-CR1 mAb 6B1. This was observed through both methods of retrieval but heat-mediated pretreatment by citrate/mw was chosen as the optimum method. The lack of immunoreactivity demonstrated by the other CR1 antibodies cannot be solely attributed to inadequate antigen retrieval. The CR1 antibodies used in this study target a variety of epitopes however 6B1 was the only antibody to solely target LHR-D. LHR-D has been suggested as the functional domain in the CR1 protein with a coding variant in the LHR region of

the CR1 gene. This variant is able to alter the conformation of CR1 (threonine for serine substitution) influencing a decline of episodic memory, however this was evaluated to be mediated by an increase in AD neuropathology (Keenan et al. 2012). Haematoxylin staining was positive for nuclei near CR1 immunoreactivity suggesting that CR1 has cellular presence in the brain (Fig. 5.2). Further investigation into the tissue quality as a cause for the absence of immunoreactivity with the other CR1 anti-bodies was investigated in serial sections of spleen tissue from an AD patient also briefly fixed for 2 h, and the presence of immunoreactivity with anti-CR1; E11, 6B1, 543 and YZ1 suggested that it was not the tissue quality that was interfering with obtaining a positive result in Fig. 5.1. Flow cytometry analysis comparing fresh and 2 h formalin fixed RBCs probed even further into determining whether the lack of immunoreactivity with some antibodies was due to fixation. The outcome from this experiment indicates that C310 and pAb are fixation sensitive, which together with IHC data from the spleen, the positive control, leaves the question open as to why YZ1, E11 and 543 do not show any immunoreactivity. All antibodies failed to produce IF in human brain tissue sections and all antibodies except E11 (Fig. 5.5) were unable to produce IF in control spleen sections therefore it was not possible to perform double immune labelling to determine the cellular presence in the tissue (see Fig. 5.2). If time permitted additional analysis into the cell type could have been carried out. This will be discussed further in chapter 6.

The Aβ peptide is able to activate two pathways of the complement system, the classical pathway through binding to C1q (Jiang et al. 1994; Velazquez et al. 1997) and

the alternative pathway by binding to C3 (Bradt et al. 1998) and as a result initiating mediators of inflammation and the MAC complex. These pathways would result in an increased production of C3b, which is required for the cleavage of C5 into C5a and one of the components of the MAC complex, C5b. An increase in C3b would result in increased binding of the complement regulatory protein, CR1, of which may be present on the cell surface of microglia, which are known to accumulate around Aβ plaques (Fetler & Amigorena 2005). This would be in line with findings from this study where there was a high volume of 6B1 immunoreactivity in areas of high plaque volume in AD tissue (Fig 5.7b). CR1 measurement from a similar Aβ plaque deposition regions in AMC's were also measured (Fig. 5.7a) and demonstrated little difference in CR1 coverage between low and high Aβ plaque burdened areas. As previously mentioned the binding to CR1 to C3b, which increased through complement pathway activation by Aβ, will generate iC3b therefore stimulating phagocytosis. Although this pathway is possibly more stimulated in the AD brain due to an increased plaque load, the beneficial phagocytosis role may be disrupted in the AD brain owing to less CR1 present. It may be hypothesized that individuals with AD-associated polymorphisms have low CR1 expression and as such, have less efficient clearance of Aβ protein.

The number of CR1 molecules per RBC is genetically determined which is typically characterised by 100 CR1 per RBC for low CR1 expressors, 500 CR1 per RBC for medium CR1 expressors and 1000 CR1 per RBC for high expressors (Wilson et al. 1986). Similarly as in human tissue it may be considered that individuals possessing an AD-associated polymorphism could result in having a reduced ability to adequately clear

Aβ protein in the blood due to having low CR1 expression on RBCs. This reduced clearance throughout life could result in the gradual accumulation of Aβ in the brain with aging and increased risk of AD. The physiological role of Aβ is not fully understood but studies identifying it as a modulator of K^+ currents in central neurons (Plant et al. 2006) and its presence in CSF of non-demented individuals (Tamaoka et al. 1997) and media from neuronal cell cultures (Haass et al. 1992) point to Aβ having a toxic role when its production and degradation are imbalanced. *In vitro* and *in vivo* animal studies have demonstrated that Aβ is capable of binding to RBCs (Nakagawa et al. 2011) and so levels of both this peptide and CR1 on RBCs were measured. Investigation into levels of CR1 on RBCs from different donors was able to demonstrate a difference in CR1 expression between these individuals however these differences did not correlate with Aβ levels in the RBCs (Fig. 5.10a-e). Upon evaluation of this result it is important to point out that the age of the donor individuals in this experiment was 24.25±4 yrs. A study measuring a correlation between RBC and plasma $A\beta_{40}$ and $A\beta_{42}$ found that levels were low on RBCs in healthy young volunteers, aged 24.2±6 but it tended to increase with age as seen in healthy older volunteers, aged 56.2±0.9 (Kiko et al. 2012). This increase over time may be due to a reduced Aβ clearance or increased Aβ production as previously suggested as a possible mechanism in AD. It could be potentially due to changes in enzymes that are associated with aging such as Aβ secretase enzymes (Nistor et al. 2007) or Aβ catabolic enzymes (Apelt et al. 2003). It would have possibly been more informative to have also investigated an inverse

correlation between CR1 and Aβ levels in older healthy donors and this could be put forward as a possible future experiment.

The number of CR1 molecules per RBC has been shown to have a significant functional impact including calcium (Ca^{++}) influx. Analysis of RBC Ca^{++} influx with the use of the fluorescent Ca^{++} indicator, Fluo-4 AM, found that antibody ligation of RBC CR1 triggered a sustained Ca^{++} influx which was observed to be more pronounced in high CR1 donors compared with low RBC CR1 expressors as measured by flow cytometry (Glodek et al. 2010). This high Ca^{++} influx was also found to promote phosphorylation of cytoskeletal proteins, α-adducin and β-spectrin, which correlated with increased RBC membrane deformability, which is important for oxygen transport. RBC morphology has been found to be more elongated in blood from individuals with AD compared to controls which typically results in RBCs being less deformable (Mohanty et al. 2008). Binding of $A\beta_{40}$ and $A\beta_{42}$ fibrils to RBCs results in decreased deformability (Jayakumar et al. 2003) suggesting that the increased availability of Aβ peptides in AD individuals lead to increased binding to RBC and as such decreased deformability and passage through the microvasculature. The decreased RBC membrane deformability correlating with the low Ca^{++} influx associated with low RBC CR1 expressors may prove detrimental in the presence of an increase in Aβ in the blood. Not only in terms of possible reduced clearance of Aβ through less CR1 molecules present but also through less oxygen transport into the brain.

5.4 Conclusion

The data presented here provide evidence for the presence of CR1 in the human brain. There has been conflicting data published as to whether CR1 is expressed in the brain however its expression has been observed in this study in four different regions of the brain, through the use of a highly specific antibody targeting LHR-D on CR1. Extensive optimisation of the range of CR1 antibodies available for this study was carried out and a summary of the outcome of the use of these in paraffin-embedded tissue and blood is demonstrated in Table. 5.1.

Antibody	Subclass	Epitope	Staining Brain (paraffin)	Staining Spleen (paraffin)	Fixed RBC	Fresh RBC
6B1	IgG1	LHR-D	+	+	+	+
543	IgG1	LHR-C, D	-	+	+	+
Rabbit Polyclonal Ab		Multiple	-	-	-	+
YZ1	IgG1	SCR 5, 6, 7 of LHR-A, B, C	-	+	+	+
IC7	IgG1		-	+	+	+
3C10	IgG1		-	n.d	-	-
E11	IgG1	LHR-C, D	-	+	+	+

Table. 5.1. CR1 antibody reactivity in tissue and blood. *n.d = not done

This study was able to identify a trend for higher CR1 coverage in the areas of the parietal, frontal, hippocampal and temporal cortex with highest Aβ plaque load. CR1 presence on microglia could account for this increase in CR1 coverage in high plaque areas due to accumulation of this glial cell around Aβ plaques.

Furthermore CR1 expression on RBCs is genetically determined and was demonstrated to vary between different individuals possibly contributing to clearance in AD. This expression was not shown to inversely correlate with Aβ levels in the blood of these young donor individuals and it is suggested that the experiment should be carried out with RBCs from older donor individuals (>60 yrs).

This is nevertheless only a preliminary investigation into CR1 expression in the brain and confirmation of the cell type associated is needed. This could be carried out by the use of laser capture microdissection to isolate the cells, and mass spectrometry which would be able to identify the cell type associated to its expression.

Chapter 6

General Discussion

6. General discussion

The research presented in this thesis was primarily focused on investigating the role of CR1 in the brain; the ramifications of how CR1 modulation influences the microglial phenotype following exposure of activators complicit in microglial responses in AD, the consequences of these responses on neuronal survival and an exploratory investigation into a possible correlation between CR1 activation and Aβ plaque load. The data presented demonstrate that microglial CR1 has a proinflammatory influence on regulating neuronal survival, plays a role in Aβ triggered phagocytosis and expression may coincide with high levels of Aβ plaques in the human AD brain.

Microglia are the immune cells of the CNS and have a crucial role in detecting and removing foreign particles in the brain. They function to protect more vulnerable cells in the CNS such as neurons, from damage, through their activation to a more immunocompetent phenotype, releasing cytotoxic substances and mediating the phagocytosis of debris. Neuroinflammation, a hallmark of which is chronic activation of microglia, is an important component of AD progression. It is initiated in response to elevated Aβ levels in attempt to slow down the disease, however, inflammatory mediators may act as a driving force for AD. In addition to Aβ plaques and NFTs, activated microglia are largely considered important hallmarks of the disease (Cameron & Landreth 2010). The complement system is also considered to play a central role in AD pathogenesis with Aβ plaques comprising of complement proteins and their expression coinciding with dementia (Kolev et al. 2009). Therefore, recent GWAS linking one of the complement regulatory proteins, CR1 to AD (Lambert et al.

2009; Corneveaux et al. 2010; Zhang et al. 2010; Carrasquillo et al. 2010) raised the question of its contribution to AD pathogenesis. It remained to be determined whether complement activation as a consequence of the inflammatory response in AD, had a protective role through phagocytosis and plaque clearance or a destructive role from chronic inflammatory tissue damage and so this thesis attempted to explore this.

Although complement can enter the brain through a compromised BBB which has been suggested as an early event in AD (Gay & Esiri 1991; Bowman & Quinn 2008), there is increasing evidence of complement mRNA in the AD brain leaning towards a possible CNS origin of synthesis (Shen, Li, et al. 1997; Van Beek et al. 2000; Walker et al. 1995). So far in the literature, there have been a number of reports of complement protein sources in the brain including neurons, astrocytes, oligodendrocytes and microglia (Gasque et al. 1993; Walker et al. 1998; Hosokawa et al. 2003; Rutar et al. 2011). However, the expression of the complement regulatory protein, CR1, in the CNS has remained controversial with conflicting reports of its expression in the brain (Singhrao et al. 1999; Singhrao et al. 2000; van Beek et al. 2005). This thesis aimed to address this question using post-mortem human brain tissue and primary rodent microglia. Following investigation with a range of highly specific CR1 antibodies that target LHR-A, -B, -C and –D, CR1 expression was present in human control and AD post-mortem brain tissue in the four regions examined. It may be possible that previous work trying to determine CR1 expression in the brain did not have highly specific antibodies as these available. Suggested links between CR1 pathways and Aβ clearance in AD have made microglia an interesting cell type to investigate due to their

phagocytic abilities and migration to Aβ plaques (Fetler & Amigorena 2005). CR1 detected at 160 kDa by Western blotting with two CR1 antibodies was increased in LPS and $A\beta_{1-42}$ activated microglia indicating a possible increase in neuroinflammation. These data suggest that further investigation into a possible role for CR1 on microglia could yield some interesting and potentially useful results.

Phagocytosis, a microglial response considered important in regulating homeostasis, occasionally through regulating Aβ plaque burden (Bard et al. 2000), was hypothesized to be impaired in microglial cultures following reduced CR1 availability. This impaired response is thought to occur in individuals with CR1 mutations, resulting in a disruption in Aβ plaque balance. Although both inflammatory treatments, LPS and $A\beta_{1-42}$, significantly reduced the phagocytosis of dextran beads, interestingly CR1 appeared in this case to play less of a phagocytic role following activation with $A\beta_{1-42}$. However, further analysis of microglial phagocytosis with fluorescently tagged Aβ curiously demonstrated a different response, which allowed us to suggest that CR1 modulation may affect microglial phagocytic pathways differently. Reduced iC3b binding to CR3 receptor on microglia, as a result of lower levels of CR1, could lead to a diminished stimulation of phagocytosis.

Microglia are responsible for protecting neurons, therefore it was interesting to measure the effect of CR1 modulation on microglial-neuronal interactions. Modelling microglial responses within CGC cultures (indirect interaction) allowed the creation of a more physiological environment. Furthermore, the use of MGCM treatment of CGC

cultures allowed us to identify the influence of soluble secreted factors, such as TNFα, on neuronal death. From both the direct and indirect interactions there was a reduction in neuronal death when CR1 was blocked, however, it was only indirect interactions that demonstrated a decrease in neuronal death following blocking microglial CR1 with $A\beta_{1-42}$ co-treatment, suggesting that soluble factors released from microglia following CR1 blockade are important in neuronal survival following Aβ stimulation. The possibility of reduced cell number accounting for death was eliminated in the live cell assays through total cell number counts and the direct treatment data was confirmed by cleaved caspase-3 ICC. The complement system is known to play an important role in the human defence system and its activation leads to the formation of the MAC complex responsible for the lysis of pathogens and cells (Shen, Halperin, et al. 1997; Leinhase et al. 2006). Impairment to the functionality of complement regulatory proteins, such as CR1, could lead to increased C3b, possibly increasing MAC lysis of cells. The decrease in neuronal death was also considered to be due to reduction in the release of microglial toxic mediators therefore this was also investigated.

Activated microglia produce neurotoxic mediators (Cameron & Landreth 2010), whereby in the healthy brain, production of cytotoxic factors is attenuated following neuronal rescue or clearance of dying neurons. However during chronic neuroinflammation these responses are not regulated which can lead to neuronal death. Cytokine production is a microglial response up-regulated during persistent activation following tissue injury, infection or inflammation (Smith et al. 2012). TNF-α

is highly expressed in neuroinflammation which is in line with increased Aβ plaque deposition in AD (Fillit et al. 1991; Paganelli et al. 2002). Release of TNFα from LPS activated microglia was reduced when CR1 was blocked with a functional blocking antibody. This was considered a possible contribution to the trend in neuronal death observed with indirect LPS and CR1 blocking treatment however further investigation blocking TNFα synthesis could not support this. $A\beta_{1-42}$ microglial activation led to increased secretion of IL-1β, another pro-inflammatory cytokine associated with the pathogenesis of AD (Licastro et al. 2004; Cacabelos et al. 1994; Cacabelos et al. 1991), which was further decreased by blocking CR1. This correlated with the indirect neuronal death data suggesting modulating CR1 can protect neurons from death following a microglial neuroinflammatory insult. This however would need to be confirmed through use of an IL-1β antagonist to measure any influence this may have on neuronal death (Minogue et al. 2012). Expression of iNOS, as measured by ICC was decreased in microglia co-treated with CR1 blocking antibody and a trend showing a decrease in Western blotting data supports this, however this data was not significant. Further investigation inhibiting iNOS activity in neuronal cultures did not provide any subsequent findings to support a role for CR1 in iNOS activity.

Interestingly blocking CR1 and the murine homolog, Crry, in primary microglia and BV2 cultures attenuated $O_2^{\cdot -}$ production. Increased C3b expression, which could be triggered by the accelerated activation of the alternative pathway by TNFα, IL-1β or IL-6 has been previously demonstrated to have a positive correlation with an increase in respiratory burst (Hoogerwerf et al. 1990). Furthermore, decreased C3b-CR1 binding

due to lack of CR1 availability, as demonstrated in this thesis is thought to ultimately lead to reduced generation of iC3b, which in turn reduces binding to CR3, resulting in impaired NADPH oxidase activity shown in leukocytes through reduced activation of RAC2 (Wymann et al. 2000). In addition, the data presented here demonstrated that CR1 activation induced superoxide production in primary microglia and BV2 cell cultures was attenuated by the addition of the NADPH oxidase inhibitor, apocynin, thus attributing NADPH oxidase involvement in CR1 mediated $O_2^{\cdot -}$ production. It could be suggested that this correlation between reduced in $O_2^{\cdot -}$ production and blocking microglial CR1 may contribute to the reduced neuronal death. In AD, microglial activation may be the predominant source of oxidative stress suggesting that targeting this mechanism and disrupting its signalling cascade may prove a useful mechanism in the reduction of neuronal damage over the course of the disease.

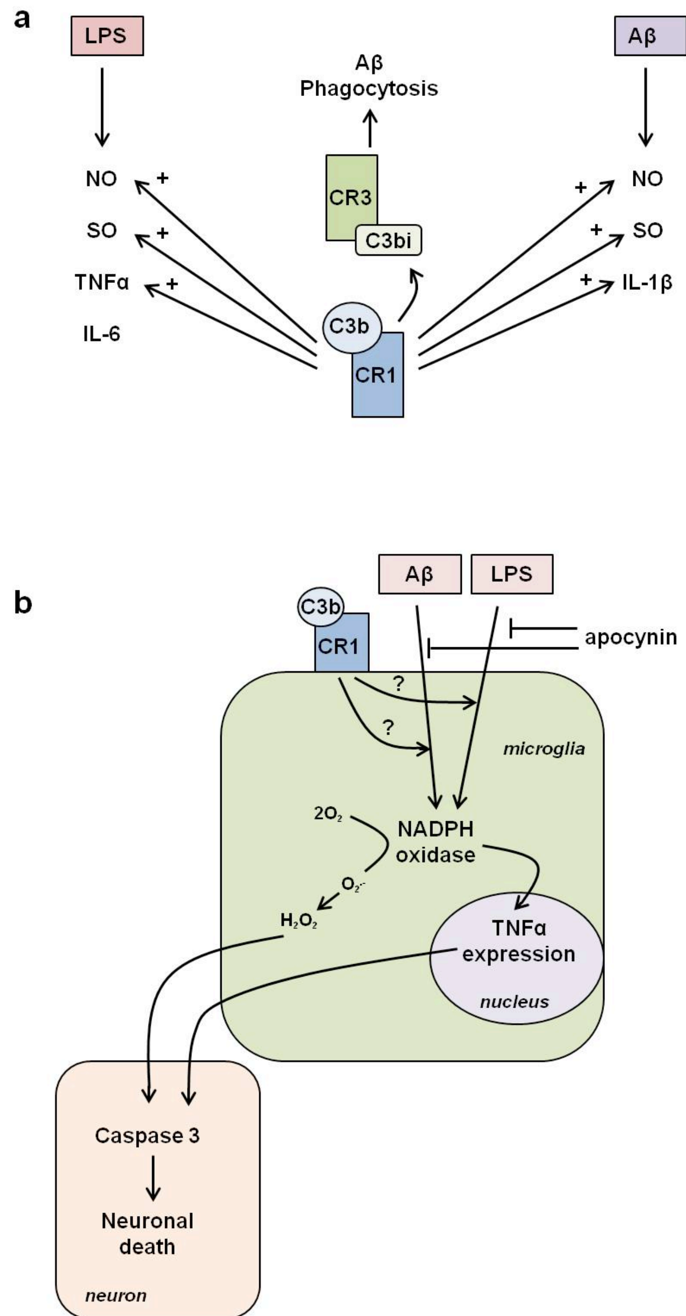


Figure 6.1: Summary of results.

(a) CR1 contributed to an increase in a number of toxic mediators released from microglia such as production of NO, SO and TNFα released from microglia following activation with LPS and NO, SO and IL-1β released from microglia following activation with $A\beta_{1\text{-}42}$. CR1 may also contribute to microglial phagocytosis of Aβ. (b) Overview of the proposed mechanism by which microglial CR1 contributes to the observed neuronal toxicity.

The last section of this thesis investigated differences in CR1 expression in human RBCs and post-mortem brain tissue. As previously discussed, the presence of CR1 in the brain has not been definitively agreed upon, and so with that there is no literature published investigating a possible correlation between CR1 expression and Aβ plaque deposition in the AD brain. This thesis provides a preliminary investigation into this due to the small size of the study but nonetheless it provides a glimpse into a possible interrelationship. Compared to AMC, CR1 expression in post-mortem brain tissue appeared lower in regions of low plaque deposition in the AD brain. In the AD brain, this may lead to reduced phagocytic clearance and thus an accumulation of Aβ plaque. Activation of the complement system by Aβ (Jiang et al. 1994; Bradt et al. 1998) contributes to the cycle of events leading to AD pathogenesis possibly though increased neuronal toxicity. However, this study would need to be repeated to increase the volume of brain tissue investigated and to determine the cell type expressing CR1. In this thesis, results following investigation of CR1 expression on RBCs between individuals supported data from previous work (Glodek et al. 2010), however the analysis determining any correlation with Aβ levels in the blood, would require additional work investigating levels in older donor individuals.

6.1 Future Work

The observations made here of CR1 mediated induction of an activated microglial phenotype are novel, however further work could be done to investigate this is greater detail. Listed below are future studies that would improve this research to enable a greater understanding of the role of CR1 in AD.

- Soluble Aβ oligomers and protofibrils have been demonstrated *in vitro* to be neurotoxic (Walsh et al. 1999; Walsh et al. 2002), and studies have shown that there are conformation-dependent differences contributing to the disease process (White et al. 2005). Therefore, it would be interesting to determine what form of Aβ generated the results presented in this thesis. Assessing the structure and conformation of Aβ oligomers, has been a contentious area of investigation. Recently there have been some interesting results following characterising α-synuclein using single-molecule fluorescence resonance energy transfer (smFRET) (Cremades et al. 2012), which may prove a favourable method for the investigation of Aβ conformation present in microglial conditioned medium at different time-points following activation. Also, electron microcopy could be used to examine the structure of the peptide.

- Scavenger receptors have been reported to mediate microglial phagocytosis of fAβ (El Khoury et al. 1996; Paresce et al. 1996) therefore the phagocytosis assays in this thesis could control for this with pre-incubation with fucoidan, a class A and B scavenger receptor ligand.

- The siRNA knockdown experiments could be further controlled by carrying out a rescue experiment, where the protein is re-introduced by transfection with the recombinant protein.

- High performance liquid chromatography (HPLC) measurement could be carried out to conclusively determine whether superoxide or H_2O_2 was produced in chapter 3.

- Measurement of neurotoxicity by lactate dehydrogenase released into the culture medium, may enhance the data in chapter 4.

- As previously mentioned, Flow cytometry analysis to comparatively measure CR1 and Aβ levels on RBCs needs to be carried out with fresh blood from donor individuals >60yrs.

- From the data presented in this thesis, it is not possible to state which cell type was expressing CR1 in the human AD brain due to the inability to carry out immunofluorescence using the CR1 antibodies provided. However, analysis using laser capture microdissection to isolate particular cells, followed by mass spectrometry may be able identify the cell type.

6.2 Conclusion

In conclusion, the data presented in this thesis provides, for the first time a possible role for CR1 in the brain and a mechanism with potentially important consequences in AD. The data presented here using two phagocytic assays identified that CR1 modulation influenced the phagocytic role of microglia, but it also put forward the prospect of CR1 contributing differently to microglial phagocytic pathways. Its role, nonetheless contributes to previous suggestions of participation in Aβ clearance (Rogers et al. 2006; Lambert et al. 2009). This thesis also provides evidence suggesting that modulation of microglial CR1 can affect neuronal survival, which is possibly mediated through superoxide production. Production of which, via NADPH oxidase activity has been previously shown to play a role in Alzheimer's disease (Shimohama et al. 2000; Ansari & Scheff 2011). Furthermore, this work explored the expression of CR1 in human post-mortem brain tissue, and was able to provide for the first time evidence for its presence in the AD brain. It attempted to explore a potential correlation between the expression of this complement regulatory protein and Aβ deposition in the AD brain and provided preliminary results featuring a correlation between areas of high plaque deposition having high expression of CR1. Finally, CR1 expression on RBCs was shown to vary between individuals with no correlation observed with Aβ levels in control blood from young donors. It is understood that basic neuroscience does not directly or immediately offer therapeutic benefit to AD suffers, however, it is hoped that further elucidation of the pathways involved will lead to a

greater understanding of AD pathogenesis which may in turn lead to more effective treatments for patients.

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