An Investigation of Inflammatory and Metabolic Blood-Based Biomarkers of Cognitive Decline

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I. Declaration of Authorship

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II. Abstract

Alzheimer's disease (AD) and other dementias are the main cause of cognitive impairment in older adults. AD is fast becoming a global epidemic, yet the development of a disease-modifying therapy remains elusive. The identification of earlier preclinical stages of disease offers the best possibility of slowing down disease progression, and the use of blood-based biomarkers for this purpose is crucial. However, studies so far have shown poor reproducibility and few studies have examined asymptomatic individuals before significant cognitive deficits arise.

Inflammation is a key feature of AD pathogenesis, and recently a close link between inflammation and metabolism has been demonstrated. The aim of this study was to investigate inflammatory and metabolic blood-based biomarkers that could be indicative of cognitive decline. This was accomplished through examining markers in monocytes incubated with plasma from cohorts with cognitive dysfunction, and by assessing markers in pro-inflammatory stimulated monocyte-derived macrophages (MDMs) and peripheral blood mononuclear cells (PBMCs) from a group of healthy older adults described as having a subtle cognitive deficit based on their performance on a story recall test relative to their estimated IQ (IQ-discrepant).

The age-related inflammatory phenotype of C57/BL6 mice was initially assessed and CXCL1 mRNA was identified in plasma-treated monocytes as a potential peripheral marker of neuroinflammation. Expression of IL-8, the human homolog of CXCL1, was revealed to be upregulated in monocytes incubated with plasma from IQ-discrepant participants and mild cognitive impairment and AD patients compared with their respective controls. In addition, monocytes treated with plasma from AD patients exhibited an increase in glycolysis. The data suggest that IL-8 mRNA, and perhaps glycolysis, in plasma-treated monocytes could be useful as a potential biomarker assay for cognitive dysfunction.

MDMs from IQ-discrepant, compared with IQ-consistent, participants displayed an exacerbated increase in TNFα mRNA and protein expression of TLR2 and CD206 following stimulation with amyloid-beta (Aβ), as well as an exaggerated response to Aβ + lipopolysaccharide (LPS) as demonstrated by the increase in TNFα mRNA and secretion and shift towards glycolysis. The effect of Aβ+LPS stimulation was also greater in PBMCs from IQ-discrepant participants, as shown by an increase in TNFα, IL-6 and IL-8 production, increased glycolysis and upregulation of PFKFB3 mRNA, an enzyme which drives glycolysis. The data suggest that cells from IQ-discrepant participants have a heightened response to pro-inflammatory stimulation.

Overall, the findings from this study show that IL-8 mRNA in plasma-treated monocytes and inflammatory and metabolic markers in MDMs and PBMCs from subjects with an IQ-discrepant memory may be useful indicators of cognitive decline.

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2-DG	2-Deoxy-D-glucose
AA	Alzheimer's Association
AD	Alzheimer's disease
AMPK	AMP activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein 1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APP/PS1	Amyloid precursor protein/presinilin 1 double mutation
Arg-1	Arginase-1
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
Aβ	Amyloid-beta
BBB	Blood-brain barrier
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
CCL	Chemokine C-C motif ligand
CCR	Chemokine C-C motif receptor
CD	Cluster of differentiation
cDMEM	Complete Dulbecco's modified Eagle medium
cDNA	Complementary deoxyribonucleic acid
Chi3l	Chitinase-3-like
CLU	Clusterin
CNS	Central nervous system
CO_2	Carbon dioxide
CR1	Complement receptor 1
cRPMI	Complete Roswell Park Memorial Institute medium
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
CXCL	Chemokine C-X-C motif ligand
CXCR	Chemokine C-X-C motif receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetertra-acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
F-2,6-BP	Fructose-2,6-bisphosphate
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FDG	Fluoro-2-deoxy-D-glucose
GLUT	Glucose receptor

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association studies
HD	Huntington's disease
HIF-1α	Hypoxia-inducible factor 1-alpha
IFNγ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factors
JAK	Janus kinase
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinases
MCI	Mild cognitive impairment
MDM	Monocyte-derived macrophage
MDP	Monocyte-macrophage DC progenitor
MMSE	Mini-mental state examination
MoCA	Montreal Cognitive Assessment
MPS	Mononuclear-phagocyte system
MRC-1	Mannose receptor
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NART	National Adult Reading Test
NFL	Neurofilament light
NFT	Neurofibrillary tangles
NIA	National Institute on Aging
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NK	Natural killer
NK-κB	Nuclear factor-kappa B
NLRP3	NOD-like receptor family, pyrin domain containing 3
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NSAIDs	Non-steroid anti-inflammatory drugs
OCR	Oxygen consumption rate
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD	Parkinson's disease
PDH	Pyruvate dehydrogenase
PET	Positron emission tomography
PFK	Phosphofructokinase

PFKFB	6-phosphofructo-2-kinase/fructose-2,6-bisphosphate
PGC-1β	Peroxisome-proliferator-activated receptor-γ co-activator-1β
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol-12-myristate-12-acetate
PPP	Pentose phosphate pathway
PRR	Pathogen recognition receptor
PSEN	Presenilin
P-tau	Phosphoryated-tau
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RQ	Relative quantity
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
SEM	Standard error of the mean
STAT	Signal transducers and activations of transcription
TBI	Traumatic brain injury
TBS-T	Tris-buffered saline-Tween
TCA	Tricarboxylic acid
TGFβ	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
T-tau	Total tau
VLP1	Visinin-like protein 1
WMS	Wechsler Memory Scale
WT	Wild-type

Chapter 1: Introduction

1.1 Cognitive decline

Ageing is associated with a change in cognitive abilities. A gradual decline in particular aspects of cognitive function occurs over time. These include processing speed, attention, executive functioning, visuospatial abilities and memory (Harada et al., 2013). Other cognitive abilities remain largely intact during ageing, such as vocabulary and certain elements of memory e.g. procedural memory. Changes in memory, mostly related to semantic and episodic memory, is the most common cognitive complaint of older adults. Structural and functional alterations in the brain correlate with age-related cognitive changes, including a loss of grey and white matter volume and a decrease in synapses (Murman, 2015).

The rate of cognitive decline is accelerated in neurodegenerative conditions, including various dementias, Parkinson's disease (PD), Huntington's disease (HD) and multiple sclerosis. Dementia is the most common cause of later-life cognitive decline, the main subtypes of which are Alzheimer's disease (AD), vascular dementia, dementia with Lewy bodies and frontotemporal dementia (Sonnen et al., 2008). Cognitive dysfunction is also evident at earlier stages of dementia progression, and the term mild cognitive impairment (MCI) was introduced in 1988 by Reisberg and colleagues to describe the intermediate stage between normal cognition and dementia, where cognitive impairment is evident but general cognitive function and daily activities are not affected (Reisberg et al., 1988). Individuals with MCI are typically categorised into either amnestic MCI or non-amnestic MCI depending on whether they present deficits in episodic memory performance or cognitive domains other than memory respectively (Petersen, 2004). However it is important to note that, although it is the most common underlying aetiology, not all individuals diagnosed with MCI go on to develop dementia as patients can also progress to PD, HD, depression, HIV/AIDs and traumatic brain injury (TBI) (Petersen et al., 2014). The rate of progression from MCI to AD is highly variable, with studies reporting conversion rates ranging between 16-41% per year (Gauthier et al., 2006).

1.2 Alzheimer's disease

AD was first described by Alois Alzheimer in 1906. His patient, Auguste Deter (more widely known as Auguste D), suffered from memory loss, disorientation, hallucinations and delusions and died at the age of 55, 4 years after her symptoms first presented (Cipriani et al., 2011). This condition was subsequently termed 'Alzheimer's disease' in 1910 by Emil Kraepelin, who distinguished it from senile dementia. Upon examination of Auguste D's brain *post-mortem*, Alzheimer noted the loss of neurons and also the presence of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFT), although these pathological hallmarks of AD were not identified as typifying the disease until the 1980's (Glenner and Wong, 1984, Brion et al., 1985).

Today AD is the most common form of dementia, accounting for 60-80% of cases. Dementia affects approximately 46.8 million people worldwide and this number is expected to triple by the year 2050 (World Alzheimer's Report 2015). AD and other dementias are extremely costly to the economy, with the global cost of dementia estimated to reach $1 trillion in 2018, highlighting the enormous burden that these conditions place on society. Clinical symptoms of AD typically include significant progressive decline in memory and at least one other cognitive domain, along with functional impairment that interferes with activities of daily living (Chertow et al., 2013). The incidence of AD and other dementias is more prevalent in women than men; however it is not known whether this is a reflection on higher mortality rates in men from causes other than dementia/AD (Masters et al., 2015).

The symptoms of AD are caused by progressive neuronal loss, which first occurs in the hippocampus and areas of the frontal and temporal lobes and then progresses to other areas of the neocortex (Masters et al., 2015). There are two main forms of AD based on the age of onset; early onset AD (EOAD) or familial AD typically occurs before the age of 65 and accounts for 1-5% of cases, and late onset AD (LOAD) or sporadic AD occurs after the age of 65 and accounts for >95% of cases (Reitz and Mayeux, 2014). The aetiology of familial AD is associated with mutations in 3 genes involved in the processing of Aβ; amyloid precursor protein (APP) and presenilin

(PSEN) 1 and 2, which are autosomal dominantly inherited and highly penetrable. On the other hand, the aetiology of sporadic AD is much more complex with contributions from multiple genes and environmental factors.

Polymorphisms in the apolipoprotein E (APOE) gene were first identified as conferring an increased risk for sporadic AD (Giri et al., 2016). In the brain, APOE is mainly produced by astrocytes and is responsible for transporting cholesterol and other lipids to neurons for the maintenance of synapses and injury repair (Bu, 2009). There are 3 alleles of APOE (ε2, ε3 and ε4) and the ε4 allele is associated with an increased risk for sporadic AD, whereas the ε2 allele is reported to confer a degree of protection (Giri et al., 2016). The presence of one allele of APOE ε4 is reported to increase the risk of AD by 2-3 fold, and the risk further increases to approximately 12-fold in individuals with two APOE ε4 alleles (Corder et al., 1993, Michaelson, 2014). APOE ε4 status is associated with a greater rate of cognitive decline in older adults (O'Hara et al., 1998) and it is also a strong predictor of progression from MCI to AD (Petersen et al., 1995). Furthermore, individuals with an APOE ε4 allele exhibit an increase in Aβ plaque burden (Kok et al., 2009) and it has been recently demonstrated that the APOE ε4 allele has a strong impact on amyloid pathology during the early seeding stage, and may influence Aβ clearance and its subsequent accumulation (Liu et al., 2017). The advent of genome-wide association studies (GWAS) has identified numerous susceptibility genes for sporadic AD which are less frequent and less strongly associated genetic risk factors. The affected genes are broadly involved in 3 major pathways; 1) lipid metabolism e.g. clusterin (CLU) and the ATP-binding cassette transporter (ABCA7), 2) phagocytosis/endocytosis e.g. phosphatidylinositol-binding clathrin assembly protein (PILCAM) and sortilin-related receptor 1 (SORL1) and 3) the immune response e.g. CD33, triggering receptor expressed on myeloid cells 2 (TREM2) and complement receptor type 1 (CR1) (Giri et al., 2016). Non-genetic risk factors for AD include cardiovascular disease, type 2 diabetes, TBI and lifestyle factors such as diet, physical exercise and cognitive engagement, which are thought to impact on cognitive reserve (the capacity of the brain to withstand pathological insult and therefore confers a certain degree of protection) (Aisen et al., 2017).

AD is pathologically characterised by the presence of extracellular Aβ plaques and intracellular NFT. NFT are made up of aggregated filaments of the phosphorylated form of the microtubule associated protein tau, the primary function of which is to stabilise microtubules (Ballatore et al., 2007). Under normal conditions, tau is phosphorylated at particular residues (181 or 231), however in pathological states they become hyperphosphorylated and form fibrils and aggregates. The precise cause of tau aggregation is unknown, although one line of evidence suggests that it is accelerated by Aβ deposition (Ballatore et al., 2007). The aggregation of hyperphosphorylated tau is evident in other neurodegenerative diseases such as Creutzfeldt-Jakob disease and progressive supranuclear palsy, these are collectively referred to as tauopathies (Masters et al., 2015).

1.2.1 The amyloid hypothesis of AD

The amyloid hypothesis, described in the early 1990's, has been the leading theory for the pathogenesis of AD and has been a focal point for the development of potential therapies (Selkoe, 1991, Hardy and Higgins, 1992). This hypothesis states that AD is caused by the accumulation of Aβ which forms extracellular diffuse plaques leading to the formation of intracellular NFT, synaptic loss and neurotransmitter signalling deficits and ultimately cell death and cognitive impairment. This process occurs gradually over 20-25 years before the onset of symptoms. It is important to note that accumulation of Aβ does not only result from increased production due to missense mutations in APP, PSEN1 and PSEN2 as seen in familial AD, but also from an imbalance between Aβ production and clearance mechanisms which is thought to occur in sporadic AD (Selkoe and Hardy, 2016).

Aβ is produced continually as a normal product of APP metabolism (Haass et al., 1992). The function of APP is still unknown, although it suspected to have a role in synaptic plasticity (Masters et al., 2015). APP is a type-1 transmembrane protein with its amino terminus within the extracellular space and its carboxyl terminus within the cytosol and is produced in large quantities in neurons (Haass et al., 2012). It is sequentially cleaved by a family of secretase proteins via two pathways; the amyloidogenic and non-amyloidogenic pathway (Figure 1.1).

APP is preferentially processed by the non-amyloidgenic pathway, where APP is initially cleaved in the middle of the Aβ region by α-secretase, thereby preventing its production (Kummer and Heneka, 2014). The cleavage of APP by α-secretase is largely mediated by the ADAM (a disintegrin and metalloproteinase) family of proteases (O'Brien and Wong, 2011). This generates a large soluble APPα (sAPPα) ectodomain and a truncated α-C-terminal fragment (α-CTF) which is further cleaved by γ-secretase to generate p3 peptides.

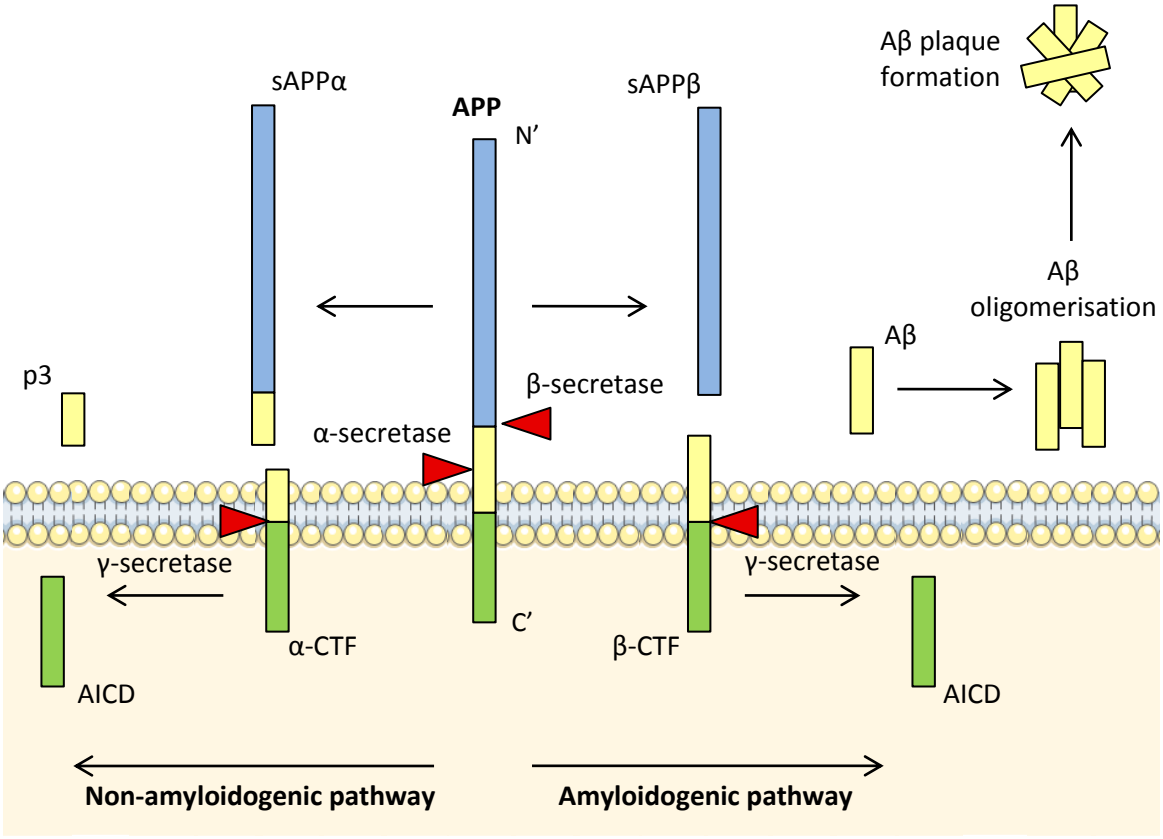

Figure 1.1 Pathways of APP processing. *APP is sequentially cleaved via the amyloidogenic or non-amyloidgenic pathway. Initial cleavage of APP by the α-secretase or β-secretase generates the sAPPα or sAPPβ ectodomains and either the α-CTF or β-CTF respectively. γ-secretase then cleaves the α-CTF into p3 and AICD peptides and the β-CTF into AICD or Aβ peptides of varying lengths. The Aβ42 species has the highest aggregation rate and forms oligomers leading to the deposition of Aβ plaques.*

In the amyloidogenic pathway, the first cleavage is instead mediated by β-secretase, which acts at a different site to α-secretase thereby producing a large ectodomain called the sAPPβ and a smaller truncated β-CTF. The enzyme responsible for this initial cleavage is the β-site amyloid precursor protein cleaving enzyme 1 (BACE1),

which can act at a minimum of two sites on APP (Kummer and Heneka, 2014). The β-CTF fragment is then cleaved by γ-secretase protein complex which results in the production of Aβ of varying lengths from 37-43 amino acids which are released into the extracellular fluids. The Aβ40 and Aβ42 species are most commonly produced and Aβ42 has the strongest tendency to aggregate to oligomers leading to neuronal injury (Haass and Selkoe, 2007). In addition, post-translational modifications of Aβ may potentiate the rate of aggregation (Kummer and Heneka, 2014). In both pathways, γ-secretase also generates the APP intracellular domain (AICD) from the cleavage of α-CFT and β-CTF fragments, which is released into the cytosol and may have a role in nuclear signalling (Haass et al., 2012).

The amyloid hypothesis is supported by the fact that genetic variants of APP, PSEN1 and PSEN2 result in an increased ratio of Aβ42/Aβ40 in patients (Masters et al., 2015). The presenilin proteins 1 and 2, encoded by PSEN1 and PSEN2, form part of the γ-secretase complex and contain the two critical aspartyl residues for the catalytic domain of the γ-secretase (Wolfe et al., 1999). Most mutations in APP occur at or near the sites cleaved by the secretases and increase the self-aggregation of the resulting peptides, rather than affecting its production (Selkoe and Hardy, 2016). An important aspect of the amyloid hypothesis is the fact that the APP gene is located on chromosome 21 which is duplicated in individuals with Down's syndrome, which is associated with the development of a premature AD-type pathology. Interestingly, individuals with rare cases of a translocation form of Down's syndrome that only involves the distal part of chromosome 21 (and therefore does not duplicate APP) do not develop AD (Prasher et al., 1998). Oligomers of Aβ42 have also been shown to induce tau hyperphosphorylation at AD-relevant epitopes and induce neuronal toxicity in culture, providing further evidence for Aβ as a key driver of pathology in AD (Selkoe and Hardy, 2016).

However, the amyloid hypothesis has been controversial as there are several important observations for which it does not account. Of most concern is the failure of numerous clinical trials in late Phase III stages that have directly targeted Aβ in some manner. Although this target is not validated and no anti-amyloid therapy has shown efficacy in human clinical trials, this is still the most popular target as 65.6% of

trials between 2002 and 2012 were directed at this target (Cummings et al., 2014). Furthermore, many studies have observed that the amyloid burden present in the brain does not always correlate with the degree of cognitive impairment, and some individuals with no clinical symptoms of AD have many cortical Aβ deposits (Hardy and Selkoe, 2002). Additionally, NFT counts have been shown to correlate better with clinical symptoms, and studies also suggest that these may precede the formation of plaques (Selkoe and Hardy, 2016). It is thought that these discrepancies could be due to the fact that 1) Anti-amyloid agents have been introduced too late in disease progression, 2) Aβ accumulates over a lifetime and downstream effects might be more proximal to clinical symptoms, 3) many of the Aβ deposits in cognitively normal adults are diffuse plaques and the Aβ oligomer content of plaques are much lower than the AD brain and 4) mutations in tau do not lead to Aβ deposition while mutations that increase Aβ accumulation leads to tau aggregation (Selkoe and Hardy, 2016). It has now been hypothesised that plaques can sequester oligomers until they reach saturation, where after they build up in synapses and other surrounding areas (Hong et al., 2014).

1.2.2 AD mouse models

The use of AD animal models has been crucial in studying the underlying mechanisms of disease and for drug development. Although no model recapitulates all aspects of the condition, each model developed allows for the examination of a particular component of the disease (LaFerla and Green, 2012). As the etiology of sporadic AD is unknown, most models have been generated using the mutations associated with familial AD. Mice transgenic for mutant human APP, such as the Tg2576 model, have been the most widely used model. They exhibit cerebral Aβ deposition and develop behavioural deficits comparable with AD, which provides another line of support for the amyloid hypothesis (Walsh and Selkoe, 2004). The APP/PS1 model of AD expresses mutant PSEN1 as well as overexpressing the APP gene containing the Swedish mutation, similar to the Tg2576 model. Due to the presence of two mutated genes these mice have a greater production of Aβ42 and a more rapid progression of pathology, with the development of plaques and

behavioural changes evident from 6-7 months (Jankowsky et al., 2004). However, APP transgenic mice fail to produce NFTs. The triple transgenic model of AD (3xTg-AD) contains mutant human tau in addition to APP and PSEN1, developing both Aβ plaques and NFTs with Aβ deposition occurring first (Oddo et al., 2003). There are many translational issues with all AD mouse models, the prime concern being the lack of any significant neuronal death and lack of concordance between the effectiveness of drugs in preclinical studies and human clinical trials. Additionally, transgenic mice are produced by introducing genetic variants to recapitulate familial AD, whereas the majority of AD cases are sporadic where genetic determinants are less evident (LaFerla and Green, 2012).

1.2.3 Inflammation in AD

An increase in baseline inflammation is evident during ageing which is reflected in the brain as well as in the periphery, a phenomenon known as inflammaging (Franceschi et al., 2017). There are several factors that probably contribute to inflammaging, including immunosenescence and dysregulation of the balance between innate and adaptive immunity. This is also seen in neurodegenerative diseases such as AD which have been shown to have an exaggerated inflammatory response and a heightened level of immune activation (Deleidi et al., 2015). Inflammation is a prominent feature of AD and there has been much debate over whether it is a driver of pathology or a consequence of disease progression (Wyss-Coray, 2006), although now it is generally accepted that it both contributes to and exacerbates AD pathology. It has already been suggested that the amyloid hypothesis should be altered to include a more central role of tau in AD pathology, and further modification is required to also incorporate the role of neuroinflammation (Heppner et al., 2015).

One major line of evidence that proposes inflammation as a key player in AD stems from the fact that prolonged use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been reported to reduce the risk of AD by approximately 50% (Wyss-Coray, 2006). In APP transgenic mice, treatment with NSAIDs was found to reduce Aβ deposition (Lim et al., 2000) and *in vitro* studies have shown that NSAIDs can reduce

Aβ production through regulation of β- and γ-secretases (Eriksen et al., 2003, Sastre et al., 2003). However, clinical trials with NSAIDs in AD have been minimally effective, which might be related to the specific target of the NSAID used (Wyss-Coray, 2006).

Numerous GWAS have implicated mutations in several genes that play a role in the immune response as a risk factor in sporadic AD. These include CR1 and CLU (also known as APOJ) (Lambert et al., 2009). CD33 and TREM2 were later identified (Naj et al., 2011, Jonsson et al., 2013), and these have been shown to play a role in phagocytosis and clearance of Aβ (Heneka et al., 2014). Interestingly, deficiency of TREM2 was reported to ameliorate Aβ and tau pathology and reduce inflammation in an AD mouse model (Jay et al., 2015). Recently, an additional variant in TREM2 and other proteins highly expressed in microglia, phospholipase C gamma 2 (PLCG-2) and abscisic acid insensitive 3 (ABI3), were identified in a study of around 85,000 AD patients and controls, further implicating microglial (and macrophage) mediated innate immunity contribution to AD pathogenesis (Sims et al., 2017). There are currently around 27 susceptibility genes for sporadic AD which strongly implicates genetic alterations in the immune system as risk factors, and genes involved in endocytosis, transport and ubiquitination are also implicated.

Microglia, the resident innate immune cells of the brain, are normally present in a quiescent 'resting' state where they survey the microenvironment for the presence of pathogens or cellular debris. Upon activation, microglia efficiently phagocytose the pathogen or cell debris to restore homeostasis. Activated microglia have been shown to surround Aβ plaques, suggesting that Aβ triggers microglial activation leading to the production of inflammatory mediators including inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)α (Lynch, 2009, Heppner et al., 2015). In AD, activated microglia are thought to have a reduced phagocytic capacity, leading to inefficient clearance of Aβ and subsequent accumulation of Aβ plaques (Heneka et al., 2015). Studies of aged animals have shown that microglia have an enhanced sensitivity to inflammatory stimuli and this microglial 'priming' is associated with increased cytokine and reactive oxygen species (ROS) production (Heneka et al., 2015). The result of microglial priming is an

exaggerated response to a secondary stimulus, which may occur peripherally and well as within the central nervous system (CNS). Indeed, systemic infection (e.g. periodontitis) in AD patients is associated with a faster rate of cognitive decline, indicating a role for peripheral inflammation in AD pathology (Holmes et al., 2003, Ide et al., 2016).

Astrocytes are also present in an activated state in AD, and are seen localised around senile plaques alongside microglia (Medeiros and LaFerla, 2013). Astrocytes are also capable of contributing to the neuroinflammatory environment by releasing pro-inflammatory cytokines, nitric oxide (NO), and other potentially cytotoxic molecules after exposure to Aβ (Heneka et al., 2015). In line with this, several inflammatory cytokines have been shown to be increased in the brains of AD patients and in transgenic mouse models of AD (Patel et al., 2005). Factors driving this neuroinflammatory response in AD might include risk factors with an associated inflammatory component, such as obesity, systemic infection and reduced physical activity (Heneka et al., 2014). Inflammatory changes are evident in MCI patients, occurring before the detection of Aβ plaques, indicating that inflammation is an early event in the pathogenesis of AD (Calsolaro and Edison, 2016).

1.3 Treatments for AD

The management of AD involves pharmacological treatment that aims to enhance cognition, address behavioural abnormalities, and treat any co-morbidities commonly encountered with AD e.g. psychiatric disorders (Masters et al., 2015). Despite numerous clinical trials over the past few decades, the current available treatments for AD only address the symptomatology of disease and do not alter disease progression. The need for a disease-modifying therapy that can slow down or halt disease progression is paramount, and yet the identification of such a treatment remains elusive. In an analysis of clinical trials carried out between 2002 and 2012, the overall success rate was 0.4% (99.6% failure) (Cummings et al., 2014). It is thought that one of the main reasons behind the failure of many potential therapeutics is the stage of AD at which the patients are enrolled in the trials – most are of moderate-severe AD, where substantial damage and neuronal loss have

already occurred. AD pathology manifests at least a decade prior to the emergence of the clinical symptoms required for diagnosis, therefore, treatment effects might be expected to be minimal in the face of the excessive neuronal damage. Identification of MCI, a prodromal stage of AD, offers the possibility of identifying those who are at risk for progression to AD and therefore earlier commencement of treatment. There is a need to develop an indicator of disease at even earlier 'preclinical' or 'asymptomatic' stages in order to enable earlier therapeutic intervention. It is believed that such an intervention could delay the onset of AD by 5 years and would result in a 57% reduction in the number of patients with AD and cut the associated health care costs almost in half (Sperling et al., 2011).

1.3.1 Approved treatments

Five drugs have been approved for the treatment of AD – four cholinesterase inhibitors (tacrine, donepezil, galantamine and rivastigmine) and the N-methyl-D-aspartate (NMDA) receptor antagonist memantine. Tacrine was the first drug approved by the US Food and Drug Administration (FDA) in 1993, followed by donepezil (1996), rivastigmine (1998) and galantamine (2001) (Cummings et al., 2014). Tacrine has since been discontinued in the US (Nygaard, 2013). The availability of cholinesterase inhibitors varies from country to country in Europe, with only rivastigmine being granted for use centrally by the European Agency for the Evaluation of Medical Products (EMEA) (Scarpini et al., 2003). Memantine was first approved for use in Europe in 2003, followed by the FDA in 2004 for patients with moderate to severe AD (Cummings et al., 2014).

The use of cholinesterase inhibitors is based on the findings that there is a deficit in cholinergic transmission in AD. These drugs decrease the catabolism of acetylcholine thereby prolonging the length of time it is present in the synapse and enhancing postsynaptic stimulation, which translates to beneficial effects on cognitive, behavioural and functional symptoms of AD (Masters et al., 2015). While donepezil and galantamine selectively inhibit acetylcholinesterase, rivastigmine additionally inhibits butyrylcholinesterase (Scarpini et al., 2003). Memantine targets the glutamatergic system by partially antagonizing the NMDA receptor, which alleviates

glutamate-mediated excitotoxicity by normalising neurotransmitter levels. Memantine is commonly used in combination therapy with a cholinesterase inhibitor, which confers additive benefits (Tariot et al., 2004).

For all approved drugs, the magnitude of the clinical response is limited. A delayed rate of decline in cognition is observed in the majority of patients for 6-9 months following treatment commencement, but only a minority of patients experience cognitive improvement (Takeda et al., 2006, Di Santo et al., 2013). Prolonged use suggests a certain degree of continued benefit compared with patients not receiving treatment, despite a decline in cognition (Masters et al., 2015). The use of donepezil has been investigated in MCI and is reported to confer a small benefit in APOE ε4 carriers by slightly improving cognition and delaying the onset of AD by approximately 1 year (Nygaard, 2013).

1.3.2 Aβ-immunotherapy trials

Many anti-Aβ therapeutics have been trialled, which aim to both immunise against Aβ to increase removal of Aβ and inhibit aggregation, and inhibit the processing of APP by secretases. Initial studies employed active immunisation; however a Phase II clinical trial of the AD vaccine AN-1792 which targeted Aβ42 was terminated after 2-3 doses due to the development of meningoencephalitis in 6% of patients, attributed to cytotoxic T cells (Orgogozo et al., 2003). To overcome this, a new generation of vaccines have been developed which target B-cell epitopes without activating T cells (Mangialasche et al., 2010). Many potential agents have been developed through passive administration of Aβ-specific monoclonal antibodies. Bapineuzumab progressed to Phase III trials in mild-to-moderate AD but did not confer any cognitive benefit and drug development was ceased in 2012 (Salloway et al., 2014). The highly anticipated Solanezumab failed to achieve its clinical endpoints in two large Phase III trials of mild and moderate AD, however the rate of cognitive decline was reported to decrease by 34% in mild AD patients of the two trials combined (Doody et al., 2014, Selkoe and Hardy, 2016). As no benefit was observed in moderate AD, the findings support the idea that therapies should be commenced at earlier stages of disease. However, a subsequent Phase III trial of mild AD patients failed to show any

cognitive benefit, with the company Eli Lilly declaring that it would not be pursuing Solanezumab any further as a potential treatment (Abbott and Dolgin, 2016). Excitement has now been building around Aducanumab from Biogen, which was shown to reduce Aβ plaques in patients with prodromal or mild AD and was accompanied by a slowing of cognitive decline, and is now progressing to Phase III trials (Sevigny et al., 2016).

Clinical trials initially focused on γ-secretase inhibitors as drug candidates; however trials of both semagacestat and avagacestat were halted at Phase III and Phase II trials respectively due to a worsening of clinical symptoms (Nygaard, 2013). The focus therefore has turned to inhibition of β-secretase by targeting BACE1, the activity of which is reportedly increased in the AD brain (Stockley et al., 2006). Initial trials were first conducted by Eli Lilly, however Phase I trials of two potential compounds LY2811376 and LY2886721 were discontinued due to toxic side effects (Moussa, 2017). Encouraging early Phase I results from Merck's verubecestat (MK-8931) resulted in a large combined Phase II/III trial of 2,000 patients with mild-to-moderate AD, referred to as the EPOCH study (Moussa, 2017). However, the company decided to conclude the trial at an earlier stage due to a lack of clinical efficacy. A Phase III trial of verubecestat is still underway in patients with prodromal AD, with results expected in 2019 (Mullard, 2017). Four other companies are still pursuing clinical trials with BACE1 inhibitors at mid or late-stage trials, including AZD3293 from AstraZeneca/Eli Lilly and Elenbecestat from Biogen/Eisai (Mullard, 2017).

The increased number of trials targeting Aβ highlights the need for the validation of biomarkers to improve diagnostic accuracy and identify treatment benefits (Masters et al., 2015). It is anticipated that success of an anti-Aβ therapy that modifies disease progression will provide compelling evidence for the amyloid hypothesis (Sevigny et al., 2016). Despite the failure of many Aβ-targeted therapies, Aβ continues to be an attractive target for drug candidates due to the fact that it is detected early in disease as shown by biomarkers.

1.3.3 Other therapeutic approaches

Tau has gained interest as a potential candidate for drug development, perhaps due to the failure of Aβ trials. Two main approaches have been adopted to target tau, which is to either promote tangle disassembly or modulate tau phosphorylation through inhibition of kinases and tau aggregation (Mangialasche et al., 2010). The AADvac1 was the first tau immunotherapy to enter clinical trials and its safety has recently been reported in a Phase I trial and further trials will assess its clinical efficacy (Novak et al., 2017). An anti-histamine, latrepiridine, was shown to significantly improve the clinical outcome of patients with mild-to-moderate AD in Phase II trials, however this was not replicated in further Phase III trials (Doody et al., 2008). Interestingly, the most potent actions of latrepiridine are attributed to its ability to enhance mitochondrial function, and mitochondrial dysfunction is thought to occur early on in AD pathogenesis (Mangialasche et al., 2010). A considerable interest in exercise as a non-pharmacological therapeutic for AD has developed. Exercise has been shown to reduce amyloid pathology and improve cognitive function in mouse models of AD, and a meta-analysis of exercise studies in MCI and AD patients concluded that physical activity has the potential to improve cognition (Ryan and Kelly, 2016, Strohle et al., 2015). Other non-pharmacological approaches include cognitive training and cognitive stimulation therapy, which has been reported to have beneficial effects in AD patients (Ballard et al., 2011).

1.4 Diagnostic utility of biomarkers in AD

The first clinical concept of AD was made in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) working groups (McKhann et al., 1984). Commonly referred to as the NINCDS-ADRDA criteria, these guidelines were used worldwide for 27 years to assist in the diagnosis of possible AD. It largely focused on the use of neuropsychological tests to evaluate cognitive function, and postulated that if patients presented with clinical symptoms this would correlate with the presence of AD pathophysiology upon autopsy (Jack et al., 2011). These criteria were revised in 2007 when it became apparent that it was not sufficiently

adequate to support advances in the field, and the use of biomarkers was first incorporated into a clinical-biological concept (Dubois et al., 2007). The criteria were again revised in 2011 by the National Institute on Aging (NIA) and the Alzheimer's Association (AA) into a three stage clinical-biological concept of AD pathogenesis: the asymptomatic preclinical phase, the symptomatic predementia phase, and the dementia phase of AD (Jack et al., 2011). This resulted in a proposed set of guidelines and diagnostic criteria for each stage of dementia due to AD, which includes differential diagnosis from other dementias and incorporates the diagnostic utility of biomarkers (Albert et al., 2011, McKhann et al., 2011, Sperling et al., 2011). This was further refined in 2014, which placed more focus on cerebrospinal fluid (CSF) Aβ/tau concentrations and imaging of amyloid through positron emission tomography (PET) as biomarkers rather than markers from magnetic resonance imaging (MRI) (Dubois et al., 2014).

A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Group, 2001). Biomarkers for AD have mainly been developed through analysis of CSF or by neuroimaging techniques and 5 are well established enough to be used in diagnostic criteria and clinical trials (Jack et al., 2013). The development of biomarkers has had a huge impact in facilitating correct diagnosis of AD and distinguishing AD subtypes and is crucial for predicting potential decline in cognitively normal individuals. Biomarkers also have significant utility in drug development for patient selection, monitoring of disease progression and assessing the drug target engagement.

1.4.1 Neuroimaging biomarkers

The main neuroimaging techniques employed in the search for a reliable biomarker for AD have been MRI and PET. MRI techniques include structural MRI, functional MRI and diffusion tensor imaging. Structural MRI focuses largely on atrophy of particular brain regions including the hippocampus, parahippocampus, amygdala, entorhinal cortex and medial temporal lobe (Fiandaca et al., 2014). Hippocampal atrophy was integrated into the 2007 and 2011 diagnostic criteria for AD, and an

overall cerebral atrophy is accepted as a biomarker indicative of progression from MCI to AD.

The use of radioactive compounds or probes in PET imaging has enabled imaging of Aβ, tau, and brain metabolism. Fluoro-2-deoxy-D-glucose (FDG) PET measures glucose utilisation in the brain and therefore areas of cerebral activity can be identified. A decrease in cerebral glucose metabolism is evident in AD, with a regional pattern of posterior temporoparietal > frontal hypometabolism (Cohen and Klunk, 2014). The development of the ^{11}Carbon-labelled Pittsburgh compound B (^{11}C-PiB) made the *in vivo* imaging of Aβ possible (Klunk et al., 2004), with many other tracers being subsequently developed. Aβ PET provides information about the extent of plaque burden in the brain and is a validated pathophysiological marker for fibrillary Aβ (Dubois et al., 2014). Concordance between positive Aβ PET and *post-mortem* analysis is over 90%, thus is it a good marker of AD pathology (Clark et al., 2011). It has also been an accurate predictor of progression from MCI to AD (Rowe et al., 2013). Aβ PET has confirmed that the deposition of Aβ occurs prior to the emergence of cognitive decline and brain atrophy with the detection of abnormalities in Aβ PET presenting a decade before (Villemagne et al., 2013). Additionally, it has improved cohort selection for clinical trials, for example in the Phase Ib trial of Aducanumab, patients for the trial were selected on the basis of a positive Aβ PET result, and a reduction in Aβ plaque load following treatment was demonstrated using Aβ PET (Sevigny et al., 2016).

PET ligands for tau have also been developed and most studies have been carried out using the tracer T807. Studies have suggested that tau correlates better with the neuronal loss and cognitive symptoms in AD than Aβ, therefore the development of a tau imaging biomarker would be of great benefit (Sperling et al., 2014). Investigation of the use of Tau PET is underway, with preliminary studies showing good discrimination between control and AD patients and correlations with degree of cognitive impairment (Saint-Aubert et al., 2017).

1.4.2 CSF biomarkers

Biomarker development through CSF analysis is an appealing approach due to the fact that the CSF communicates freely with the brain interstitial fluid and it therefore offers the possibility of directing assessing the brain neurochemistry (Fiandaca et al., 2014). The concentrations of Aβ and tau species have been most widely studied, but other markers such as neurofilament light polypeptide (NFL), visinin-like protein 1 (VLP1) and YKL-40 (chitinase-3-like (Chi3l) 1) have also been examined.

Concentrations of Aβ42 (and the Aβ42/40 ratio) have consistently been shown to be decreased in CSF from AD patients and correlates well with plaque deposition by *post-mortem* examination and with Aβ PET imaging (Masters et al., 2015). It has been suggested that the ratios of Aβ42/40 and Aβ42/38 are more appropriate in identifying prodromal stages of AD and in distinguishing AD from other dementias (Janelidze et al., 2016b). Interestingly, it has been revealed that in familial AD, CSF Aβ42 is an earlier indicator of disease than analysis of Aβ by PET, as concentrations of Aβ42 appear to decline 25 years prior to the onset of clinical symptoms, whereas abnormalities in Aβ assessed by PET, along with CSF tau, are detected 15 years before symptom onset (Bateman et al., 2012). Tau is measured in the CSF through total tau (T-tau), which directly reflects neuronal degeneration although it is not specific for AD, and phosphorylated tau (P-tau), which is thought to be a direct marker of tangle pathology (Dubois et al., 2014). The levels of both T-tau and P-tau are increased in the CSF of AD patients, and the levels of P-tau are reported to correlate with NFT pathology *post-mortem* as well as hippocampal atrophy and a fast clinical progression (Masters et al., 2015). In addition, a meta-analysis performed recently concluded that, when analysed separately, Aβ42, T-tau and P-tau were strongly associated with AD and with MCI that subsequently progressed to AD (Olsson et al., 2016). The benefit of combining these markers in predicting MCI progression to AD was demonstrated in a separate meta-analysis, which reported that Aβ42 with either T-tau or P-tau had the highest predictive accuracy compared with the markers individually (van Rossum et al., 2010). An AD CSF profile of low Aβ42 and high T-tau/P-tau has been proposed, which is reported to reach a sensitivity of 90-95% and a specificity of 90% in AD and is the recommended protocol

for assessment of AD patients (Dubois et al., 2014). This accommodates the findings that while CSF Aβ42 is an early indicator of disease, T-tau and P-tau improve the prediction of progression (Masters et al., 2015). However, it is recommended that CSF biomarkers should be used to support the findings from other clinical measures and should not be used as standalone tests (Dubois et al., 2014).

Aside from Aβ and tau, NFL is a neuronal protein which has also been strongly associated with AD, while other similarly emerging biomarkers such as YKL-40 and VLP1 were only moderately associated (Olsson et al., 2016). The diagnostic value of additional markers remains to be seen, as while YKL-40 (a marker of neuroinflammation) and neurogranin (a marker of synaptic generation) were found to accurately distinguish AD from other dementias, it did not improve the diagnostic accuracy when compared with the core biomarkers i.e. Aβ42, T-tau and P-tau (Janelidze et al., 2016a).

1.4.3 The continuum of AD

The advances in biomarkers have altered the view of AD from a disease of defined clinical stages to a process that is moving along a continuum from an asymptomatic phase to preclinical which is then followed by the symptomatic phase (Aisen et al., 2017). The use of CSF and PET biomarkers in particular has been crucial in this regard. A model proposed by Jack and colleagues describes the temporal evolution of biomarkers throughout the continuum that is thought to parallel the pathophysiological sequence of AD, where biomarkers are hypothesised to have a sigmodal shape trajectory that reaches a plateau over time (Jack et al., 2013). The earliest detectable change is in Aβ pathogenesis detected first through CSF Aβ42 concentrations then Aβ PET, and is followed by CSF tau concentrations (and perhaps tau assessed by PET) and changes in brain atrophy which has been detected 15 years prior to clinical onset (Bateman et al., 2012). Last are changes in cerebral metabolism by FDG PET and subtle impairments in episodic memory which begin approximately 10 years before expected symptoms (Bateman et al., 2012). This continuum of AD and the proposed sequence of biomarkers is summarised in Figure 1.2.

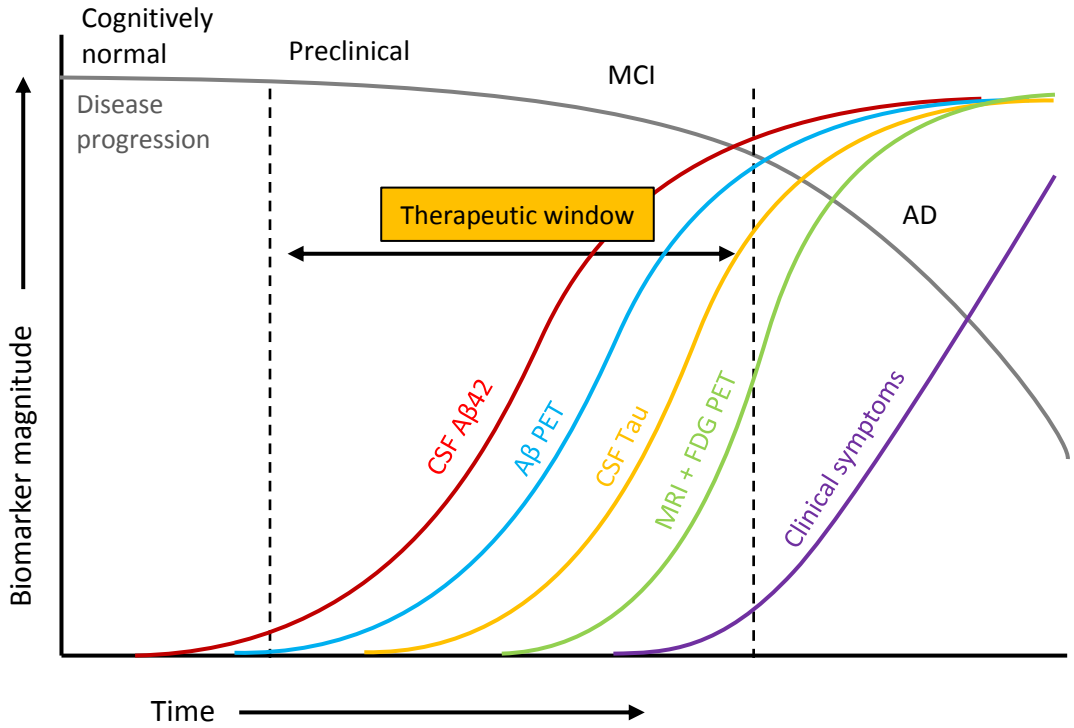

Figure 1.2 The continuum of AD and temporal change in biomarkers. *The pathogenesis of AD begins years prior to the onset of clinical symptoms, and changes in the preclinical and MCI stages of disease can be detected through CSF and neuroimaging biomarkers. During the preclinical phase, decreases in CSF Aβ42 levels are the first detectable change, followed by increase in Aβ PET and CSF Tau concentrations as the disease progresses into MCI. Abnormalities in structural MRI and FDG PET are also detected in MCI before the onset of clinical symptoms in MCI and AD. Identification of the preclinical and MCI stages through the use of biomarkers offers the possibility of commencing treatments at an earlier stage during the 'therapeutic window', in the hopes of modifying disease outcome and delaying disease progression. Adapted from Jack et al (2013).*

An "A/T/N" system has recently been proposed as a common framework to describe the biomarker profiles of an individual where "A" refers to an Aβ biomarker (Aβ PET of CSF Aβ42), "T" refers to tau pathology biomarkers (CSF P-tau or tau PET) and "N" refers to biomarkers of neurodegeneration or neuronal injury (CSF T-tau, FDG PET, structural MRI) (Jack et al., 2016). Each biomarker has a cut-off point and is rated as positive or negative e.g. A+/T+/N- or A+/T-/N+. This is not meant to act as a diagnostic classification system but rather to aid cognitive ageing research and will

be integrated into the research framework for investigating the AD continuum put forward by the NIA-AA in 2018.

The term preclinical is used to describe the initial phase of the disease process, which encompasses those in whom there is evidence of early AD pathological changes but do not meet the clinical criteria for MCI or AD (Sperling et al., 2011). The preclinical phase is further divided into 3 stages which reflect the suspected evolution of AD pathology as detected by biomarkers. Stage 1 is characterised by evidence of amyloidosis by Aβ PET and low CSF Aβ42 but no evidence of neurodegeneration or cognitive symptomatology. Stage 2 includes evidence of early neurodegeneration as well as amyloid positivity, such as elevated T-tau or P-tau, hypometabolism by FDG PET or cerebral atrophy on structural MRI. Finally, in addition to markers from the first two stages, stage 3 is defined by the appearance of subtle cognitive deficits, which do not significantly affect daily living and do not meet the criteria for MCI (Fiandaca et al., 2014). Subtle deficits in cognitive function are loosely defined as between 1 and 1.5 standard deviations below the mean and can be detected by sensitive cognitive measures such as those assessing episodic memory (Sperling et al., 2011, Fiandaca et al., 2014).

The identification of the preclinical stage is important as it presents a window of opportunity for potential disease-modifying therapy intervention, highlighted in Figure 1.2 (Fiandaca et al., 2014). Preclinical AD is common in cognitively normal elderly people and, although it is associated with future cognitive decline and mortality (Vos et al., 2013, Burnham et al., 2016), it is important to note that it does not necessarily mean that all individuals at this stage will process to clinical AD (Sperling et al., 2011).

1.5 Blood-based biomarkers

Neuroimaging and CSF biomarkers have been extremely valuable in the diagnosis of AD and identifying earlier stages of disease, however there are major drawbacks associated with both approaches. CSF is collected by lumbar punctures, an invasive procedure which poses significant health risks particularly in the elderly population,

and while neuroimaging techniques are not invasive they are very costly procedures which are not widely available (Fiandaca et al., 2014). Additionally, both require significant training and technical skill. Therefore, neither is appropriate for implementation into everyday practice in a clinical setting. An ideal biomarker for AD should be cost-effective, minimally invasive, easily translated to general use and have a high degree of sensitivity and specificity. Blood-based biomarkers have therefore been investigated as an alternative biomarker for AD and earlier cognitive decline, as it meets the specified requirements and lends itself well to repeated sampling. It has the potential to represent the first stage in a multistage screening and diagnostic process, which could also be useful for recruitment into clinical trials (Henriksen et al., 2014).

Blood is comprised of plasma, platelets, erythrocytes and leukocytes and contains a wide range of substances including proteins, lipids, peptides and metabolites that reflects physiological activity and pathology (Song et al., 2009). However, it is important to note that blood composition is influenced by all tissues in the body and will therefore not necessarily be specific for brain related pathology (Henriksen et al., 2014). The majority of studies to date have focused on the analysis of plasma and have investigated AD-related changes in a wide range of analytes including Aβ and tau, proteins, lipids, transcriptome, autoantibodies and micro RNA.

1.5.1 Plasma Aβ and tau

The level of detection of Aβ and tau in peripheral blood is substantially lower than in CSF owing to the restriction of movement of proteins due to the blood brain barrier (BBB) (Fiandaca et al., 2014). In fact until recently, the concentration of tau in the plasma was below the limit of detection for the majority of available technologies (Song et al., 2009), therefore Aβ has been the most extensively examined peripheral marker for AD, with conflicting results emerging (Henriksen et al., 2014). A meta-analysis carried out by Koyama and colleagues (2012) found that in 13 studies, a lower ratio of Aβ42/Aβ40 was significantly associated with the development of AD. In contrast to this, the Alzheimer's Disease Neuroimaging Initiative (ADNI) reported that plasma Aβ levels did not significantly correspond to the clinical state (Toledo et

al., 2011). The extent to which cell-bound and free forms of Aβ relate to the cognitive state is unclear (Snyder et al., 2014). In addition, the circulating concentrations of Aβ are derived from both peripheral tissues and the brain (Schneider et al., 2009), making it difficult to specifically relate Aβ concentrations in plasma to cognitive function and this might account for the variability and unsatisfactory results. To investigate plasma Aβ as a preclinical biomarker, an extensive longitudinal study of older adults without dementia was undertaken. The data showed that a lower ratio of Aβ42/Aβ40 was associated with a higher rate of cognitive decline over 9 years (Yaffe et al., 2011). It was also found that this association is greater among those with less education, lower literacy, and with the presence of an APOE ε4 allele. Additional longitudinal studies of preclinical AD states are needed to examine this further as a potential indicator of early cognitive decline. A conclusion on the diagnostic value of Aβ can perhaps be drawn from a recent meta-analysis in 2016, which revealed that the plasma Aβ42 levels were not associated with AD (Olsson et al., 2016). However, this study also reported a significant association of T-tau plasma levels with AD although, as only 5-6 studies were included in this analysis, more studies are required to investigate the diagnostic value of plasma tau concentrations.

1.5.2 Plasma proteomic signatures

Many studies have investigated the plasma proteome and several signatures have been proposed as potential biomarkers for AD and of progression from MCI to AD. One of the first studies conducted by Ray and colleagues proposed a set of 18 plasma proteins involved mostly in the immune response, haematopoiesis and apoptosis, that distinguished AD patients from controls with almost 90% accuracy and predicted the conversion from MCI to AD with 91% accuracy (Ray et al., 2007). This pivotal publication laid the foundation for subsequent studies which further examined this protein signature in separate cohorts. Studies performed shortly afterwards showed that 5 of the 18 proteins were sufficient to differentiate AD and controls with the same accuracy (Gomez Ravetti and Moscato, 2008), while another revealed a lower diagnostic accuracy of 61% using the whole panel (Soares et al.,

2009). Two additional studies also examined the reproducibility of this signature. Marksteiner and colleagues reported an increased concentration of 5 out of the 16 proteins in the panel which distinguished MCI or AD patients from controls with 65-75% sensitivity and 52-63% specificity, but could not differentiate between MCI and AD patients (Marksteiner et al., 2011). Similarly, Bjorkqvist and colleagues only detected an increased concentration in 3 out of the 18 proteins in AD patients compared with controls, and the panel predicted AD with 63% accuracy (Bjorkqvist et al., 2012). Interestingly, the concentrations of epidermal growth factor (EGF) and CCL15 were increased in both studies, which perhaps warrant further investigation in separate cohorts, but overall findings demonstrate that reproducibility of the 18 protein signature identified by Ray et al. is poor.

Numerous other profiling platforms have been explored. O'Bryant and colleagues (2010) developed an algorithm using a 30-protein signature to detect AD with a reported 80% sensitivity and 91% specificity. Many of the proteins identified were inflammatory in nature, but it was also noted that the panel had minimal overlap with the study conducted by Ray et al. in 2007. A subsequent study by the same group devised a model using an 11-protein signature that is valid in both serum and plasma in two independent cohorts, and which overlapped with their previously proposed model (O'Bryant et al., 2011). Similarly, a separate group identified yet another 18 protein panel which distinguished AD patients from healthy controls with high accuracy and which also featured numerous inflammatory proteins (Doecke et al., 2012). This group later also proposed CLU and chemokine-309 as diagnostic and predictive markers of AD respectively (Gupta et al., 2017). The SOMAscan assay has been employed in some studies which allows for large scale analysis of proteins, which so far has revealed prediction of AD with 67% sensitivity and 64% specificity using a subset of 13 proteins which is disappointingly low, where CLU was found to have the strongest association with cognitive decline (Sattlecker et al., 2014). Another study by the same group assessed 94 out of the 163 identified candidate protein biomarkers reported from over 21 studies, and using the SOMAscan technology revealed that 9 of the 94 proteins were associated with an AD-related phenotype (Kiddle et al., 2014). Fewer studies have examined protein signatures as

indicators of early cognitive decline, however Hye and colleagues identified 10 plasma proteins that predicted progression from MCI to AD with 87% accuracy (Hye et al., 2014). Additionally, a panel of 11 proteins has been successful in identifying preclinical AD but this was strongly influenced by APOE status (Johnstone et al., 2012).

A wide range of proteins were examined in the above studies, yet many of the proteins implicated in these panels have been inflammatory in nature, highlighting the likely role of inflammation in AD. Many studies have assessed plasma/serum concentrations of inflammatory cytokines and chemokines, but results are quite varied. A meta-analysis carried out in 2010 of 40 studies concluded that the concentrations of IL-6, TNFα, IL-1β, IL-12 and IL-18 were increased in the plasma of AD patients compared with controls and no differences in IL-4, IL-10, IL-8 and interferon (IFN)γ were detected (Swardfager et al., 2010). A more recent meta-analysis of 175 studies revealed that the concentrations of IL-1β, IL-2, IL-18, IL-6, IFNγ, TNFα were increased in the plasma of AD patients and that IL-6 was inversely correlated with the degree of cognitive impairment (Lai et al., 2017). Inflammatory cytokines showed some predictive value of MCI conversion to AD; however this was improved by the addition of neuroimaging markers (Furney et al., 2011). Interestingly, Leung and colleagues reported that increased levels of IL-4, IL-10 and granulocyte-colony stimulating factor were associated with a fast rate of decline in AD patients compared over 1 year (Leung et al., 2013). Plasma cytokine levels vary substantially between studies, perhaps due to the presence of co-morbidities, and therefore might have limited use for identification of earlier stages of cognitive decline.

1.5.3 Plasma lipids profiles

Analysis of plasma lipid concentrations has also yielded varied biomarker signatures. One such study successfully predicted conversion of MCI to AD with 90% accuracy using a panel of 10 lipids (Mapstone et al., 2014), which included phospholipids, acylcarnitines and phosphatidylcholines that are reflective of cell membrane integrity. However, Casanova and colleagues failed to replicate these findings in two

independent cohorts and reported poor sensitivity and specificity with the 10 lipid signature (Casanova et al., 2016). This panel was reviewed by the original authors and a new 24 metabolite signature proposed, the majority of which were lipids and included 7 of the metabolites previously featured in the 10 lipid signature, and it also had a higher degree of accuracy (Fiandaca et al., 2015). A separate signature of 10 metabolites is reported to discriminate between controls and AD patients with almost 80% accuracy, where 6 of the metabolites were identified as long chain cholesteryl esters, which were reduced in AD patients (Proitsi et al., 2015). Cholesterol is the precursor of these lipids and this was not altered in the study, which is in contrast with previous reports of elevated cholesterol in MCI patient plasma and an associated risk of plasma high-density lipoprotein cholesterol levels with the risk of MCI (He et al., 2016a). Further longitudinal studies are required to investigate the reliability of these proposed plasma lipid profiles. Interestingly, increased levels of sphingomyelins and ceramides, which are lipids directly related to apoptosis, have been found to be predicators of memory impairment in asymptomatic individuals over a period of 9 years (Mielke et al., 2010) and could represent an early indicator of cognitive impairment.

1.5.4 Unmet need for blood-based biomarkers

Despite numerous studies, to date there has been no consensus for a defined blood-based signature, with poor reproducibility between studies as one of the major obstacles. As a result, blood-based biomarkers are not yet ready for use in the clinic as they have not been sufficiently evaluated for their intended purpose of use (Dubois et al., 2016). The wide range of analytical methods used makes comparison between studies difficult, and only a handful of studies have examined pre-symptomatic individuals in a longitudinal design to identify possible predictive markers of MCI or AD. In addition, plasma concentrations of analytes are variable as all bodily tissues can potentially contribute to changes and the extent to which brain pathology is reflected in the blood cannot be discerned. Analysis of the cellular component of the blood has been largely neglected, and it has been suggested that future studies should also examine the *ex vivo* stimulation of peripheral immune

cells as well as the concentrations of circulating proteins and lipids (Snyder et al., 2014). As depicted in Figure 1.3, blood is a rich source of immune cells of both innate and adaptive immunity including granulocytes (basophils, eosinophils and neutrophils), lymphocytes (B, T and natural killer (NK) cells) and monocytes, the latter of which can be differentiated into either macrophages or dendritic cells (DCs). The cellular component of the blood offers much opportunity for blood-based biomarker development which has not yet been thoroughly explored.

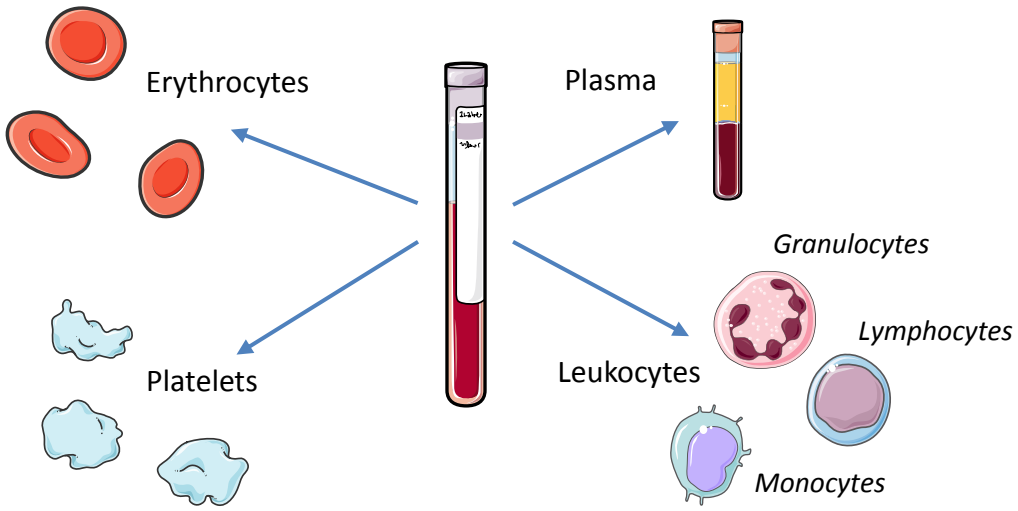

Figure 1.3 Components of the blood. *Blood is comprised of plasma, platelets, erythrocytes and leukocytes. The majority of blood-based biomarker research has so far focused on analysis of plasma; however there are many leukocyte cell types present in the blood that presents much opportunity for biomarker development, including granulocytes, lymphocytes and monocytes.*

1.6 Macrophages

Macrophages are the professional phagocytes of the innate immune system and were first described as such by Elie Metchnikoff in 1905 who received a Nobel Prize for his description of phagocytosis. Macrophages are highly plastic and heterogeneous cells that perform a range of functions in order to maintain homeostasis. These include clearance of erythrocytes from the circulation for recycling, tissue repair and remodelling, removal of apoptotic cells and defence against invading pathogens (Mosser and Edwards, 2008). The concept of macrophage polarisation emerged almost two decades ago in an attempt to mirror

the T helper (Th) Th1/Th2 paradigm of T cells (Mills et al., 2000). Thus, macrophages have been described as either M1 (classical) or M2 (alternative) and display distinct phenotypic differences in response to various environmental cues in order to carry out specific functions.

1.6.1 Origins and monocytes

Immune cells are derived from haematopoetic stem cells (HSCs) resident in the bone marrow which gives rise to myeloid, lymphoid, megakaryocyte and erythroid cell lineages (Epelman et al., 2014). Monocytes, macrophages and DCs share a common bone-marrow derived precursor called the monocyte-macrophage DC progenitor (MDP) (Fogg et al., 2006). More recently a restricted monocyte-macrophage progenitor cell derived from the MDP has been discovered which is present in the bone marrow and spleen and produces the major monocyte subsets (Hettinger et al., 2013). Monocytes and macrophages have traditionally been described as the mononuclear-phagocyte system (MPS), which encompasses all macrophage-like cells resident in various tissues. The MPS was proposed by van Furth in the 1960's, who hypothesised that tissue macrophages are derived from circulating monocytes in the bloodstream (van Furth and Cohn, 1968). This has been the prevailing view until recently, where mounting evidence has demonstrated (largely through fate-mapping techniques) that in fact tissue macrophages are established during embryonic development and persist into adulthood (Ginhoux and Jung, 2014). However, this has been largely investigated in animal models and it is not yet known whether human macrophages also have distinct ontological origins (Epelman et al., 2014). Many distinct macrophage populations exist in peripheral tissues, such as alveolar macrophages, Kupffer cells in the liver and splenic macrophages. The CNS also contains macrophage subpopulations including perivascular and choroid plexus macrophages and microglia, which are the resident immune cell in the brain and are thought to derive solely from progenitors in the yolk sac during embryonic development, although it is also debated that monocytes are capable of crossing the BBB and differentiating into microglia (Gordon and Taylor, 2005).

Human peripheral blood monocytes are a heterogeneous population that can be divided into two main subsets on the basis of CD14 and CD16 expression. The majority of monocytes, termed classical monocytes, highly express CD14 and are negative for CD16 (Shi and Pamer, 2011). Monocytes expressing CD16 are further subdivided into two subtypes based on whether CD16 levels are high or intermediate and are thus described as non-classical or intermediate monocytes respectively. Distinct expression of chemokine receptors further defines monocyte subsets as classical monocytes express CCR2 and non-classical express CCR5 (Gordon and Taylor, 2005). The classification of monocyte subsets in humans is not recognised in mice, where monocytes are classified on the basis of Ly6C expression, however their role in the immune response has been described as similar (Shi and Pamer, 2011).

Monocytes are present in the blood, spleen and bone marrow and constitute approximately 10% of total leukocytes in humans (Italiani and Boraschi, 2014). If monocytes in the circulation are not recruited to peripheral tissues after 1-2 days they die and are removed. Monocyte recruitment to the site of inflammation is mediated through a complexity of chemokine receptors and cellular adhesion molecules. Exposure to granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 differentiates monocytes to DCs (Gordon and Taylor, 2005), whilst differentiation to macrophages can occur in response to numerous stimuli and polarises cells into distinct phenotypes.

1.6.2 Classical activation of macrophages

So-called M1-polarising stimuli include pro-inflammatory cytokines, primarily IFNγ, TNFα and GM-CSF, which are released from T or NK cells, and microbial products such as lipopolysaccharide (LPS). M1 macrophages are distinguished by an IL-12^{high} IL-23^{high} IL-10^{low} phenotype and are efficient producers of ROS and NO as well as a range of pro-inflammatory cytokines and chemokines (e.g. IL-1β, IL-6, IL-8, IL-23) (Mantovani et al., 2013). M1 activated macrophages play an important role in host defence as they have high microbicial and tumoricidal activity, however, if the inflammatory mediators they produce are not tightly regulated, they can also cause damage to the host (Wilcock, 2012). For example, the production of pro-

inflammatory cytokines has been associated with the development and expansion of Th17 cells, which produce IL-17 that contributes to autoimmune disorders through the recruitment of polymorphonuclear leukocytes (Mosser and Edwards, 2008).

IFNγ signals through the IFNγ receptor (IFNGR), which is present in the plasma membrane and comprised of the IFNGR-1 and IFNGR-2 chains. Janus kinase (JAK) adaptor proteins are recruited which activate signal transducers and activation of transcription (STAT)1 and interferon regulatory factors (IRFs) that control genes associated with cytokine receptors, cell activation markers and cell adhesion molecules (Martinez and Gordon, 2014). IFNγ is initially produced by NK cells however, as this is only transient, sustained levels of IFNγ are provided by Th1 cells (Mosser and Edwards, 2008). Mice deficient in IFNγ or its receptors are more susceptible to bacterial infections, as are humans with mutations in these signalling pathways, indicating the importance of IFNγ in the immune response (Filipe-Santos et al., 2006).

The response of macrophages to pro-inflammatory stimuli is also mediated largely through a family of pattern recognition receptors (PRRs), toll-like receptors (TLRs). TLRs recognise a wide range of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). LPS is a component of the cell wall of gram-negative bacteria and is recognised by TLR4, the most widely studied TLR. LPS-induced activation of TLR4 initiates a signalling cascade through the adaptor protein myeloid differentiation primary response 88 (MyD88) that leads to production of pro-inflammatory cytokines and chemokines and co-stimulatory molecules, mediated via nuclear factor- kB (NF-kB), activator protein 1 (AP-1), IRFs and STAT1 signalling (Martinez and Gordon, 2014).

A defining feature of the M1/M2 classification of macrophages is the metabolism of arginine. M1 macrophages metabolise arginine to NO and citrulline by inducible nitric oxide synthase (iNOS), which has become an archetypal marker of the M1 state. However the translation of this to human macrophages has been met with some difficulty with many studies failing to detect iNOS expression and NO production, therefore it is possible that additional cues or the pre-existence of an

inflammatory environment is necessary to observe its expression (Thomas and Mattila, 2014).

Increased IL-1β and TNFα production are important markers of the M1 phenotype, as well as also being inducers of M1 activation in macrophages. Both are potent inflammatory cytokines and critical regulators of the immune response. IL-1β is a member of the IL-1 family and is produced through activation of a complex of proteins termed the inflammasome. The receptor for IL-1β consists of an IL-1R1 and IL-1R accessory protein and intracellular signalling is mediated by MyD88, while TNFα mediates its effects through binding to one of two receptors, TNF receptor 1 and 2 (Turner et al., 2014). Both intracellular signalling cascades involve the activation of mitogen-activated protein kinases (MAPKs) and NF-κB, resulting in increased pro-inflammatory gene expression.

1.6.3 Alternative activation of macrophages

The M2 activation state was initially described via the actions of the anti-inflammatory cytokine IL-4, which is released from Th2 cells and upregulates the expression of mannose receptor (MRC-1) and the major histocompatibility class II complex (MHC II) and inhibits inflammatory cytokine production (Stein et al., 1992). Shortly following this, the same group also revealed that the effects of IL-4 were closely mimicked by IL-13 (Doyle et al., 1994). Henceforth, IL-4 and/or IL-13 have become the most widely studied inducers of macrophage alterative activation.

The similar actions of IL-4 and IL-13 can be explained partly due to their overlapping signalling mechanisms. IL-4 binds to the IL-4Rα chain and recruits either the IL-2Rγ chain or IL-13Rα1 to form the type I or type II receptor in the plasma membrane respectively (Gordon, 2003). IL-13 binds to the three receptor complexes, either IL-13Rα1 alone or with subsequent recruitment of IL-4Rα, and the IL-13Rα2 complex. Both cytokines elicit signalling pathways involving the activation of JAKs and phosphorylation of STAT6 which translocates to the nucleus and regulates gene transcription (Gordon and Martinez, 2010).

In contrast to the M1 state, M2 macrophages are generally identified by an IL-12^{low} IL-23^{low} IL-10^{high} phenotype (Mantovani et al., 2013). However the diversity of M2-activated macrophage function and the discovery of other M2-stimuli resulted in the identification of three subtypes – M2a, M2b and M2c (Mantovani et al., 2004).

IL-4 and IL-13 polarised macrophages were classified as M2a and are characterised by the increased expression of the IL-1 receptor antagonist, MRC-1 and arginase (Arg-1) activity. Arg-1 catalyses the hydrolysis of arginine to ornithine and urea and the subsequent production of polyamines and collagen promotes cell proliferation and repair (Italiani and Boraschi, 2014). Like iNOS, Arg-1 is considered one of the main markers of the M2 state but identifying this marker in human macrophages has been problematic (Gordon and Martinez, 2010). MRC-1, which is involved in endocytosis and phagocytosis, is a commonly used marker of M2a activation and is expressed more abundantly in human macrophages (Gordon, 2003). M2a macrophages are also known as wound-healing macrophages, consistent with their role in contributing to the production of the extracellular matrix for remodelling and repair. Thus, genes associated with tissue reconstruction including found in inflammatory zone 1 (Fizz1, or Retnla) and Chi3l3 (YM1) are upregulated in this state (Mosser and Edwards, 2008). However, as they lack human homologs they cannot be used as markers of human macrophage alternative activation (Raes et al., 2005). M2a macrophages are also implicated in allergic responses (Wilcock, 2012).

In response to immune complexes or agonists of TLRs and IL-1R, macrophages adopt an M2b state, which is a combination of both M1 and M2a activation states as it produces pro-inflammatory cytokines as well as high levels of IL-10 and promotes Th2 differentiation and humoral antibody production (Mantovani et al., 2004). Finally, M2c or regulatory macrophages are polarised by IL-10 (released from T regulatory (Treg) cells and glucocorticoids. These macrophages are responsible for suppressing the immune response by producing high levels of IL-10 and transforming growth factor β (TGFβ) (Wilcock, 2012).

1.6.4 Revised view on macrophage polarisation

The original concept of M1/M2 polarisation was derived from observations of differential metabolism of arginine in C57/BL6 and BALB/c mice by Mills and colleagues, however it has since been discovered that the genetics of these strains differ substantially which might impact on the differences observed. In addition, this concept failed to take into account macrophage activation in the presence of lymphocytes, which is an important consideration (Murray et al., 2014). A common framework for macrophage nomenclature and experimental guidelines was proposed by Murray and colleagues in 2014, although it continues to make use of a combination of markers to describe distinct activation states (Hume, 2015). Much of the research on macrophage polarisation has been carried out *in vitro* and it is likely that the *in vivo* setting is much more complex. It is now appreciated that the M1/M2 dichotomy is too simplistic and it is more likely that the M1 and M2 state represent the extremes of a spectrum, as such that macrophages may display markers and characteristics of both states and exist at some intermediate stage along the continuum. This model, proposed by Mosser and Edwards, depicts three main macrophage subtypes based on their function (classical, wound healing and regulatory) which are analogous to a colour wheel with a significant overlap between populations. This view also supports the highly plastic nature of macrophages, which have been shown to switch phenotypes in response to environmental cues (Mosser and Edwards, 2008). It is also confirmed further by Xue et al. (2014) who examined the transcriptional response of human monocyte-derived macrophages (MDMs) to a plethora of stimuli and identified 49 different co-expressed clusters of genes, indicating a significant degree of overlap in response to stimuli. It has even been suggested that single cell profiling might eventually reveal that every macrophage is in fact unique (Hume, 2015). Thus, it is more suitable to refer to M1 macrophages as ‘M1-like’ or simply ‘pro-inflammatory’ and M2 macrophages as ‘M2-like’ or ‘anti-inflammatory’. A simplified depiction of macrophage polarisation is shown in Figure 1.4.

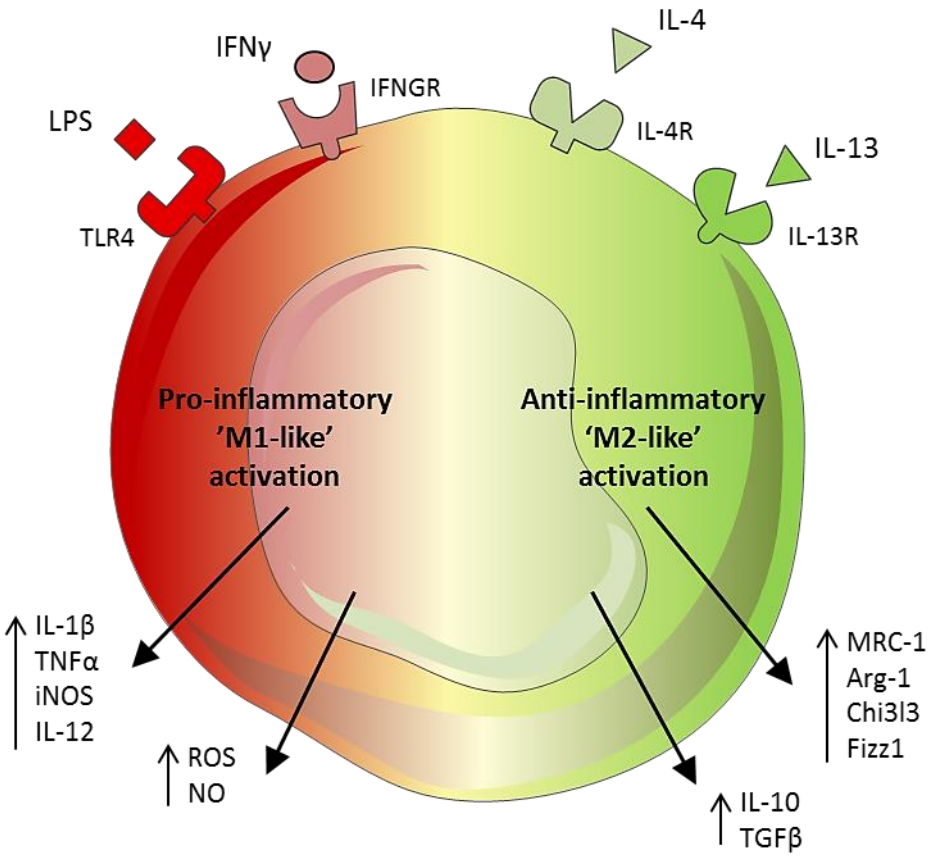

Figure 1.4 Macrophage activation states. *Monocytes can be differentiated into a wide spectrum of macrophage phenotypes, which are broadly termed either pro-inflammatory (M1-like) or anti-inflammatory (M2-like). Pro-inflammatory macrophages are activated in response to pro-inflammatory stimuli such as LPS or IFNγ signalling through TLR4 or IFNGR respectively. This results in an increased production of inflammatory mediators, ROS and NO, and an upregulation of markers typically associated with this state including IL-1β, TNFα, iNOS and IL-12. The anti-inflammatory cytokines IL-4 and IL-13 signalling through IL-4R and IL-13R polarise macrophages to an anti-inflammatory phenotype, which increases the secretion of anti-inflammatory cytokines IL-10 and TGFβ and results in the upregulation of markers associated with this state including MRC-1, Arg-1, Chi3l3 and Fizz1. The current view of macrophage polarisation depicts these two phenotypes as the extremes of a spectrum where a cell may express markers of either stage at any given time depending on the particular environmental cues.*

1.6.5 Monocytes/macrophages as potential biomarkers for AD

Monocytes and macrophages have been investigated as potential biomarkers for MCI and AD, although examination of earlier preclinical states remains largely neglected. Phagocytosis is defective in monocytes from AD patients, which have shown poor ability to phagocytose Aβ *in vitro* (Fiala et al., 2005), and macrophages from AD patients show decreased transcription levels of TLRs during phagocytosis compared with controls (Fiala et al., 2007). A flow cytometric test of Aβ phagocytosis was reportedly successful in identifying 60% and 94% of MCI and AD patients respectively (Avagyan et al., 2009).

Aβ uptake is reportedly inhibited by increased expression of CD33, and a decrease in CD33 expression is associated with increased production of TNFα, IL-1β and IL-8 from monocytes (Lajaunias et al., 2005). AD patients show a decrease in the percentage of CD33 positive monocytes compared with controls which was correlated with mini-mental state exam (MMSE) scores, and the diagnostic sensitivity and specificity was 61% and 60% respectively showing that CD33 levels can distinguish between AD patients and controls to some extent although this is too low for clinical utility (Hu et al., 2014).

Genetic variants in CD33 have been associated with an increased risk for AD through GWAS, and increased expression of CD33 is reportedly associated with the CD33 risk allele in young and old subjects, but it was not associated with a diagnosis of AD (Bradshaw et al., 2013). Although these studies report conflicting findings, it is possible that markers on immune cells may provide the basis of a biomarker for AD.

Markers of monocyte activation are also increased from AD patients, although interestingly CCR2, which has been shown to mediate monocyte trafficking across the BBB, is decreased on AD monocytes (Zhang et al., 2013). AD leukocytes also display higher levels of NOS activity, suggesting that the immune cells of AD patients are more pro-inflammatory compared with controls (De Servi et al., 2002). This is consistent with the finding that monocytes from MCI and AD patients have higher basal levels of cytokine secretion including TNFα, IL-1β, IL-6 and IL-12 (Guerreiro et al., 2007). Interestingly, monocytes from AD patients differentiated into DCs exhibit

higher levels of IL-6 production (Ciaramella et al., 2010). The response of cells to *ex vivo* stimulation by pro-inflammatory stimuli has also been assessed. Monocytes isolated from AD patients stimulated with LPS show an increased expression of intracellular adhesion molecule 1 (ICAM-1) and human leukocyte antigen – antigen D related (HLA-DR), which suggests that cells from AD patients have a heighted response to inflammatory stimuli (Ciaramella et al., 2010). Furthermore, monocytes/macrophages from AD patients stimulated with Aβ42 exhibit higher levels of TLR2 and TLR4 expression and an increase in IL-6 and CCR2 (Saresella et al., 2014). This study also revealed that cells from both MCI and AD patients displayed increased levels of TLR3 and TLR8 and IL-23 production in response to Aβ stimulation. These studies demonstrate that monocytes and macrophages show an increased inflammatory profile in AD, however further studies examining preclinical cohorts are needed.

1.7 Metabolic reprogramming of macrophages

The phenomenon of metabolic reprogramming is a key regulatory event in the response of macrophages to environmental cues. A change in cellular metabolism of macrophages is linked with alterations in macrophage function and also the plasticity of macrophages is associated with their ability to reprogram cellular metabolism (El Kasmi and Stenmark, 2015). An important aspect in this regard is the observation that macrophages not only alter their metabolism in response to nutrient availability and oxygen conditions, but that this also occurs in response to PRR and cytokine receptor activation or antigens (O'Neill and Pearce, 2016). The term 'immunometabolism' therefore describes the ability of metabolism to determine the phenotype of immune cells.

Glucose is metabolised through two interconnected pathways to generate adenosine triphosphate (ATP) to supply energy for cellular function. Glucose is transported into the cell by a family of transmembrane glucose receptors (GLUTs) and is initially converted into pyruvate through the process of glycolysis. This takes place in the cytosol, does not require oxygen and generates 2 molecules of ATP. Pyruvate is then either converted into lactate or acetyl CoA which enters the tricarboxylic acid cycle

(TCA) in the matrix of the mitochondria (otherwise known as the Krebs or citric acid cycle), generating nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2). These donate electrons to the electron transport chain of oxidative phosphorylation, where oxygen acts as an electron acceptor. Electrons are passed through a series of complexes (the respiratory chain) located in the inner mitochondrial membrane resulting in a proton gradient that drives ATP production via the ATP synthase (El Kasmi and Stenmark, 2015). This results in the net production of 36 ATP molecules. Cells can also metabolise fatty acids and glutamine in order to maintain TCA cycle activity and oxidative phosphorylation if pyruvate supply is limited.

Under homeostatic conditions, macrophages are thought to respire predominately through mitochondrial oxidative phosphorylation. Inflammatory macrophages, on the contrary, exhibit an increase in glycolytic metabolism and a corresponding decrease in TCA cycle activity and oxidative phosphorylation (Kelly and O'Neill, 2015). This phenomenon was first described by Otto Warburg in the 1920's, who reported an increase in glycolysis in tumour cells despite the availability of oxygen (Warburg et al., 1927), and is termed the 'Warburg effect' or 'aerobic glycolysis'. Glycolysis is thought to dominate as it rapidly generates ATP to meet the cellular requirements, although it is an inefficient means of glucose metabolism (Kelly and O'Neill, 2015). While the increase in glycolysis aides proliferation of cancer cells, it is thought to serve other functions in macrophages, such as providing energy for the synthesis of inflammatory mediators, increased production of ROS for enhanced bacterial killing, and maintenance of the mitochondrial membrane potential to ensure cell survival (El Kasmi and Stenmark, 2015, Garedew et al., 2010).

1.7.1 Metabolic signature of pro- and anti-inflammatory macrophages

Polarised macrophages display different metabolic phenotypes, as such the M1-like macrophages are biased towards glycolysis and M2-like macrophages utilise oxidative phosphorylation and fatty acid oxidation, which is suitable for sustained energy for tissue remodelling and repair (Galvan-Pena and O'Neill, 2014). A number of factors have a role in regulating this switch that both enhances and deactivates

the opposing metabolic pathway, which are summarised for pro-inflammatory activated macrophages in Figure 1.5.

One of the key mechanisms regulating the increase in glycolysis in LPS-activated macrophages is a switch in expression of isoforms of 6-phosphofructose-2-kinase/fructose-2,6-bisphosphatase (PFK2) from the liver form (L-PFK2) to the ubiquitous form (u-PFK2) (Rodriguez-Prados et al., 2010). These enzymes catalyse both the synthesis and degradation of fructose-2,6-bisphosphate (F-2,6-BP), as it contains both kinase and bisphosphatase domains (Pilkis et al., 1981). The role of F-2,6-BP is to allosterically activate 6-phosphofructo-1-kinase (PFK1), which catalyses the conversion of fructose-6-phosphate to the next intermediate in the glycolytic chain, fructose-1,6-bisphosphate. The more active form of PFK2, u-PFK2, encoded by the PFKFB3 gene, has a kinase:bisphosphatase ratio of 740:1 and therefore drives glycolytic flux through maintaining high levels of F-2,6-BP (Sakakibara et al., 1997). Under anti-inflammatory conditions, L-PFK2 encoded by PFKFB1 is expressed, which maintains lower levels of F-2,6-BP as it has a balanced kinase:bisphosphatase ratio (Galvan-Pena and O'Neill, 2014). Thus, this is a crucial rate-limiting step in determining the rate of glycolysis.

Interestingly, PFKFB3 is also a target gene of the transcription factor hypoxia-inducible factor (HIF)-1α, which is a central component of Warburg metabolism of pro-inflammatory macrophages (Minchenko et al., 2002). As the name suggests, HIF-1α is upregulated in hypoxic conditions which is observed in tumour cells, and it is also expressed during aerobic glycolysis in macrophages (Blouin et al., 2004). Other target genes of HIF-1α include GLUT1, and lactate dehydrogenase (LDH) and pyruvate dehydrogenase (PDH), both which limit the availability of acetyl CoA for the TCA cycle (Kelly and O'Neill, 2015). Overexpression of GLUT1 in a mouse macrophage cell line RAW264.7 increased glucose uptake and resulted in increased ROS and pro-inflammatory cytokine production (Freemerman et al., 2014). HIF-1α is stabilised as a result of succinate, an inhibitor of prolylhydroxylases (PHD) which can occur in response to TCA intermediates, which also regulates the production of IL-1β (Tannahill et al., 2013).

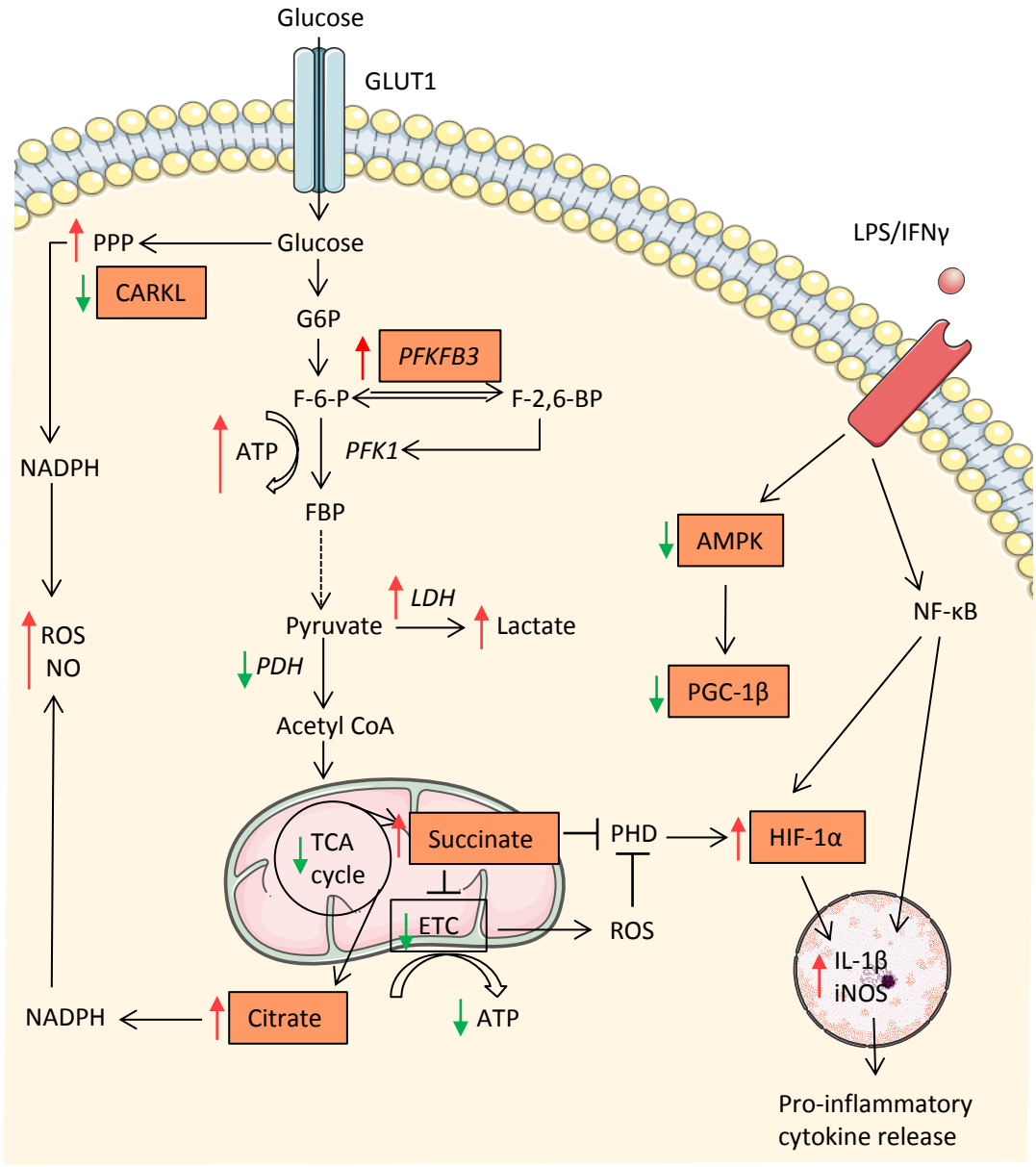

Figure 1.5 Metabolic reprogramming in pro-inflammatory macrophages. *Glucose is transported into the cell via the GLUT1 receptor and enters either 1) the pentose phosphate pathway (PPP) where CARKL is downregulated leading to the generation of NADPH for ROS and NO production, or 2) glycolysis, which is driven by an upregulation of PFKFB3 leading to increased ATP production. The conversion of the end product of glycolysis, pyruvate, into lactate is increased by upregulation of LDH and its conversion into acetyl CoA is reduced by a decrease in PDH. The activity of the TCA cycle is therefore decreased, although there is an increase in the TCA cycle intermediates succinate and citrate, which inhibit the electron transport chain (ETC) resulting in decreased ATP and increased ROS and NO production. Succinate and ROS also indirectly increase HIF-1α expression, which leads to an increase in pro-inflammatory markers and cytokine production. LPS and IFNγ can also activate HIF-1α through NF-κB signalling, in addition to attenuating activity of AMPK and therefore suppressing PGC-1β mediated transcription in order to promote the pro-inflammatory phenotype.*

Pro-inflammatory macrophages display a build-up of succinate and other intermediates of the TCA cycle such as citrate, despite a decrease in overall TCA cycle activity (El Kasmi and Stenmark, 2015). As well as stabilising HIF-1α, succinate can also inhibit complex I in the respiratory chain, increasing ROS production, which can also inhibit PHDs (Drose, 2013). Conversely, inhibition of succinate dehydrogenase reduces succinate oxidation and produces an anti-inflammatory phenotype (Mills et al., 2016). LPS also induces expression of the mitochondrial citrate carrier which increases citrate export and metabolism of citrate generates NADH phosphate (NADPH) for ROS and NO production (O'Neill and Hardie, 2013).

The production of NO is generated through increased expression of iNOS, which is upregulated in pro-inflammatory macrophages. NO inhibits complexes in the respiratory chain to decrease mitochondrial respiration, and inhibition of iNOS in LPS-stimulated macrophages rescues normal mitochondrial respiration and improves repolarisation to an anti-inflammatory phenotype by IL-4, indicating that NO is an important mediator in the switch to glycolysis (Kelly and O'Neill, 2015, Van den Bossche et al., 2016). iNOS is a target gene for HIF-1α, demonstrating another way by which HIF-1α can regulate glycolysis (Galvan-Pena and O'Neill, 2014). The inhibition of complexes in the respiratory chain further increases ROS production, a critical feature of the pro-inflammatory response for pathogen clearance (O'Neill and Hardie, 2013).

NADPH is generated through an increase in the pentose phosphate pathway (PPP) (Jha et al., 2015). An increase in glucose flow through the PPP can contribute to an increase in lactate production, which is a hallmark of aerobic glycolysis. The PPP is regulated by carbohydrate kinase-like protein (CARKL), which is decreased in macrophages upon LPS stimulation and increased by IL-4 (Haschemi et al., 2012). Additionally, the anti-inflammatory macrophage phenotype is promoted through a transcriptional co-activator peroxisome-proliferator-activated receptor-γ co-activator-1β (PGC-1β), which leads to increased fatty-acid oxidation, mitochondrial biogenesis and oxidative phosphorylation (Vats et al., 2006). PGC-1β is in turn induced by AMP activated protein kinase (AMPK). AMPK is an energy sensing enzyme, thus its role is to inhibit ATP-consuming pathways and promote ATP-

producing pathways to converse energy. Its activity is inactivated in LPS-stimulated macrophages whereas it is activated in IL-10/TGFβ treated macrophages, and therefore promotes alternative activation of macrophages (Sag et al., 2008).

1.7.2 Bioenergetic dysfunction as a potential biomarker for AD

Metabolic dysfunction is evident in AD as well as other neurodegenerative conditions. Ageing in general is associated with an overall decline in bioenergetic function and FDG PET scans of aged individuals show a decrease in glucose metabolism (Wilkins et al., 2014, Chetelat et al., 2013). A reduction in brain metabolism has also been well documented in AD patients and FDG PET has been a useful marker in predicting progression from MCI to AD and identifying those at risk of developing AD (Cohen and Klunk, 2014, Kennedy et al., 1995).

Mitochondria in particular have been shown to be defective in AD, showing decreased ATP production, impaired respiratory chain function and increased ROS production (Cabezas-Opazo et al., 2015). Aβ directly affects mitochondrial function through impairment of the electron transport chain (Cabezas-Opazo et al., 2015). Evidence from AD mouse models suggests that these events occur early in disease pathogenesis, with decreases in mitochondrial respiration and PDH expression evident before the development of Aβ plaques (Yao et al., 2009). Reductions in the expression of PDH and other enzymes involved in oxidative phosphorylation such as cytochrome c oxidase (complex IV of the mitochondrial respiratory chain) are reported in the brains of AD patients (Gibson et al., 1998). A number of genes involved in oxidative phosphorylation as well as glycolysis are decreased in the AD hippocampus, which might account for the overall decrease in brain metabolism (Brooks et al., 2007). Other proteins involved in mitochondrial biogenesis such as PGC-1α are also decreased in the hippocampus of AD patients (Sheng et al., 2012). Interestingly, many genes involved in mitochondrial function that were decreased in the AD brain were found to be upregulated in the brains of MCI patients (Berchtold et al., 2014). The expression of genes and proteins involved in glycolysis have been less well studied, but increases in lactate levels in the brain and CSF of AD patients

have been reported suggesting that intermediates in this pathway might be altered (Gu et al., 2012).

Abnormalities in metabolic function are also reported in the periphery in AD. Increases in markers of oxidative stress are reported in the blood of AD patients (Schrag et al., 2013) and the expression of genes in the mitochondrial respiratory chain are decreased in whole blood samples from MCI and AD patients (Lunnon et al., 2012). A few studies have also examined the metabolic profile of peripheral immune cells. Lymphocytes from AD patients show elevated basal levels of ROS production (Leutner et al., 2005) and reduced basal rates of mitochondrial respiration (Leuner et al., 2012, Maynard et al., 2015). Cytoplasmic hybrids (cybrids) cell lines generated using platelet mitochondria from MCI and AD patients also show a decrease in mitochondrial respiration, along with reduced PGC-1α protein levels (Silva et al., 2013). However, there are few studies that have assessed the metabolic signature of monocytes/macrophages, or indeed PBMCs, in AD. Similarly, metabolic function has not been examined as a potential marker of early cognitive decline in asymptomatic individuals and the majority of research has focused on abnormalities in mitochondrial function, therefore assessment of alterations in the glycolytic pathway have been largely neglected. Therefore this, and analysis of the metabolic response of peripheral immune cells from asymptomatic cohorts to an inflammatory challenge has not been investigated, and represents a novel approach for biomarker development. This is the focus of the work in this thesis.

1.8 Study aims

1. To assess age-related changes in central and peripheral inflammation of C57/BL6 mice in order to determine if a peripheral marker can be developed through incubation of a monocyte cell line with plasma.

2. To investigate the effect of plasma from various cohorts on markers of gene expression and metabolism in a monocyte cell line as a biomarker assay for cognitive dysfunction.

3. To examine the phenotype of peripheral immune cells from a cohort of older adults described as having an IQ-discrepant episodic memory and to assess the response to pro-inflammatory stimulation as a potential biomarker for early cognitive decline.

Chapter 2: Materials and Methods

2.1 Aseptic technique

All cell culture work was carried out in a laminar flow culture hood (SafeFAST Classic class II A1/A2 Microbiology Safety Cabinet, FASTER, Italy). The laminar flow hood maintains a sterile environment by filtering air to prevent air-borne pathogens from entering the work area. The interior of the hood was generously sprayed with 70% ethanol (EtOH) before and after use to ensure sterile conditions. Following use it was exposed to ultraviolet (UV) light for 30 min. Any materials passed into the hood were also sprayed with 70% EtOH. Filter serological pipettes and pipette tips were consistently used to avoid possible contamination. Disposable latex gloves were worn at all times and were sprayed with 70% EtOH before entering the work area. Dissection instruments were sterilised by thorough cleaning with Virkon® (DuPont, USA) followed by overnight baking at 200°C. Cells were cultured in an incubator at 37°C in a 5% carbon dioxide (CO_2) humidified atmosphere. The water reservoir was supplemented with AutoClean (Wak-Chemie Medical GmbH, Germany) to prevent the growth of aqueous organisms. To maintain a sterile environment, the laminar flow hood and incubator were cleaned regularly with Biocidal ZF™ (Wak-Chemie Medical GmbH, Germany) and 70% EtOH.

2.2 Culture media preparation

Dulbecco's modified Eagle's medium (DMEM; GIBCO®, Life Technologies; 500 ml) and Roswell Park Memorial Institute 1640 culture medium (RPMI; HyClone®, Thermo Fisher scientific, USA; 500 ml) were supplemented with 2.05 mM L-glutamine and 10% fetal bovine serum (FBS) containing 50 U/mL penicillin/streptomycin (GIBCO®, Life Technologies). The resulting complete DMEM (cDMEM) and complete RPMI (RPMI) were used in all cell culture experiments to maintain either mouse- or human-derived cells respectively.

2.3 Cell counting

Cells were counted by diluting cells in Trypan Blue (1:10; Sigma-Aldrich, UK) and a glass haemocytometer (Marienfeld-Superior, Germany) used to count the cells under

a light microscope. Viable cells exclude the dye and therefore appear bright under a light microscope. The number of cells/ml was determined using the formula: cell count x dilution factor x 10^4.

2.4 Animals

Animals were maintained under veterinary supervision in a specific pathogen-free environment at the Bioresources Unit of Trinity College Dublin, and were housed in groups of 4 or 5 per cage, at 20-22°C with a 12 h light/dark cycle. All animals had free access to food and water and were fed a standard laboratory diet. All mice were maintained according to European Union regulations, and experiments were performed under license from the Department of Health and Children and with approval from the Trinity College Dublin Bioresources Ethics Committee.

2.4.1 Isolation of CNS mononuclear cells

Female C57/BL6 mice aged 3 months (n=5) and 18 months (n=4) were sacrificed by decapitation whilst under anaesthesia induced by euthatal and animals were perfused with ice-cold 1 X phosphate buffer saline (PBS) prior to sacrifice to remove any contaminating cells in the brain from peripheral blood. Mice were sacrificed with assistance from Dr. Aedin Minogue. Brains were isolated and placed in medium A (see Appendix III) on the lid of a Petri dish containing ice. The cerebellum was removed and cortex and hippocampus isolated. Portions of the hippocampus were snap-frozen in liquid nitrogen and stored at -80°C in RNase-free microcentrifuge tubes for later mRNA analysis. The remaining tissue was cross-chopped using a sterile scalpel and placed in a hand-held homogeniser where it was mechanically dissociated. Using a 3 ml Pasteur pipette the tissue was dissociated through a 70 µM cell strainer and the flow-through collected and brought to 25 ml with medium A and centrifuged (200 x *g*; 10 min; 4°C) with the centrifuge brake turned off. The resultant pellet was re-suspended in 75% Percoll (10 ml; see Appendix III) and overlaid with 25% Percoll (10 ml) and 1 X PBS (6 ml) and centrifuged (800 x *g*; 30 min; 4°C) with the centrifuge brake turned off. The result of this density-dependent centrifugation is the separation of the myelin which is collected at the 0-25% Percoll interface from

the mononuclear fraction which can be seen at the 25-75% interface (Figure 2.1). The myelin was gently removed using Pasteur pipettes in 1 or 2 aspirations and mononuclear cells were subsequently collected and transferred to 50 ml Falcon tubes. Cells were washed with 1 X PBS (30 ml) and centrifuged (200 x *g*; 10 min; 4°C) with the centrifuge brake turned off. The supernatant was discarded carefully and the pellet re-suspended in 1 X PBS (1 ml) for flow cytometry staining as described in section 2.19.

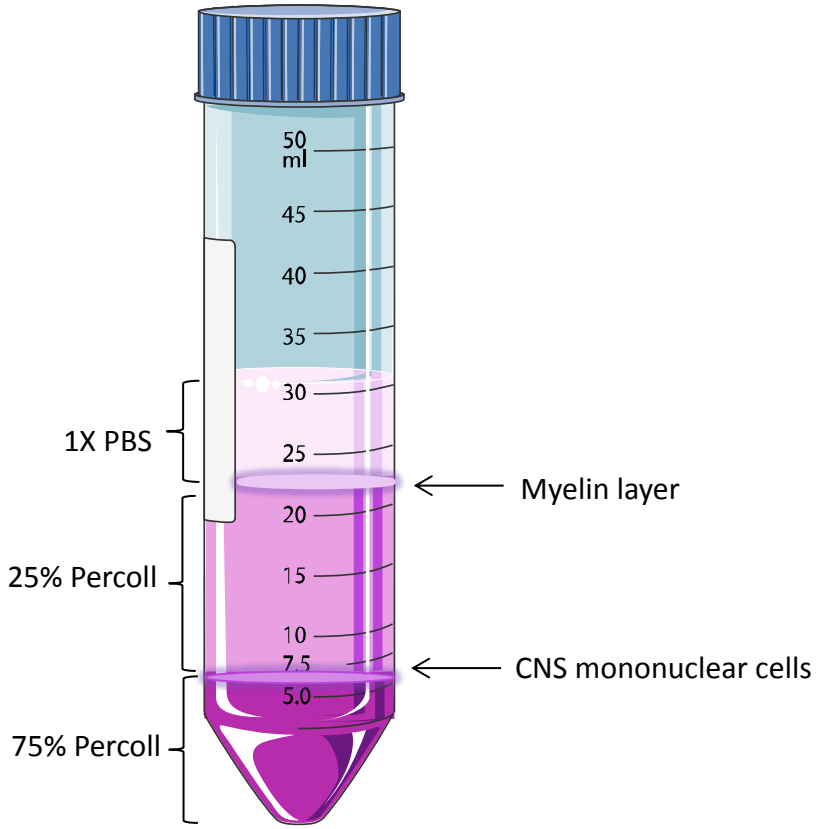

Figure 2.1 Percoll gradient for isolation of CNS mononuclear cells by density gradient centrifugation.

2.4.2 Preparation of plasma from mice

Blood samples from female C57/BL6 mice aged 3 months (n=5) and 18 months (n=4) were obtained under euthatal-induced anaesthesia via cardiac puncture using EDTA-coated syringes and 1.5 ml sterile Eppendorf tubes. In a series of separate experiments, blood samples from wild-type (WT, n=11) and APP/PS1 (n=9) mice aged 22 months were obtained in the same manner. All blood samples collected were

centrifuged (2000 x *g*; 10 min; 22°C) and the resulting plasma fraction aliquoted and stored at -80°C until further use.

2.5 J774.2 cell culture and treatment

Mouse J774.2 monocytes were kindly donated by Professor Luke O'Neill, TCD. J774.2 cells (passage 14-18) were maintained in cDMEM at 37°C in a humidified 5% CO_2; 95% air environment. J774.2 cells were counted in a Trypan Blue cell suspension (10%) and seeded onto a 24-well cell culture plate (density: $1x10^6$ cells/ml) or onto a 24-well Seahorse XF24 cell culture microplate (density: $1.2x10^6$ cells/ml) pre-coated with poly-D-lysine (50 ng/ml; Merck Millipore, Germany). For characterisation of pro-inflammatory and anti-inflammatory phenotypes, cells were treated with either recombinant mouse IFNγ (20 ng/ml) or IL-4 (10 ng/ml; R&D Systems, UK) for 4 h. Separately, J774.2 cells were treated with plasma from either young and aged mice (1:5) or WT and APP/PS1 mice (1:10) for 4 h. Control cells did not receive any cytokine or plasma treatment and were incubated in culture media alone. Cells were subsequently harvested and assessed for mRNA expression and bioenergetic profile as described in sections 2.14, 2.15 and 2.20.

2.6 MCI, AD and control participants

Patients with a clinical diagnosis of AD (NINCDS criteria; 2 female, 4 male) and MCI (Petersen criteria; 5 female, 12 male) with a mean age of 64.56 years (SD = 6.34) were recruited from the BIOMARKAPD project at St. James's and St. Vincent's Hospitals, Dublin, Ireland and Bon Secours Hospital, Tralee, Kerry, Ireland. Control participants (6 female, 14 male) with a mean age of 65.03 years (SD = 8.52) were recruited from the Santry Sports Centre, Dublin, Ireland. Cognitive function was assessed by the MoCA and showed a significant difference between controls and MCI/AD patients and also a significant difference between MCI and AD patients (Table 4.1). All control subjects had a MoCA score of >23. Blood samples (20 ml) were collected in EDTA tubes and centrifuged (2000 x *g*, 10 min, 22°C). Aliquots of plasma were stored at -80°C. This study was approved by the Adelaide and Meath Hospital, Dublin, incorporating the National Children's Hospital/St. James's Hospital

Joint Research Ethics Committee and all participants provided informed consent. Participants were excluded from the study if they were smokers, had significant active medical conditions, were currently taking psychoactive medication, had a history of major psychiatric or neurological condition, epilepsy, diabetes, heart attacks, traumatic brain injury, immunodeficiency, or were taking immunosuppressants or corticosteroids.

2.7 IQ-memory consistent and IQ-memory discrepant participants

2.7.1 Overview

Adults were recruited from the NEIL Memory Research Unit (MRU) at the Trinity College Institute of Neuroscience, Dublin, Ireland on 3 separate occasions (Hannigan et al., 2015). Each session comprised of a battery of neuropsychological tests focusing on memory and executive function and a blood assessment. The MMSE and MoCA were used as a screening measure, the National Adult Reading Test (NART) (Nelson, 1982) as an estimate of premorbid intelligence levels and memory was assessed using the Logical Memory I and II subsets of the Wechsler Memory Scale IV (WMS-IV) (Wechsler, 1998).

For the WMS-IV, participants were administered either the Adult Battery (for ages 16-69, consists of a Story B and C each administered once) or the Older Adult Battery (for ages 70+, consists of a Story A (administered twice) and a Story B which is administered once). The tests were carried out (Logical Memory I) and a period of 20 minutes was allowed to elapse before the tests were carried out a second time (Logical Memory II), which had not been previously disclosed to the participants. The total recall score for the Logical Memory II subset was calculated and results scaled to account for age using a table included. A z-score was subsequently calculated with the scaled Total recall using the following formula: (Total scaled z-score – 10)/3. This had been previously determined by members of the MRU.

Following this, an asymmetry index was used to classify participants into two subgroups, which was calculated by subtracting each participant's standardised

score (z-score) for the Logical Memory II delayed recall measure from their standardised score (z-score) for NART-IQ. Participants were classified as IQ-memory discrepant (IQ-discrepant) if their delayed recall z-score was more than 1 standard deviation below their z-score for NART-estimated IQ; and all other participants were classified as IQ-memory consistent (IQ-consistent). The classification of participants in this manner is represented in Figure 2.2.

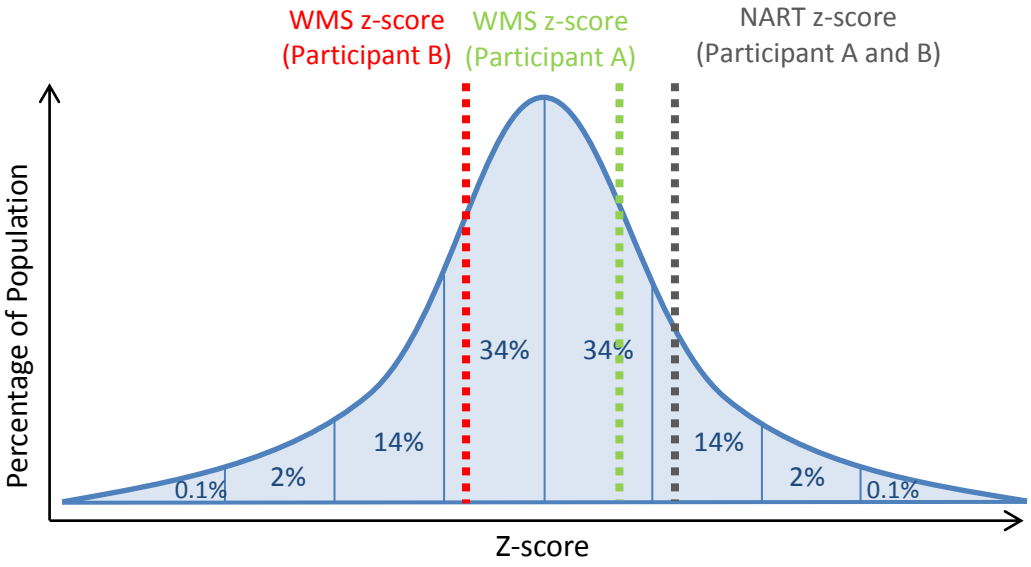

Figure 2.2 Representation of classification of participants into IQ-consistent and IQ-discrepant groups.

Healthy older adults were classified as either IQ-consistent or IQ-discrepant on the basis of their performance on the WMS relative to their estimated premorbid IQ measured by the NART. For example, for two participants (A and B) with the same NART z-score represented by the dashed grey line, participant A is classified as IQ-consistent as their WMS z-score (green dashed line) is within 1 standard deviation of their NART z-score. However, as the WMS z-score for participant B (red dashed line) is more than 1 standard deviation from their NART z-score, this participant is classified as IQ-discrepant.

The groups of participants are described further in sections 2.7.2 – 2.7.4. Participants who had psychiatric illnesses, normal pressure hydrocephalus, seizure disorders, brain tumours, alcohol or drug abuse/dependence (within the past 2 years), had a history of stroke disease or significant head trauma followed by persistent neurologic defaults, known structural brain abnormalities, multiple lacunes or lacunes in a critical memory structure shown on neuroimaging, evidence of infection,

infarction or other focal lesions shown on screening/baseline MRI scan, any significant systemic illness or unstable medication condition which could lead to difficulties and those currently using warfarin were excluded from the study.

2.7.2 Group 1

Adults (20 female, 6 male) with a mean age of 63.26 years (SD = 6.42) were classified by the criteria described in section 2.7.1, resulting in 13 individuals (10 female, 3 male; mean age 63.30 years, SD = 0.43) classified as IQ-consistent and 13 individuals classified as IQ-discrepant (10 female, 3 male; mean age 63.23 years, SD = 0.34). There was no significant difference in MMSE or MoCA scores between the groups (Table 3.1). Participants donated whole blood (45 ml) which was collected in heparinised BD Vacutainer tubes, diluted 1:1 with sterile 1 X PBS and layered over Lymphoprep™ (Axis-Shield, Norway) before centrifugation (800 x *g*, 30 min, 22°C). Aliquots of plasma were stored at -80°C. Informed consent was provided by all participants and the study was approved by the Faculty of Health Sciences at Trinity College Dublin. All neuropsychological assessments were carried out by members of the MRU.

2.7.3 Group 2

Adults (39 female, 36 male) with a mean age of 65.18 years (SD = 8.35) were classified by the criteria described in section 2.7.1, resulting in 46 individuals (28 female, 18 male; mean age 65.78 years, SD = 8.82) classified as IQ-consistent and 29 classified as IQ-discrepant (11 female, 18 male; mean age 64.24 years, SD = 7.59). There was no significant difference in MMSE or MoCA scores between the groups (Table 5.1). Participants donated whole blood (45 ml) which was processed as described in sections 2.8 and 2.9. Informed consent was provided by all participants and the study was approved by the Faculty of Health Sciences at Trinity College Dublin. All neuropsychological assessments were carried out by members of the MRU.

2.7.4 Group 3

Selected participants from Group 2 were invited for an additional assessment 2 years after their initial assessment. Adults (13 female, 12 male) with a mean age of 68.32 years (SD = 7.59) were classified by the criteria described in section 2.7.1, resulting in 15 individuals (5 female, 10 male; mean age 70.27 years, SD = 7.14) classified as IQ-consistent and 10 classified as IQ-discrepant (8 female, 2 male; mean age 65.40 years, SD = 7.66). There was no significant difference in MMSE or MoCA scores between the groups (Table 7.1). Participants donated whole blood (45 ml) which was processed as described in sections 2.8 and 2.9. Informed consent was provided by all participants and the study was approved by the Faculty of Health Sciences at Trinity College Dublin.

2.8 Isolation of PBMCs from whole blood

Whole venous blood (45 ml) was collected in EDTA tubes (Vacutette®, Greiner Bio-One, Austria). Blood was diluted 1:1 with sterile 1 X PBS and layered over sterile Lymphoprep™ (Axis-Shield, Norway). Samples were centrifuged (3000 x *g*; 15 min; 22°C) with the brake and accelerator off. Lymphoprep™ has a density of 1.077 g/mol, which is greater than mononuclear cells and lower than erythrocytes and polymorphonuclear cells. Therefore, as mononuclear cells have a lower buoyant density than erythrocytes and polymorphonuclear cells, the result of this density separation is the formation of a distinct band or 'buffy coat' at the sample/medium interface which is rich in mononuclear cells (Figure 2.3). Plasma (12 ml) was removed from above the interface, aliquoted and stored at -80°C until further analysis. The buffy coat containing the PBMCs was removed carefully and transferred to a fresh falcon tube which was made up to 50 ml with sterile 1 X PBS and centrifuged (1200 rpm; 10 min; 22°C). The supernatant was discarded and the pellet re-suspended in sterile 1 X PBS (25 ml) and centrifuged (1200 rpm; 10 min; 22°C). Supernatant was discarded and the pellet re-suspended in 10 ml of cRPMI media. Cells were counted using Trypan Blue solution (10%) and total number of PBMCs calculated.

For cryopreservation, PBMCs (1 x 10^6) were removed from the cell suspension, centrifuged (1200 rpm; 5 min; 22°C) and re-suspended in FBS (2 ml) containing DMSO (10%) and added to 2 cryopreservation tubes and placed in a 'Mr Frosty' freezing container with isopropanol and stored at -80°C overnight. Samples were transferred to a liquid nitrogen container the following day. The remaining PBMCs were used for isolation of monocytes to be differentiated into MDMs as described in section 2.9.

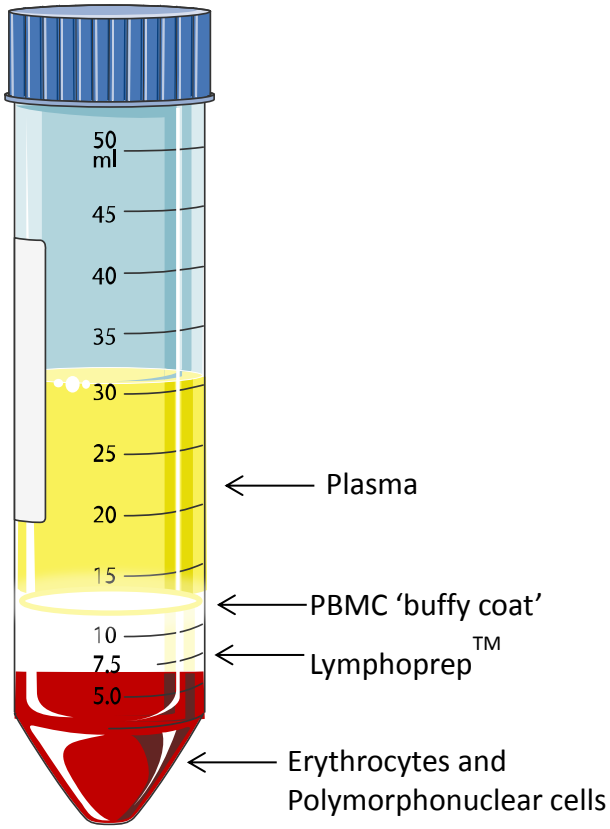

Figure 2.3 Separation of mononuclear cells from peripheral blood by density gradient centrifugation.

2.9 Isolation of MDMs

$CD14^+$ monocytes were isolated from PBMCs using magnetically-activated cell sorting (MACS; Miltenyi Biotec, Germany). The principle of this technique is to magnetically-label cells with CD14 MACS MicroBeads which are loaded into a column placed in the magnetic field of a MACS separator. The magnetically-labelled $CD14^+$

cells are retained in the column while unlabelled $CD14^-$ cells run through. Once the column is removed from the magnetic field, the magnetically retained $CD14^+$ cells can be eluted.

The remaining PBMCs were centrifuged (1200 rpm; 5 min; 22°C), washed with MACS buffer (5 ml; see Appendix III) and centrifuged (1200 rpm; 5 min; 22°C). All supernatant was carefully removed to ensure the pellet was dry and cells re-suspended in the appropriate volume of MACS buffer (80 µl per $1x10^7$ cells) and CD14 MACS MicroBeads (10 µl per $1x10^7$ cells) and incubated for 15 min at 4°C. Cells were washed with MACS buffer (1 ml per $1x10^7$ cells), centrifuged (1200 rpm; 5 min; 22°C), re-suspended in MACs buffer (1 ml) and added slowly to a MACS LS^+ column placed in the MACS separator. The column was washed three times with MACs buffer (3 ml), removed from the magnet and $CD14^+$ cells eluted in cRPMI (5 ml). Cells were centrifuged (1200 rpm; 5 min; 22°C), re-suspended in cRPMI (3 ml) and counted using Trypan Blue solution (10%).

Freshly isolated monocytes were seeded in 24-well culture plates (density: $0.6x10^6$ cells/ml) or onto a 24-well Seahorse XF24 cell culture microplate (density: $0.4x10^6$ cells/ml) pre-coated with poly-D-lysine (50 ng/ml), allowed to differentiate into MDMs in cRPMI supplemented with GM-CSF (10 ng/ml; R&D Systems, UK), and incubated for 7 days at 37°C in a humidified 5% CO_2; 95% air environment. Cells were treated and harvested as described in sections 2.12 and 2.14.

2.10 Preparation of Aβ peptides

Lyophilised amyloid β_{1-40} and amyloid β_{1-42} (Aβ40, Aβ42; Life Technologies) peptides were dissolved in high performance liquid chromatography (HPLC) grade water at 6 mg/ml and diluted to 1 mg/ml with sterile 1 X PBS. The peptide content of each batch was taken into account when reconstituting to ensure a 1 mg/ml concentration. The stock solutions were allowed to aggregate for 48 h at 37°C with continuous rotation at 220 revolutions rpm. Stock solutions were frozen at -20°C until use. The Aβ aggregation state was assessed by western immunoblotting described in section 2.16 and Aβ42 was aggregated to a higher degree than Aβ40 as

shown in Figure 2.4. For all cell culture treatments a combination of Aβ40 (4.2 µM) and Aβ42 (5.8 µM) was used, and this cocktail will be referred to throughout as Aβ (10 µM).

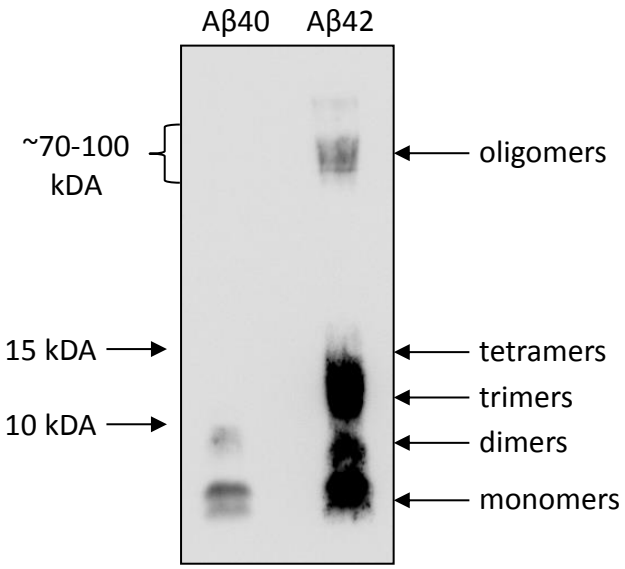

Figure 2.4 Aggregated Aβ40 and Aβ42 used in cell culture experiments.

2.11 Stimulation of PBMCs

Cryopreserved PBMCs isolated from IQ-consistent and IQ-discrepant participants (Group 2, section 2.7.3) were thawed for 1 min in a water bath and RPMI (1 ml) added slowly before transferring to a fresh falcon of RPMI (5 ml) and centrifuged (300 x *g*; 10 min; 22°C). Cells were re-suspended in RPMI (1 ml) and counted using Trypan Blue solution (10%). Cells were then seeded onto a 24-well tissue culture plate (density: $1x10^6$ cells/ml) or onto a 24-well Seahorse XF24 cell culture microplate (density: $2x10^6$ cells/ml) pre-coated with poly-D-lysine (50 ng/ml). Cells were incubated overnight and subsequently stimulated with Aβ (10 µM) and LPS (100 ng/ml; Enzo Life Sciences, USA) for 24 h and harvested for mRNA and bioenergetic analysis as described in sections 2.14, 2.15 and 2.20. Supernatant samples were assessed for cytokine concentrations as described in section 2.18.

2.12 Stimulation of MDMs

MDMs isolated from IQ-consistent and IQ-discrepant participants from Group 2 (section 2.7.3) and Group 3 (section 2.7.4) were stimulated in separate series of experiments. For participants in Group 2, cells that had been allowed to differentiate for 6 days were stimulated with either Aβ (10 μM) or LPS (100 ng/ml) for 24 h and assessed for mRNA and cell surface expression by flow cytometry as described in sections 2.14, 2.15 and 2.19. For participants in Group 3, after 6 days in culture cells were stimulated with a combination of LPS (100 ng/ml) and Aβ (10 μM; LPS was added 3 h prior to Aβ) for a total of 24 h and assessed for mRNA and protein expression and bioenergetic analysis as described in sections 2.14, 2.15, 2.16 and 2.20. For both groups, supernatant samples were assessed for cytokine concentrations as described in section 2.18.

2.13 THP-1 cell culture and treatment

Human THP-1 monocytes were kindly donated by Professor Andrew Bowie, TCD. THP-1 cells (passage 12-15) were maintained in cRPMI at 37°C in a humidified 5% CO_2; 95% air environment. THP-1 cells were counted in a Trypan Blue cell suspension (10%) and seeded onto a 24-well cell culture plate (density: $1x10^6$ cells/ml) or onto a 24-well Seahorse XF24 cell culture microplate (density: $1.2x10^6$ cells/ml) pre-coated with poly-D-lysine (50 ng/ml). For characterisation of pro-inflammatory and anti-inflammatory phenotypes, cells were treated with either recombinant human (rh) IFNγ (20 ng/ml; R&D Systems, UK) or a combination of both rhIL-4 (10 ng/ml) and rhIL-13 (10 ng/ml; Biolegend, USA) for 4 h. In a series of separate experiments, THP-1 cells were treated with plasma (1:40) from either control, MCI and AD patients and IQ-consistent and IQ-discrepant participants (Groups 1, 2 and 3) for 4 h. THP-1 cells were also treated with plasma from IQ-consistent and IQ-discrepant participants (Group 1) for 24 h. This dilution was previously identified as the optimal concentration (Dr. Eric Downer, pers. comm.). Control cells did not receive any cytokine or plasma treatment and were incubated in culture media alone. Cells were subsequently harvested and assessed for mRNA expression as described in sections 2.14 and 2.15. THP-1 cells were also treated with plasma (1:10) from either control,

MCI and AD patients for 4 h and bioenergetic profile assessed as described in section 2.20.

For additional experiments using plasma from IQ-consistent and IQ-discrepant participants (Group 1) the following conditions were used: 1) cells were pre-treated with an antibody for TLR2 (αTLR2; 2.5 µg/ml; 30 min) prior to treatment of THP-1 cells with Aβ and plasma; 2) cells were pre-treated with a phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (10 µM; 30 min) prior to treatment of THP-1 cells with plasma; 3) plasma was incubated at 56°C for 1 h prior to treatment of THP-1 cells. In all experiments THP-1 cells were incubated with plasma for 4 h and control cells were incubated in either IgG or culture media alone. Cells were subsequently harvested and assessed for mRNA expression as described in sections 2.14 and 2.15.

2.14 Cell Harvesting and Supernatant Collection

For semi-adherent cells supernatants were aspirated and dispensed several times in each well to dislodge the cells and transferred into 1.5 ml RNase-free microcentrifuge tubes (Eppendorf, Germany) and centrifuged (1500 rpm; 5 min; 22°C). The supernatants were collected and transferred to sterile 1.5 ml Eppendorf tubes. Cell lysis buffer was prepared for harvesting cells for either mRNA or protein analysis. For mRNA analysis, RA1 buffer (Machery-Nagel, Germany) and β-mercaptoethanol (Sigma-Aldrich, UK) was made up at a ratio of 100:1 and 350 µl added to each well. For protein analysis, lysis buffer was prepared (Appendix III) and 50 µl added to each well. The wells containing the cell lysis buffer were scraped using a 1 ml pipette tip and the contents of each well transferred to the corresponding 1.5 ml RNase-free microcentrifuge tube and the cell pellet re-suspended. For protein analysis, samples were immediately centrifuged (15000 rpm; 10 min; 4°C) and supernatant gently removed. All cell lysates and supernatant samples were stored at -20°C until further analysis.

2.15 Real-Time Polymerase Chain Reaction (RT-PCR)

2.15.1 RNA Extraction

RNA was extracted from harvested cells and brain tissue using the NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany). When extracting RNA from brain tissue, lysis buffer (350 µl) was first added to the tube containing the brain section and homogenised using a hand-held homogeniser. For all extractions, the cell or tissue lysate was applied to the NucleoSpin® Filter units (violet) placed in a 2 ml collecting tube and centrifuged to filtrate the lysate (11000 x *g*; 1 min; 22°C). The violet NucleoSpin® Filter units were discarded and 70% molecular-grade ethanol (350 µl) was added to each collecting tube to adjust RNA binding conditions and aspirated and dispensed 5-8 times. The lysate was loaded into NucleoSpin® RNA II column units (blue) placed in a 2 ml collecting tube and centrifuged (11000 x *g*; 30 sec; 22°C) to bind RNA to the column filter. The columns were placed into new 2 ml collecting tubes and membrane desalting buffer (350 µl) applied to the column to remove residual salt from the column's silica membrane. The samples were centrifuged (11000 x *g*; 1 min; 22°C) to dry the membrane. In order to digest the DNA, DNase reaction mixture (1:10 ratio of rDNase and rDNase reaction buffer; 95 µl) was applied directly onto the centre of the silica membrane of the column and incubated at room temperature for 15 min. The silica membrane was washed 3 times; RAW2 buffer (200 µl) was first added to the column and centrifuged (11000 x *g*; 30 sec; 22°C). The columns were transferred to new collecting tubes and RA3 buffer (600 µl) was loaded and the samples centrifuged (11000 x *g*; 30 sec; 22°C). The flow-through was discarded and the column placed back into the collecting tube. The column was washed a final time with RA3 (250 µl) and centrifuged (11000 x *g*; 2 min; 22°C), and the membrane dried completely. The column was placed in a pre-labelled nucleasefree 1.5ml microcentrifuge tube and RNase-free water (40 µl) applied to elute the RNA. Samples were centrifuged (11000 x *g*; 1 min; 22°C) and the columns discarded and the eluted RNA collected in the microcentrifuge tubes stored at -20°C short term or -80°C long term until quantification of RNA concentration.

2.15.2 Quantification of RNA Concentration

RNA quality and concentration was assessed by the Nanodrop™ 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). A blank reading was first taken with RNase-free water before making a sample measurement using 1 µl aliquots of each sample. RNA concentrations (ng/µl) were given based on absorbance at 260 nm and the selected analysis constant. The ratio absorbance at 260 and 280 nm was used to assess the purity of RNA. A ratio of ~2.0 is indicative of pure RNA and all samples used had an A260:A280 ratio of >1.5. RNA in each sample was equalised with RNA-free water (20 µl).

2.15.3 Reverse transcription for cDNA synthesis

Equalised RNA was subjected to reverse transcription into cDNA using the High Capacity cDNA RT kit (Applied Biosystems, UK). The Master Mix was prepared according to Table 2.1 and stored on ice.

Solution	Volume per sample (µl)
10x reverse transcription buffer	4
25x dNTPs	1.6
10x random primers	4
Multiscribe™ reverse transcriptase	2
RNase-free water	8.4

Table 2.1 Components of Master Mix (for 20µl sample).

RNA sample (20 µl) and Master Mix (20 µl) were added to labelled PCR tubes (Sarstedt, Germany). The contents were mixed and bubbles removed using a mini-centrifuge. The samples were placed in a thermal cycler (PTC 200 Peltier Thermal Cycler, BioRad, USA) and subjected to the following cycles: 10 min at 25°C, 2 h at 37°C cycle and 5 min at 85°C. The resulting cDNA was stored at -20°C until RT-PCR analysis.

2.15.4 cDNA amplification by RT-PCR

An aliquot of each cDNA sample (5 µl) was added to each well of a 96-well PCR plate (Thermo Scientific, USA) along with the Master Mix containing Kapa Polymerase (10 µl; Kapa Biosystems, USA), RNase-free water (3 µl), 18S primer (1 µl), and the appropriate target primer (1 µl). Assessment of target genes was performed using the real-time PCR dye-labelled primers TaqMan® Gene Expression Assays (Applied Biosystems, Germany) for the mouse genes listed in Table 2.2 and human genes listed in Table 2.3. The 96-well plate was covered with an adhesive seal and centrifuged (1200 rpm; 1 min; 22°C) and RT-PCR was carried out using an Applied Biosystems ABI Prism 7300 Fast Track Sequence Detection System v1.4.1. The samples were subjected to 1 run composed of 4 stages: 50°C for 2 min, 95°C for 10 min; 95°C for 15 sec and 60°C for 1 min. This was repeated for 40 cycles after which the plate was removed and data analysed.

2.15.5 PCR Quantification

The expression of each target gene was determined using the CT method (2^{-DDCT}, where Ct is the threshold cycle) for all RT-PCR analysis using the 7500 Fast system V1.3.1 relative quantitative study (Applied Biosystems, Germany). This method assesses the relative gene expression of target genes in different samples compared to the endogenous control (18S). The values were normalised to control samples and the relative differences in expression of the target gene between the samples quantified giving a relative quantity (RQ) value.

Gene Name	TaqMan Gene Expression Assay
Arginase-1	Mm00475988_m1
IL-6	Mm00446190_m1
iNOS (NOS2)	Mm00440502_m1
KC (CXCL1)	Mm00433859_m1
Mannose receptor-1	Mm00485148_m1
MCP-1 (CCL2)	Mm00441242_m1
MIP1-α (CCL3)	Mm00441259_g1
RANTES (CCL5)	Mm01302427_m1
TNFα	Mm00443258_m1

Table 2.2 Mouse TaqMan Gene Expression Assay numbers.

Gene Name	TaqMan Gene Expression Assay
Arginase-1	Hs00968979_m1
CD11b (ITGAM)	Hs00355885_m1
IL-1β	Hs01555410_m1
IL-6	Hs00985639_m1
IL-8	Hs01567912_g1
iNOS (NOS2)	Hs 01075529_m1
Mannose receptor-1	Hs00267207_m1
PFKFB1	Hs00997227_m1
PFKFB3	Hs00998700_m1
TLR2	Hs00610101_m1
TLR4	Hs00152939_m1
TNFα	Hs99999043_m1

Table 2.3 Human TaqMan Gene Expression Assay numbers.

2.16 Western Immunoblotting

2.16.1 Pierce® BCA protein assay

Samples were harvested as per section 2.14. A Pierce® BCA protein assay was carried out on samples as per manufacturer's instructions to determine protein concentrations (Fisher Scientific, UK). Briefly, a standard curve was produced by carrying out serial dilutions of bovine serum albumin (BSA) from a stock solution (2 mg/ml in dH_2O) in 1:10 lysis buffer in dH_2O to give a range of standards up to 1 mg/ml. Samples were diluted in dH_2O (1:10) and both samples and standards were added to a 96-well plate in duplicate (25 µl). Pierce® BCA Reagents A and B were prepared in a 50:1 ratio and added to each well (200 µl). The plate was covered and incubated in the dark for 30 min at 37°C and the absorbance was read at 562nm using a BioTek Synergy HT Multi-Mode Plate Reader. Protein concentrations were determined relative to the standard curve (expressed as µg/ml) and equalised in lysis buffer. Samples were diluted with 4 X Tris-glycine sample buffer (Appendix III) and heated at 70°C for 5 min to denature the proteins and stored at -20°C. For determining aggregated states of Aβ, 2 X sample buffer (Appendix III) was added to 1 µg of Aβ40 and Aβ42 protein and samples were heated at 70°C for 5-10 min.

2.16.2 Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

Polyacrylamide gels (1 mm thick) were prepared with a monomer concentration of 10% or 12% (Appendix III) and cast between two glass plates (10 cm; BioRad Laboratories, USA), and overlaid with 4% stacking gel (Appendix III) with a comb inserted into the stacking gel to create loading wells. Gels were mounted in an electrophoresis unit (BioRad Laboratories, USA) and electrode running buffer (Appendix III) was added to the inner and outer compartments. Tris-tricine gels were prepared for determining Aβ aggregated states (16.5% separating gel, 10% spacing gel, 4% stacking gel; Appendix III) and anode and cathode buffer added to the outer and inner compartments of the electrophoresis unit respectively (Appendix III). A pre-stained molecular ladder (10 µl; Spectra, Fisher Scientific, UK) was added to the first loading well followed by samples (15 or 20 µg of protein, or 1 µg of each Aβ

species) which had been heated for a further 5 min at 70°C before loading onto the gel. The proteins were separated across the gel by applying 90-120 mV across the unit for between 90-120 mins.

2.16.3 Western Immunoblotting

Gels were removed from the electrophoresis unit and the stacking gel discarded. The separating gel was placed on a nitrocellulose membrane (20 or 45 µM; Sigma-Aldrich, UK) between filter paper (Sigma-Aldrich, UK) and fibre pads, all pre-soaked in transfer buffer (Appendix III) and ensuring no bubbles were present between the layers. The cassettes were inserted into a blotting unit (BioRad Laboratories, USA), filled with transfer buffer and run at 90 mV for 90 min. Gels were discarded and nitrocellulose membranes immersed briefly in Ponceau S solution (Sigma-Aldrich, UK) to ensure sufficient transfer of proteins. Membranes were washed until clear with tris-buffered saline with Tween-20 (TBS-T; Appendix III) and blocked for 1 h in 5% non-fat dried milk in TBS-T (10 ml). All incubations were carried out on a see-saw rocker (SSL4, Stuart Scientific, UK) to ensure the membranes were covered fully by solutions. Membranes were incubated overnight at 4°C with primary antibody (10 ml) diluted in 5% BSA containing 0.1% or 0.05% Tween-20 (Table 2.4). The membranes were washed 4 times with TBS-T (15 min) and incubated with the appropriate secondary antibody (Goat anti-rabbit IgG or goat anti-mouse IgG; 1:5000 in 5% non-fat dried milk in TBS-T; 10 ml; 22°C) conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, USA). Membranes were washed 4 times with TBS-T and incubated in chemiluminescent detection solution (2 ml; 5 min; 22°C; WesternBright^{TM} ECL, Advansta, USA) and imaged using a FujiFilm LAS-4000 imaging system (Fuji, UK). Following imaging, membranes were stripped using Re-Blot Plus strong solution (10 ml; 5-10 min; 22°C; Merck Millipore, Germany) and re-probed for β-actin and imaged.

2.16.4 Densitometry analysis

Imaged membranes were quantified by densitometric analysis using ImageJ (Rasband W.S., National Institutes of Health, USA). Values were expressed as a ratio of total protein to β-actin.

Protein (MW)	Antibody	Supplier	Dilution
IL-1β (17, 31 kDa)	Mouse monoclonal anti-IL-1β (3A6)	Cell Signalling Cat # 12242	1:1000
Caspase-1 (20, 48 kDa)	Rabbit monoclonal anti-Caspase-1 (D7F10)	Cell Signalling Cat # 3866	1:1000
NLRP3 (112 kDa)	Mouse monoclonal anti- NLRP3 (Cryo-2)	Adipogen Cat # AG-20B-0014	1:1000
ASC (25 kDa)	Mouse polyclonal anti-ASC (AL177)	Adipogen Cat # AG-25B-0006	1:1000
PFKFB1 (55 kDa)	Rabbit polyclonal anti-PFKFB1	Abcam Cat # 71626	1:200
PFKFB3 (60 kDa)	Rabbit monoclonal Anti-PFKFB3	Abcam Cat # 181861	1:2000
Aβ	Anti-β-amyloid$_{1-16}$ clone: 6E10	Biolegend Cat # 803001	1:1000

Table 2.4 Primary antibodies used for Western Immunoblotting.

2.17 Mesoscale multiplex assay

Analysis of cytokine and chemokine concentrations in young and aged C57/BL6 mouse plasma was assessed using the mouse Proinflammatory Panel 1 Kit V-PLEX (Meso Scale Discovery, USA) as per manufacturer's instructions. Briefly, standards and samples were added in duplicate to the plate in Diluent 41 reagent (50 µl; samples diluted 1:2) and incubated for 2 h on a shaker at room temperature (Orbital Shaker SO3, Stuart Scientific, UK). The plate was washed 3 times with wash buffer (150 µl per well; Appendix III) and detection antibody for all analytes (IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, CXCL1, IL-10, IL-12p70, TNFα) was added (25 µl) and incubated for 2

h on a shaker at room temperature. The plate was washed 3 times with wash buffer (150 µl) and 2 X MSD read buffer T was added to each well (150 µl). The plate was read using a Mesoscale Sector Imager and cytokine/chemokine concentrations were calculated relative to the standard curve (expressed as pg/ml of protein).

2.18 ELISA (Enzyme-Linked Immunosorbent Assay)

The concentrations of IL-1β, IL-6, IL-8 and TNFα were measured in supernatant samples from human-derived cells using commercially available kits as per manufacturer's instructions (R&D Systems, UK). A 96-well microplate (Sigma-Aldrich/NUNC, UK) was coated with capture antibody (50 µl) at the appropriate dilution (Table 2.5) in 1 X PBS and incubated overnight at room temperature. After each step, the microplate was covered with a microplate sealer (Cruinn Diagnostics, Ireland) and all incubations were carried out on a shaker at room temperature (Orbital Shaker SO3, Stuart Scientific, UK). The capture antibody was aspirated and the plate was washed 3 times with wash buffer (Appendix III; 300 µl). Any excess wash buffer was removed from the wells by blotting the plate dry on a paper towel. The plate was blocked for 1 h with reagent diluent (1% BSA in 1 X PBS; 150 µl) at room temperature. The plate was washed and dried as before. An 8-point standard curve was prepared through a series of 2-fold dilutions from a top standard appropriate for the analyte examined (Table 2.5). The optimal dilution of sample was determined and dilutions for both standards and samples were prepared in RPMI and added to the plate (50 µl) in duplicate. The plate was sealed, left to incubate for 2 h at room temperature and washed and dried as before. Detection antibody (50 µl) was added to the plate at the appropriate dilution in reagent diluent (Table 2.5) and incubated for 2 h at room temperature. The plate was washed as before and streptavidin-horseradish peroxidase prepared in reagent diluent in the appropriate dilution as per manufacturer's instructions was added to each well (50 µl). The plate was incubated for 20 min in the dark at room temperature. Following this the plate was washed a final time and 3,3'5,5'-tetramethylbenzidine substrate solution (TMB, Sigma-Aldrich, UK) was added to each well (50 µl) and incubated in the dark for 20 min at room temperature. At this point if the reaction had occurred sufficiently as

determined by the intensity of the colour change, stop solution (H_2SO_4; 1 M) was added to each well (50 µl) to stop the reaction. The absorbance was read at 450nm using a BioTek Synergy HT Multi-Mode Plate Reader. A standard curve was constructed by plotting known standards against absorbance values and the concentration of analyte in sample was calculated relative to the standard curve (expressed as pg/ml of protein).

Cytokine	Capture Antibody	Standards	Detection Antibody	Catalogue number
IL-1β	4 µg/ml	0-250 pg/ml	200 ng/ml	DY201
IL-6	2 µg/ml	0-600 pg/ml	50 ng/ml	DY206
IL-8	4 µg/ml	0-1000 pg/ml	20 ng/ml	DY208
TNFα	4 µg/ml	0-1000 pg/ml	400 ng/ml	DY210

Table 2.5 Cytokine expression using ELISA.

2.19 Flow cytometry

Cells isolated from young and aged mice as described in section 2.4.1 were surface-stained to identify neutrophil, macrophage and microglial populations. MDMs stimulated with Aβ and LPS from IQ-consistent and IQ-discrepant participants (Group 2) as described in section 2.12 were stained for analysis of inflammatory markers and PBMCs from IQ-consistent and IQ-discrepant participants (Group 2) were stained for identification of lymphocyte populations.

For mouse samples, the cell suspension in 1 X PBS was transferred to FACS tubes and centrifuged (1200 rpm; 5 min; 22°C). The PBS was discarded and the top of the tubes dabbed on a sheet of clean tissue paper to dry completely. Live/Dead stain (1 ml; diluted 1:1000 with PBS, Life Technologies, USA) was added to all tubes, vortexed, and incubated in the dark for 30 min at room temperature. For human samples, cells were harvested gently using a cell-scraper (Sarstedt, Germany), transferred to FACS tubes and centrifuged (1200 rpm; 5 min; 22°C). Both mouse and human samples were washed in 1 ml FACS buffer (see Appendix III) and centrifuged (1200 rpm; 5 min; 22°C). The supernatant was discarded and tubes dabbed as before. Blocking

solution (1:50; 50 µl; Purified Rat Anti-Mouse CD16/CD32, BD Pharmingen, USA (mouse); Human TruStain FCX™, Biolegend, USA (human)) was added to all samples, vortexed and incubated in the dark for 15 min at room temperature. The antibody Master Mix (50 µl) was added to all sample tubes (Table 2.6 and Table 2.7). Tubes were vortexed and incubated in the dark for 15 min at room temperature. Samples were washed in FACS buffer (1 ml), centrifuged (1200 rpm; 5 min; 22°C) and the supernatant discarded and tubes dabbed as before and re-suspended in a final volume of FACS buffer (300 µl). Propidium iodide (PI; Sigma-Aldrich, UK; 1 µl) was added to human samples before reading to distinguish between live and dead cells.

FACS antibody	Fluorescent Label	Dilution	Company
CD11b	Pe-Cy7	1:100	Biolegend
CD45	APC-Cy7	1:80	Biolegend
CCR1	PE	1:20	R&D Systems
CXCR2	CCR1	1:20	R&D Systems
LY6G	FITC	1:100	Biolegend

Table 2.6 Mouse antibodies used for flow cytometry.

FACS antibody	Fluorescent Label	Dilution	Company
CD3	FITC	1:50	Biolegend
CD11b	FITC	1:100	Biolegend
CD14	Pe-Cy7	1:50	Biolegend
CD16	PerCP	1:50	Biolegend
CD19	APC	1:50	Biolegend
CD206	PE	1:100	Biolegend
TLR2	APC	1:100	eBiosciences
TLR4	Pe-Cy7	1:100	Biolegend

Table 2.7 Human antibodies used for flow cytometry.

In order to account for the overlap in fluorescence emitted by fluorochromes when performing flow cytometry, compensation beads (BD Biosciences, USA) for each fluorochrome used were prepared. Positive and negative compensation beads were added to a FACS tube, diluted with FACS buffer and antibody added at the same concentration used in the stain. For Live/Dead and PI compensation, cells that were stained only with Live/Dead or PI were used for compensation instead of beads. Fluorescence minus one (FMO) controls were also prepared, which is necessary for identification of gating boundaries during analysis. During the staining process, FACS tubes containing cells were stained with every fluorochrome except for the one under investigation (at the same concentration as the samples). Flow cytometric analysis was performed on all samples using a DAKO CyAn-ADP 7 colour flow cytometer (Beckman Coulter, USA) and analysed with FlowJo v7.6.5 software (Tree Star Inc., USA).

2.20 Bioenergetic analysis using the Seahorse XF Analyser

2.20.1 Seahorse XF Glycolytic Stress Test

The Seahorse Extracellular Flux (XFe24) Analyser (Seahorse Bioscience, USA) was used to assess bioenergetics of cells by performing a Seahorse XF Glycolytic Stress test to assess different parameters of glycolysis. Optimal cell numbers and concentrations of compounds/inhibitors were determined before the start of experiments as shown in Table 2.8 for J774.2, PBMCs, MDMs and THP-1 cells prepared and treated as per sections 2.5 and 2.11-2.13.

Cell type	Cell density	Glucose concentration	Oligomycin concentration	2-DG concentration
J774.2	$1.2x10^6$ cells/ml	10 mM	2 µM	50 mM
PBMC	$2x10^6$ cells/ml	10 mM	0.75 µM	50 mM
MDM	$0.4x10^6$ cells/ml	10 mM	7.5 µM	50 mM
THP-1	$1.2x10^6$ cells/ml	10 mM	7.5 µM	100 mM

Table 2.8 Optimised conditions for performing glycolytic stress test.

Cells were seeded at the optimised density (in the appropriate media for the cell type) in Seahorse XF24 cell culture microplates (Seahorse Bioscience, USA) pre-coated with poly-D-lysine (50 ng/ml) in 100 µl/well. One well per row was left blank for background correction when performing the run on the Analyser. The plate was left in the hood for 1 h before being incubated at 37°C for a further 1 h. Cells were then topped up with media (150 µl) and incubated overnight (J774.2, PBMCs, THP-1) or for 7 days (MDMs) at 37°C in a humidified 5% CO_2; 95% air environment.

J774.2 and THP-1 cells were treated the following day with plasma from WT and APP mice (1:10) for 4 h prepared as described in section 2.4.2 and with plasma from MCI and AD patients and controls (1:10) for 4 h prepared as described in sections 2.6. PBMCs were also stimulated the following day with either media alone or Aβ (10 µM) and LPS (100 ng/ml) for 24 h as described in section 2.11. MDMs were treated 6 days later with either media alone or a combination of Aβ (10 µM) and LPS (100 ng/ml) for 24 h (LPS was added 3 h prior to Aβ) as described in section 2.12.

The day before the assay the sensor cartridge was hydrated by adding Seahorse XF Calibrant solution (1 ml; Seahorse Bioscience, USA) to each well of the utility plate and left overnight in a CO_2-free incubator at 37°C. On the day of the assay, cells were washed twice with Seahorse XF Base Medium (supplemented with L-glutamine (2mM); pH 7.35) by gently removing all but 50 µl of media and replacing with assay media (1 ml). After the final wash, media (475 µl) was added to each well for a final volume of 525 µl/well and the plate was placed in a CO_2-free incubator at 37°C for 1 h. Working concentrations of glucose (Sigma-Aldrich, UK), oligomycin (Abcam, UK) and 2-deoxy-D-glucose (2-DG; Sigma-Aldrich, UK) were prepared in assay media as shown in Table 2.8. Compounds were loaded into ports A (56 µl), B (62 µl) and C (69 µl) of the cartridge respectively. The cartridge was loaded onto the Seahorse XF Analyser for calibration and following this the cell plate was added and extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) measured every 8 min for 96 min. After the first 3 baseline measurements the compounds were sequentially injected after every 3 time points.

2.20.2 Analysis of Seahorse XF Glycolytic Stress Test

ECAR and OCR were automatically calculated by the Seahorse XF24 software. The key parameters derived from a glycolytic stress test are shown in a sample ECAR graph in Figure 2.5. The cells are initially starved of glucose as there is no glucose present in the Seahorse XF Base Medium. The non-glycolytic acidification rate therefore measures other sources of glycolytic flux that are not due to glycolysis and is calculated by an average of the first 3 measurements. Glycolysis is calculated by the difference between the average of the second 3 values (after the addition of glucose) and the non-glycolytic acidification rate. Glycolytic capacity is calculated by the difference between the average of the third 3 values (after the addition of oligomycin) and the non-glycolytic acidification rate. Oligomycin is an ATP synthase inhibitor and suppresses mitochondrial respiration, therefore glycolytic capacity is an indication of the maximal rate of glycolysis in the cell. The OCR is an indication of oxidative phosphorylation, and the ratio of oxidative phosphorylation to glycolysis i.e. the OCR:ECAR, can also be calculated by taking the average of the second 3 values of ECAR and OCR. This will indicate whether or not there is a shift towards either glycolysis or mitochondrial respiration.

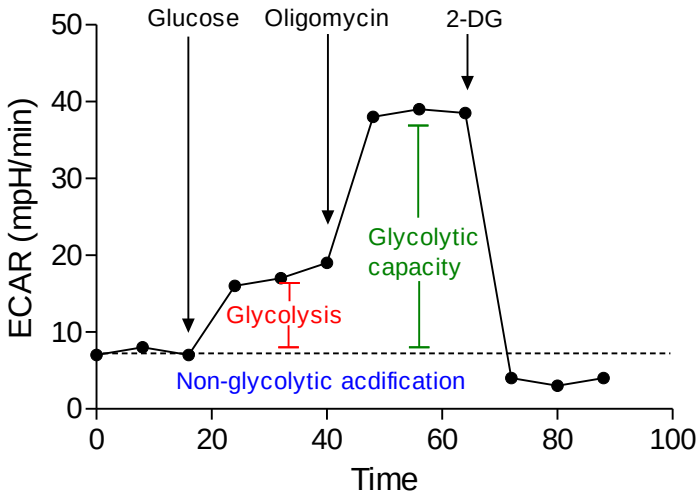

Figure 2.5 Sample ECAR graph of Glycolytic Stress Test.

2.20.3 Crystal Violet Assay

To reduce variability between MDM cells from different participants, data were normalised to cell number by performing a crystal violet assay directly after the Seahorse run. The media was removed and cells fixed in glutaraldehyde (1% in 1 X PBS; 300 µl; Sigma-Aldrich, UK) for 15 mins at room temperature. The glutaraldehyde was removed and cells washed with 1 X PBS. Crystal violet solution (0.1% in 1 X PBS; 500 µl; Sigma-Aldrich, UK) was added to each well and incubated for 30 mins at room temperature. The crystal violet solution was discarded and the plate was washed gently with dH_2O and left to air dry overnight. The following day Triton X solution (1% in 1 X PBS; 400 µl; Sigma-Aldrich, UK) was added to the plate which was placed on a shaker for 15 mins, or until the cells had lysed. An aliquot (100 µl) of solution from each well was transferred to a 96-well plate and absorbance was read at 550nm on a BioTek Synergy HT Multi-Mode Plate Reader. Seahorse parameters were calculated relative to the crystal violet assay reading.

2.21 Statistical Analysis

All data were analysed using the statistical packages Graph Pad Prism (GraphPad Software, Inc.) and SPSS (IBM). Statistical comparisons were performed using a Student's *t*-test for independent means or one- or two-way analysis of variance (ANOVA) followed by Dunnett's or Newman-Keul's *post-hoc* test where appropriate to determine which conditions were significantly different from each other. Linear regression was performed for correlation analysis. A p value less than 0.05 was deemed statistically significant. Data are expressed as means ± standard error of the mean (SEM).

Chapter 3: Inflammation during ageing and the use of plasma-treated monocytes as a biomarker of ageing and cognitive dysfunction

3.1 Introduction

Normal ageing is accompanied by a chronic low-grade increase in inflammation known as inflammaging, a term that was first coined by Franceschi and colleagues (2000). It is characterised by an increase in circulating pro-inflammatory cytokines including TNFα and IL-6, ROS, acute-phase proteins and autoantibodies (Deleidi et al., 2015). In the elderly population it is considered to be a major contributor to mortality and morbidity (Franceschi et al., 2017). Mechanisms contributing to inflammaging are thought to include the accumulation of endogenous host-derived cell debris, senescent cells which become more pro-inflammatory upon ageing, harmful products produced by the microbiota and immunosenescence (Franceschi and Campisi, 2014). These developments in ageing greatly impact on CNS function and promote neuroinflammation by modulating glial cells which adopt a more pro-inflammatory state (Di Benedetto et al., 2017), aided by a disruption in the BBB which allows the influx of immune cells into the brain parenchyma. This neuroinflammation can result in an increased susceptibility to cognitive decline and is associated with many neurodegenerative conditions.

As it is not possible to directly examine many aspects of brain physiology clinically, a marker that is indicative of the cognitive state must be derived by examining the physiology of peripheral cells. Blood-derived biomarkers have several conferred benefits over CSF and neuroimaging biomarkers as their measurement is cost-effective, minimally invasive and suitable for repeated sampling. Plasma concentrations of inflammatory mediators have been examined during ageing and in particular increasing concentrations of IL-6 have been associated with cognitive decline (Schram et al., 2007), and high levels of IL-6 have in fact been described as “the most powerful predicators of mortality and morbidity in the elderly” (Franceschi et al., 2017). However as analysis of cytokines and other mediators has produced variable results, it is possible that a functional assay involving examination of cells might give more reliable, consistent results.

Monocytes are cells of the innate immune system that play a central role in inflammation and host defence and differentiate into either dendritic cells or

macrophages depending on the stimulus encountered. Monocytes/macrophages have remarkable diversity and plasticity and can adopt a variety of phenotypes in response to environmental cues (Mantovani et al., 2013). Macrophage activation states were originally described in a similar manner to the Th1-Th2 dichotomy as either M1 (classical) or M2 (alternative) activation. Cytokines such as IFNγ and TNFα or bacterial components such as LPS induce a pro-inflammatory M1 phenotype, characterised by the production of NO and increased expression of pro-inflammatory cytokines and mediators including IL-1β. M1-like macrophages orchestrate pathogen killing, tissue destruction and anti-tumour resistance (Mantovani and Locati, 2009). In contrast, anti-inflammatory cytokines, primarily IL-4 and IL-13, polarise macrophages to an anti-inflammatory M2 state which is identified by the increase in Arg-1 and MRC-1 expression. M2-like macrophages are responsible for tissue remodelling and repair, parasite resistance and immunoregulation. This bipolar view of macrophage polarisation has been heavily criticised as there is a growing body of evidence for numerous activation states which is more likely to be reflective of the *in vivo* setting (Mosser and Edwards, 2008). Even though it is now appreciated that macrophage polarisation states exist along a spectrum, it is still useful to describe them in terms of the pro- and anti-inflammatory markers they express, which is an indication of their general state of activation.

The aims of this study are:

1. To investigate whether there is an age-dependent increase in inflammatory cytokines, hippocampal pro-inflammatory gene expression and infiltration of peripheral immune cells into the brain of C57/BL6 mice.
2. To characterise M1-like and M2- like markers in the J774.2 cell line and assess the effect of plasma from young and aged C57/BL6 mice on gene expression.
3. To characterise M1-like and M2-like markers in the THP-1 cell line and examine the effect of plasma from participants with and without subtle cognitive dysfunction (classified as IQ-consistent and IQ-discrepant) on gene expression.

3.2 Methods

To investigate neuroinflammatory events during ageing, young (3 month, n=5) and aged (18 month, n=4) female C57/BL6 mice were sacrificed by decapitation whilst under anaesthesia induced by euthatal and animals were perfused with ice-cold 1 X PBS prior to sacrifice to remove any contaminating cells in the brain from peripheral blood. CNS mononuclear cells were isolated and the percentage of peripheral immune cells in the brain investigated by flow cytometry as described in sections 2.4.1 and 2.19. Sections of hippocampal tissue were snap-frozen in liquid nitrogen and assessed for chemokine gene expression as described in section 2.15. Blood samples were collected and the concentration of circulating inflammatory mediators was investigated using a multiplex ELISA platform as described in sections 2.4.2 and 2.17 respectively.

J774.2 cells were incubated in the presence or absence of IFNγ, IL-4 or plasma from young and aged mice. Cells were harvested and investigated by RT-PCR as described in section 2.5.

THP-1 cells were incubated in the presence or absence of IFNγ or IL-4 and cells were harvested for assessment by RT-PCR as described in section 2.13.

Healthy older adults (n=26) with a mean age of 63.26 years were recruited from the MRU at Trinity College Dublin. Participants were classified as either IQ-consistent (n=13) or IQ-discrepant (n=13) based on their delayed word recall in relation to their estimated IQ as described in section 2.7.1 and 2.7.2. No difference in MMSE or MoCA scores was observed between the groups. This work was carried out by members of the MRU. Blood samples were processed and plasma isolated and stored as described in section 2.7.2. Phlebotomy was carried out by Ms. Grainne Fleming. Plasma was incubated with THP-1 cells as described in section 2.13 and harvested for analysis of gene expression by RT-PCR.

3.3 Results

3.3.1 Aged mice show an increase in circulating cytokines

The inflammatory phenotype of young and aged mice was characterised by assessing the concentration of circulating cytokines (IFNγ, IL-1β, IL-2, IL-4, IL-2, IL-6, IL-10, IL-12p70, CXCL1 and TNFα) by multiplex ELISA. Analysis of plasma showed a significant increase in CXCL1, TNFα, IL-1β and IL-10 concentrations in plasma from aged mice compared with young mice (Figure 3.1 A-D, $p<0.05$, Student's *t*-test for independent means). An increase in IL-12p70 and IL-6 plasma concentrations was also observed in aged mice but this did not reach statistical significance (Figure 3.1 E, F). No difference was seen in IL-5 plasma concentration (Figure 3.1 G) between the groups and the concentrations of IFNγ, IL-2 and IL-4 were undetectable in plasma from both young and aged mice.

3.3.2 Neutrophils and macrophages infiltrate the CNS in aged mice

In order to assess whether peripheral immune cells infiltrated the brain with age, mononuclear cells were isolated from the brains of young and aged mice and flow cytometry performed to identify $CD11b^+$ cells. The expression of Ly6G, a neutrophil specific marker, was assessed on the $CD11b^+$ population and a significant increase in neutrophils, identified as $CD11b^+Ly6G^+$ cells, was observed in aged mice (Figure 3.2 A, $p<0.05$, Student's *t*-test). No difference in the expression of chemokine receptors CCR1 and CXCR2 on neutrophils was observed (Figure 3.2 B, C).

The expression of CD45 on the $CD11b^+$ population was used to distinguish between macrophages (high expressing CD45) and microglia (low expressing CD45). Aged mice showed a significant increase in macrophages, identified as $CD11b^+CD45^{high}$, compared with young mice (Figure 3.3 A; $p<0.001$; Student's *t*-test) and no difference in microglia identified as $CD11b^+CD45^{low}$ was seen between the groups (Figure 3.3 B). No differences in CCR1 and CXCR2 receptor expression on macrophages was observed (Figure 3.4 A, B), however expression of both receptors was significantly increased on microglia from aged, compared with young, mice

(Figure 3.4 C, D; $p<0.05$, Student's *t*-test). The data indicate that peripheral immune cell infiltration occurs during ageing.

3.3.3 Hippocampal chemokine expression is increased in aged mice

The increase in infiltrating neutrophils and macrophages in the CNS of aged mice suggests that peripheral cells might be migrating to the brain along a chemotactic gradient. The expression of chemokines in the hippocampus of young and aged mice was therefore examined by RT-PCR. CXCL1 and CCL2 mRNA expression were significantly upregulated in the hippocampus of aged, compared with young, mice (Figure 3.5 A, B; $p<0.05$, $p<0.01$; Student's *t*-test). An increase in CCL3 and CCL5 mRNA expression was also observed although these changes did not reach statistical significance (Figure 3.5 C, D).

3.3.4 CXCL1 mRNA is upregulated in J774.2 cells in response to plasma from aged mice

Having demonstrated that aged mice display an elevated neuroinflammatory phenotype, a proxy method of assessing this peripherally was investigated by examining the response of J774.2 cells to plasma. J774.2 cells are a mouse monocyte/macrophage cell line and are routinely used *in vitro*. In order to characterise these cells in terms of their response to pro- and anti-inflammatory stimuli, cells were first treated with either IFNγ or IL-4 for 4 hours. The expression of pro-inflammatory markers TNFα, iNOS and IL-6 was significantly upregulated by IFNγ treatment (Figure 3.6 A, B, C, $p<0.05$, $p<0.001$, One-way ANOVA with Dunnett's post-hoc analysis). Interestingly, the expression of CXCL1 was found to be significantly decreased in response to IFNγ (Figure 3.6 D, $p<0.05$, One-way ANOVA with Dunnett's post-hoc analysis). None of the inflammatory markers were affected by IL-4 treatment. IL-4 significantly upregulated archetypal markers of an anti-inflammatory state MRC-1 and Arg-1 (Figure 3.6 E, F, $p<0.01$, $p<0.001$, One-way ANOVA with Dunnett's post-hoc analysis). IFNγ exerted no effect on MRC-1 or Arg-1 mRNA expression.

J774.2 cells were subsequently incubated with plasma from young and aged mice for 4 hours and pro-inflammatory marker expression assessed by RT-PCR. Expression of CXCL1, the mouse homologue of IL-8, was significantly increased in J774.2 cells in response to plasma from aged, compared with young, mice (Figure 3.7 A, $p<0.05$, Student's *t*-test). No difference in the expression of TNFα, IL-6 and iNOS was observed (Figure 3.7 B, C, D). The data suggest that there are factors present in the plasma of aged mice that specifically upregulate CXCL1 mRNA in J774.2 cells.

3.3.5 IL-8 mRNA is upregulated in THP-1 cells in response to plasma from IQ-discrepant participants

Having identified an approach that might indicate the presence of an underlying neuroinflammatory phenotype through incubating a cell line with plasma in mice, the effect of plasma samples from IQ-consistent and IQ-discrepant participants was assessed on a human monocyte cell line, THP-1 cells. THP-1 cells, like J774.2 cells, are commonly used *in vitro* as a cell culture model. Participants described as IQ-discrepant were identified as having a poorer than predicted performance on their delayed verbal recall in the WMS relative to their IQ estimated by the NART, and participants who performed as predicted were termed IQ-consistent. The participant groups were age and gender matched and showed no difference in MMSE or MoCA scores. Table 3.1 shows that there was a significant difference in delayed verbal recall z-score and cognitive asymmetry z-score between the groups, the basis on which the cohorts were divided (Table 3.1, $p<0.001$).

Firstly, the ability of THP-1 cells to shift to a pro- and anti-inflammatory state was characterised. THP-1 cells were treated with IFNγ or a combination of IL-4 and IL-13 for 4 hours and pro- and anti-inflammatory marker expression was examined by RT-PCR. IFNγ increased the mRNA expression of TNFα and IL-6 (Figure 3.8 A, C, $p<0.01$, $p<0.001$ One-way ANOVA with Dunnett's post-hoc analysis) and no differences were seen in response to IL-4 + IL-13. The expression of iNOS and IL-8 remained unchanged in response to both IFNγ and IL-4 + IL-13 (Figure 3.8 B, D). IL-4 + IL-13 increased MRC-1 and Arg-1 mRNA in THP-1 cells although this did not reach

statistical significance (Figure 3.8 E, F). MRC-1 expression was not altered by IFNγ treatment, whereas the expression of Arg-1 was not detected.

Following on from this, THP-1 cells were incubated with plasma for 4 and 24 hours and pro-inflammatory marker expression assessed by RT-PCR. The mRNA expression of IL-8 was significantly upregulated in response to plasma from IQ-discrepant compared with IQ-consistent participants after 4 hours (Figure 3.9 A). A similar change was observed after a 24 hour incubation period (Appendix IV Figure S1 A). No differences in TNFα, IL-6 and iNOS mRNA expression were observed after 4 hours (Figure 3.9 B, C, D) or 24 hours (Appendix IV Figure S1 B, C, D).

3.3.6 IL-8 mRNA in THP-1 cells is correlated with cognitive function

The relationship between the increase in IL-8 mRNA (RQ) and participant's cognitive scores was investigated. A significant correlation was found between IL-8 mRNA in THP-1 cells and the cognitive asymmetry z-score (delayed verbal recall in relation to estimated IQ) (Figure 3.10 A, $p<0.001$; linear regression analysis) and the delayed verbal recall z-score component of the WMS (Figure 3.10 B, $p<0.001$; linear regression analysis).

3.3.7 Inhibition of TLR2 and PI3K does not diminish the increase in IL-8 mRNA in THP-1 cells in response to plasma from IQ-discrepant individuals

In order to investigate potential factors present in the plasma of IQ-discrepant individuals that might be responsible for the specific increase in IL-8 mRNA in THP-1 cells, THP-1 cells were pre-treated with an antibody for TLR2 (αTLR2) or an inhibitor of PI3K, LY294002, for 30 minutes prior to exposure to plasma for 4 hours. TLR2 has been shown to act as a receptor for Aβ (Costello et al., 2015) and therefore the effect of TLR2 inhibition on Aβ stimulation in THP-1 cells was investigated. The data show that αTLR2 significantly attenuated the Aβ-induced increase in IL-8, TNFα and IL-6, but not iNOS, expression (Figure 3.11 A, B, C, D, $p<0.001$, Two-way ANOVA with Newman Keuls post-hoc analysis). Inhibition of TLR2 prior to incubation of plasma with THP-1 cells increased IL-8 mRNA and decreased the expression of TNFα, IL-6 and iNOS mRNA (Figure 3.12 A, B, C, D, $p<0.05$, $p<0.01$, $p<0.001$, Two-way ANOVA

with Newman Keuls *post-hoc* analysis). However, IL-8 mRNA was increased in cells exposed to plasma from IQ-discrepant compared to IQ-consistent participants in cells pre-treated with both IgG control and αTLR2 (Figure 3.12 A, $p<0.05$, $p<0.001$, Two-way ANOVA with Newman Keuls *post-hoc* analysis).

THP-1 cells treated with LY294002 for 4 hours in the steady state show a significant decrease in IL-8 and IL-6 mRNA expression (Figure 3.13 A, C, $p<0.01$, $p<0.001$, Student's *t*-test), while the expression of TNFα and iNOS was upregulated (Figure 3.13 B, D, $p<0.001$, Student's *t*-test). THP-1 cells pre-treated with LY294002 and subsequently incubated with plasma show a significant treatment effect in the expression of IL-8, TNFα, IL-6 and iNOS (Figure 3.14 A, B, C, D, $p<0.01$, $p<0.001$, Two-way ANOVA with Newman Keuls *post-hoc* analysis). The expression of IL-8 is increased in cells without LY294002 pre-treatment in response to plasma from IQ-discrepant participants and although this trend persists with LY294002 pre-treatment it is not statistically significant (Figure 3.14 A, $p<0.001$, Two-way ANOVA with Newman Keuls *post-hoc* analysis).

To determine if the increase in IL-8 mRNA is due to a proteinaceous substance present in the plasma, plasma was heat-inactivated at 56°C for 1 hour prior to incubation with THP-1 cells for 4 hours. Plasma from IQ-discrepant individuals that either had or had not been heat-inactivated induced an increase in IL-8 mRNA compared with plasma from IQ-consistent participants; this trend was further increased with heat-inactivated plasma (Figure 3.15 A, $p<0.05$, $p<0.01$, Two-way ANOVA with Newman Keuls *post-hoc* analysis). The expression of TNFα remained unchanged and no effect of plasma heat-inactivation was observed (Figure 3.15 B, Two-way ANOVA with Newman Keuls *post-hoc* analysis).

Taken together, the data suggest that the factors present in the plasma of IQ-discrepant participants inducing an increase in IL-8 mRNA in THP-1 cells are not likely to be a proteinaceous substance or to be signalling through TLR2 or PI3K.

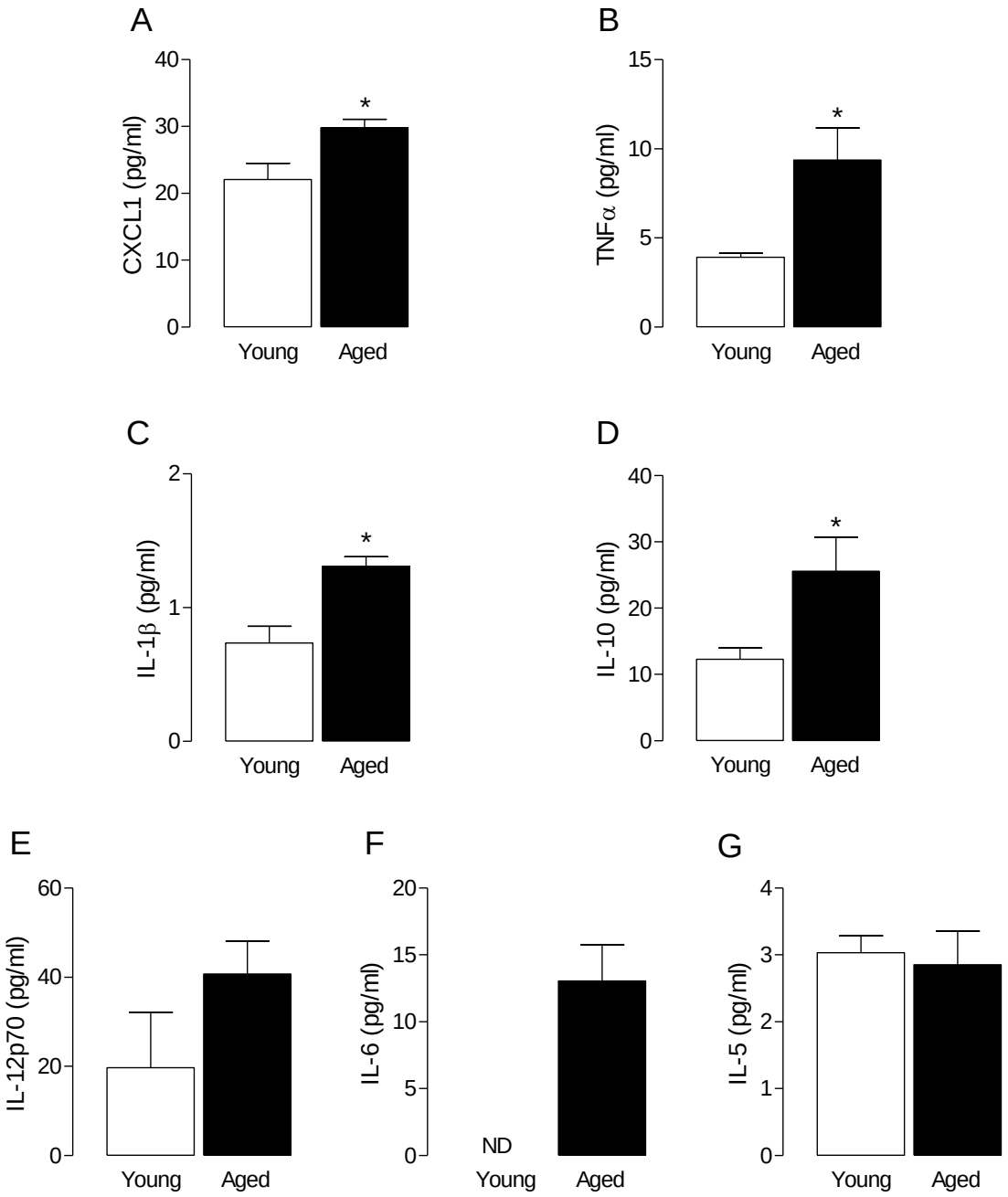

Figure 3.1 Pro-inflammatory cytokines are increased in the plasma of aged mice.

Young (3m) and aged (18m) mice were sacrificed and plasma assessed for cytokine concentrations by multiplex ELISA. The concentrations of CXCL1, TNFα, IL-1β and IL-10 (A-D) were increased in the plasma from aged, compared with young, mice. No significant differences in IL-12p70, IL-6 and IL-5 (E-G) concentrations were observed. Data are expressed as the mean ± SEM (n = 4-5). *p<0.05 vs Young; Student's t-test for independent means. ND = not detected.

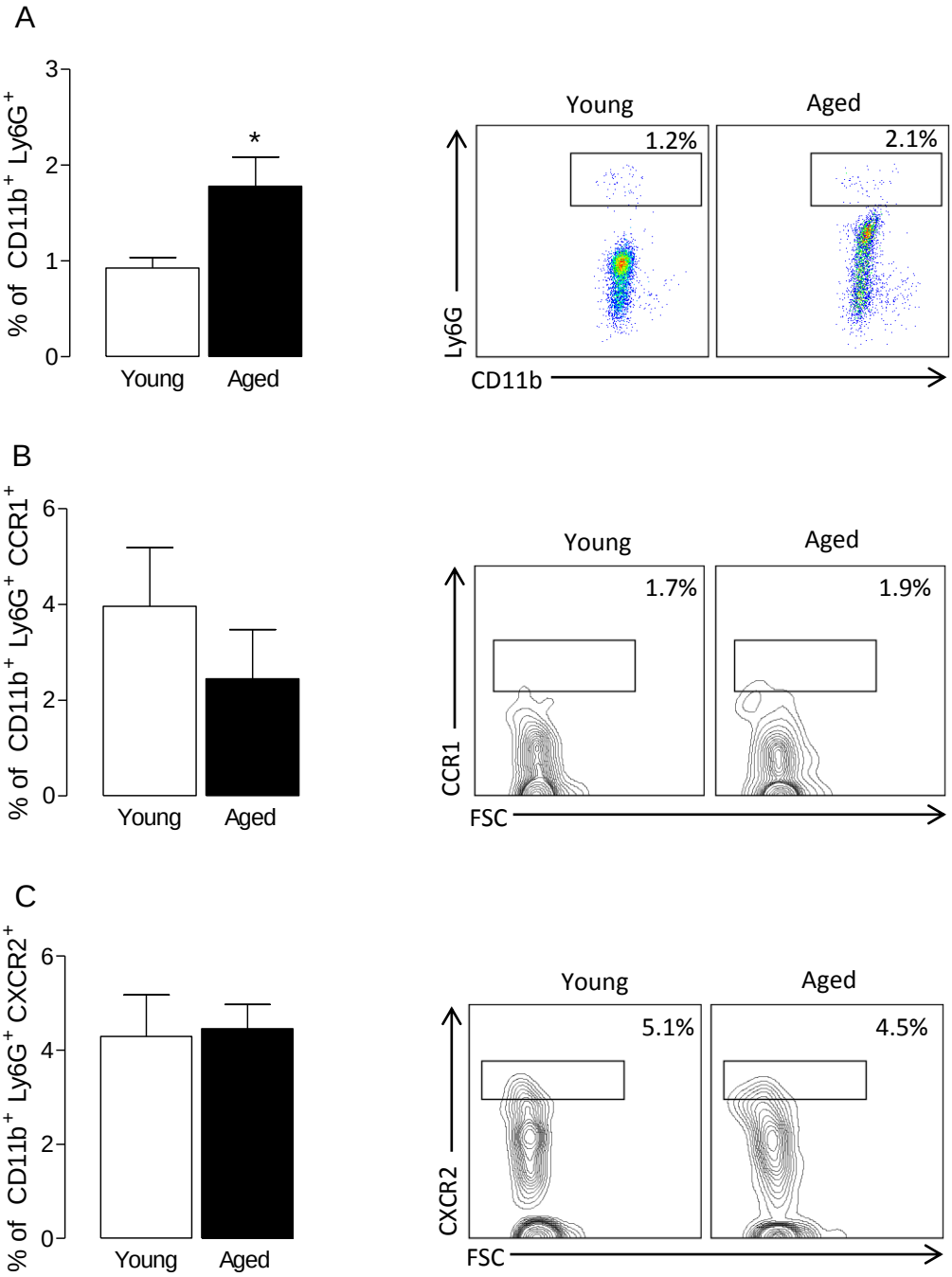

Figure 3.2 Neutrophils are increased in the brains of aged mice.

Young (3m) and aged (18m) mice were sacrificed and flow cytometry performed on brain tissue. Neutrophils, identified as $CD11b^{+}Ly6G^{+}$, were increased in the brains of aged mice compared with their younger counterparts (A). No difference was seen in the expression of CCR1 and CXCR2 on neutrophils from aged, compared with, young mice (B, C). Representative FACS plots are shown. Data are expressed as the mean ± SEM (n = 4-5). *p<0.05 vs Young; Student's t-test for independent means.

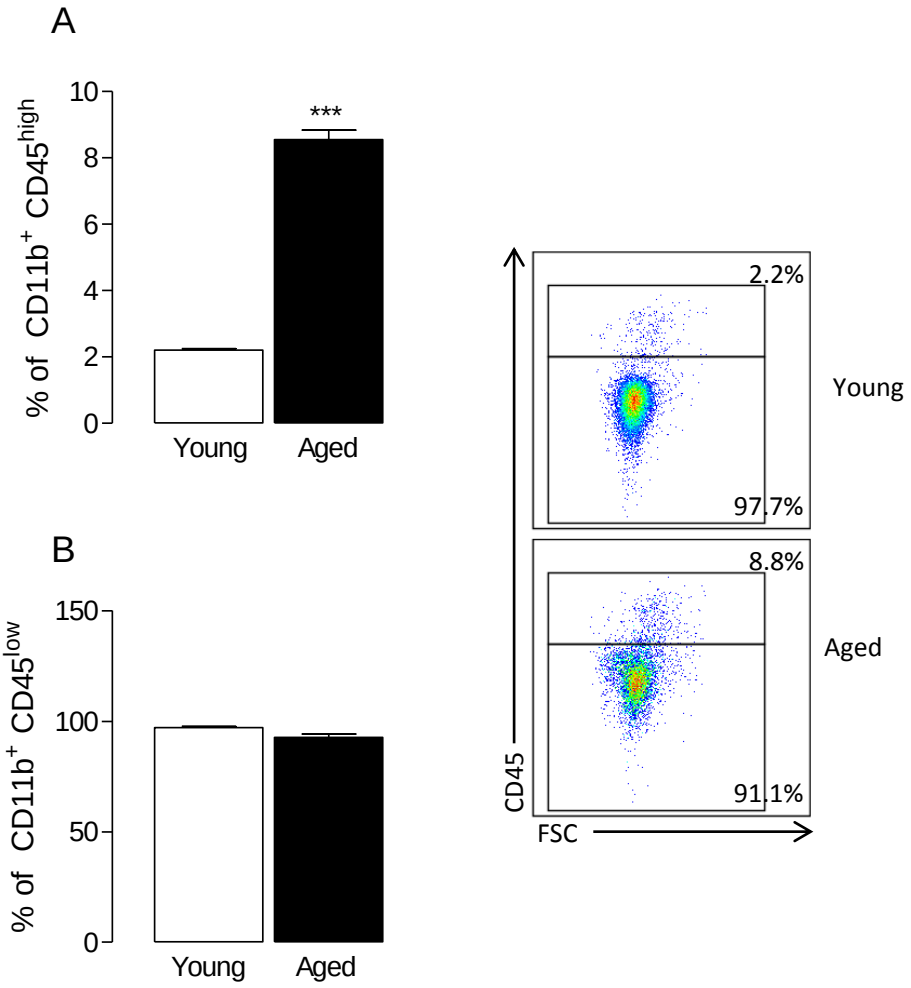

Figure 3.3 Macrophages are increased in the brains of aged mice.

Young (3m) and aged (18m) mice were sacrificed and flow cytometry performed on brain tissue. Brains from aged mice showed an increase in the percentage of macrophages, identified as expressing $CD11b^{+}CD45^{high}$, compared to brains from young mice (A) but no change in the percentage of microglia identified as expressing $CD11b^{+}CD45^{low}$ (B) was seen. Representative FACS plots are shown. Data are expressed as the mean ± SEM (n = 4-5). ***p<0.001 vs Young; Student's t-test for independent means.

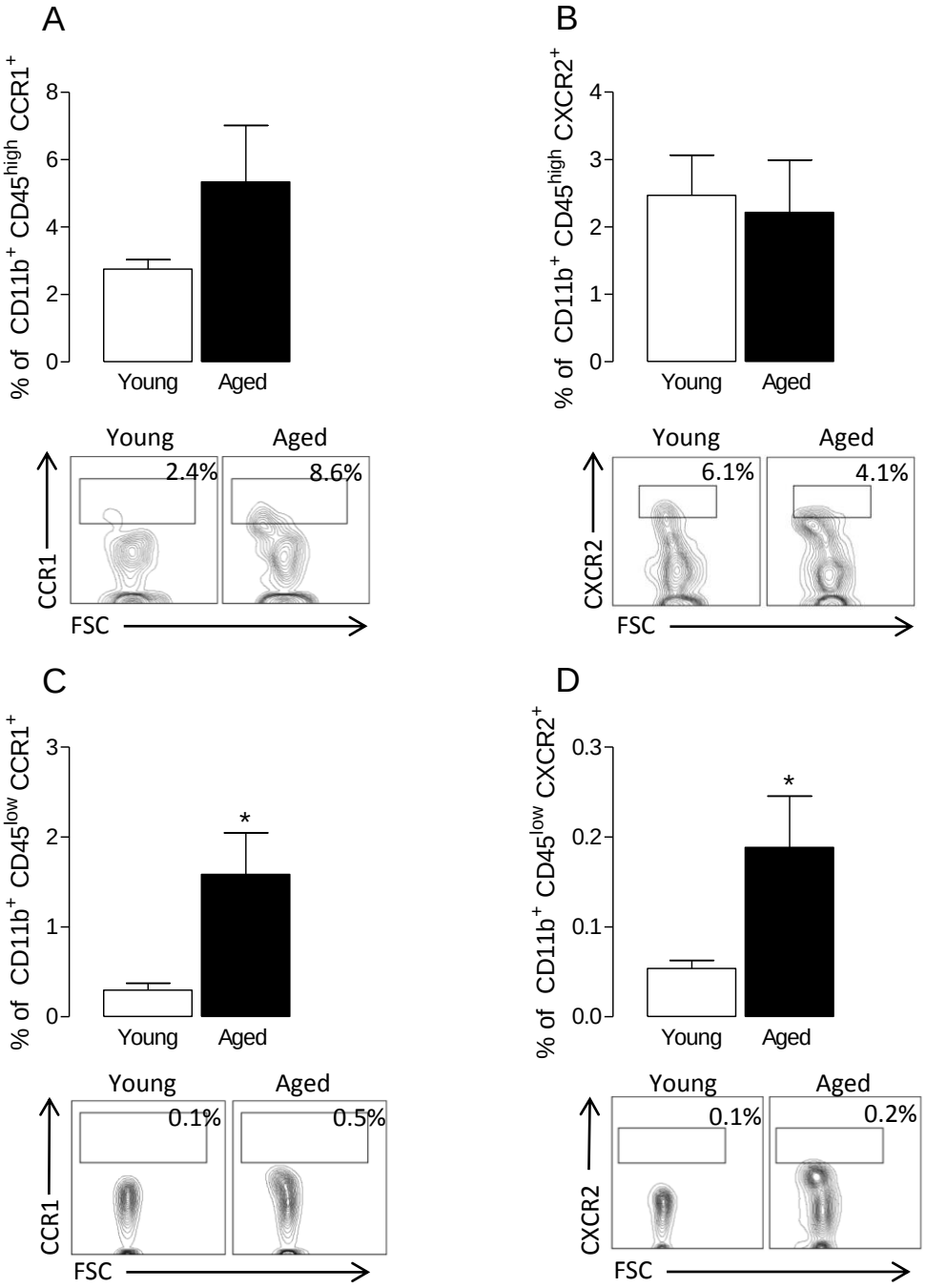

Figure 3.4 Microglia but not macrophages from aged mice show an increased in CCR1 and CXCR2 expression.

Young (3m) and aged (18m) mice were sacrificed and flow cytometry performed on brain tissue. Macrophages from aged mice, identified as $CD11b^{+}CD45^{high}$, showed no change in CCR1 (A) and CXCR2 expression (B) compared with young mice. The expression of CCR1 and CXCR2 was significantly increased on microglia from aged mice, identified as $CD11b^{+}CD45^{low}$, in comparison to microglia from young mice (C, D). Representative FACS plots are shown. Data are expressed as the mean ± SEM (n = 4-5). *p<0.05 vs Young; Student's t-test for independent means.

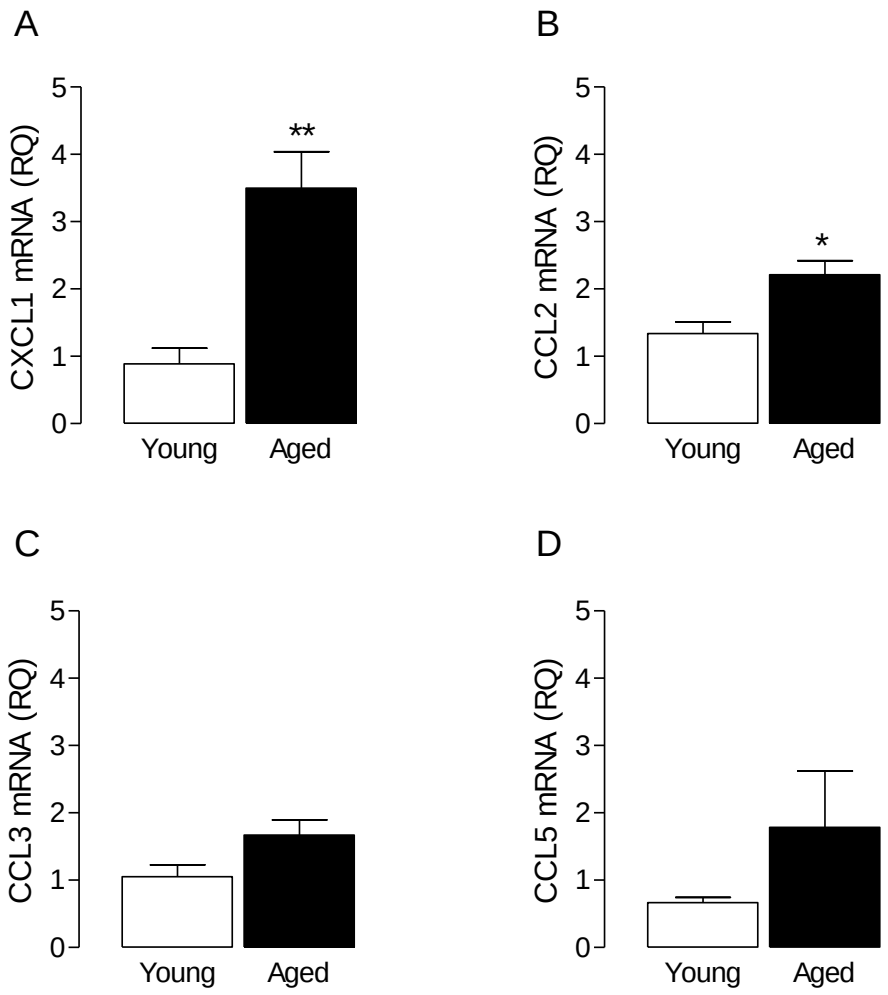

Figure 3.5 Chemokine mRNA expression is upregulated in the hippocampus of aged mice.

Young (3m) and aged (18m) mice were sacrificed and the mRNA expression of chemokines in the hippocampus was examined by RT-PCR. The expression of CXCL1 (A) and CCL2 (B) was increased in the hippocampus of aged mice compared with young mice. No difference was observed in CCL3 (C) and CCL5 (D) mRNA expression. Data are expressed as the mean ± SEM (n=4-5). *p<0.05, **p<0.01 vs Young; Student's t-test for independent means.

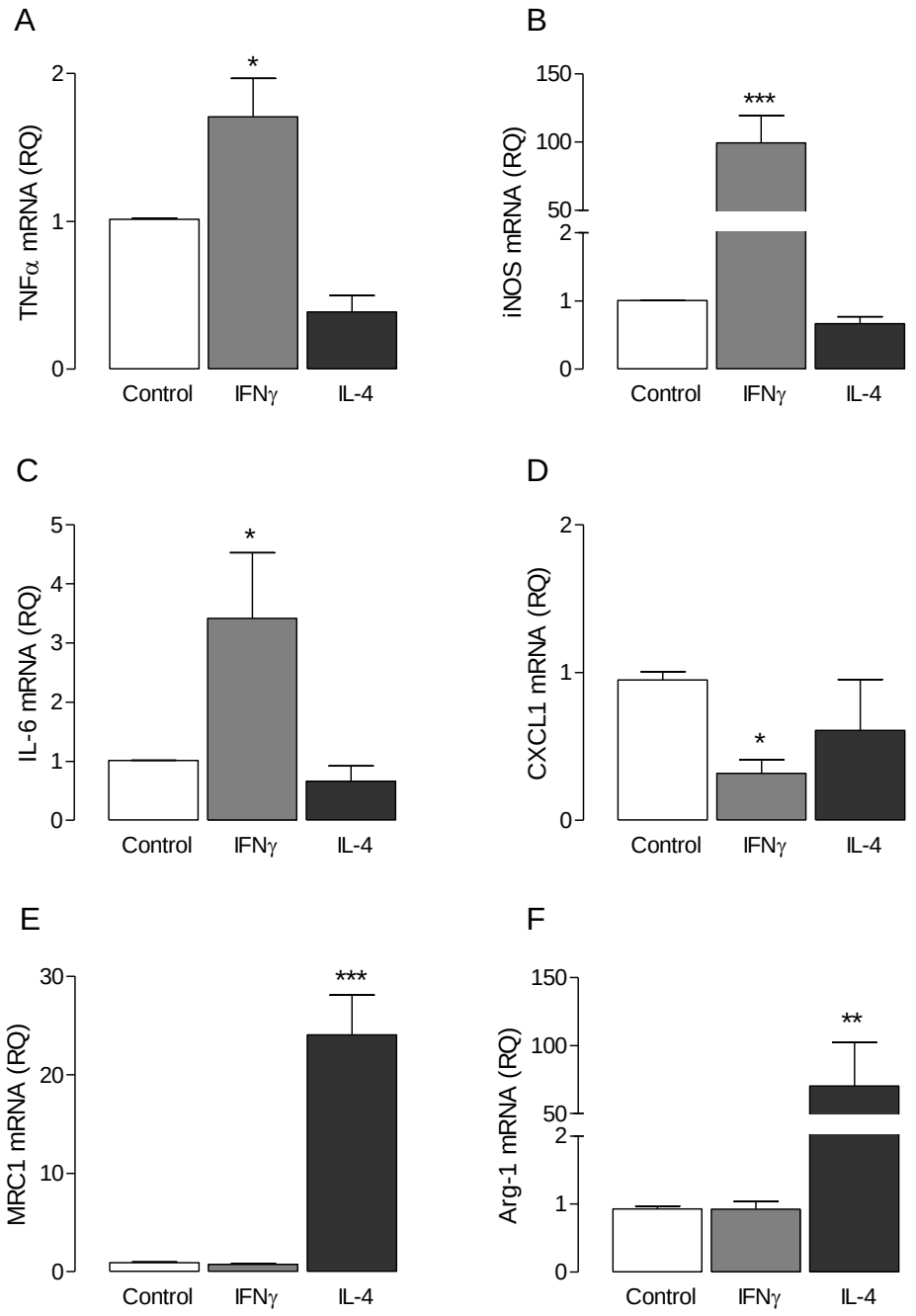

Figure 3.6 IFNγ and IL-4 polarise J774.2 cells to a pro-inflammatory and anti-inflammatory phenotype respectively.

J774.2 cells were incubated with or without IFNγ (50 ng/ml) or IL-4 (20 ng/ml) for 4 h. Cells were harvested and mRNA expression was assessed by RT-PCR. IFNγ significantly upregulated mRNA expression of TNFα (A), iNOS (B) and IL-6 (C). CXCL1 mRNA expression significantly decreased in IFNγ-incubated cells (D). IL-4 significantly upregulated the mRNA expression of MRC-1 (E) and Arg-1 (F). Data are expressed as the mean ± SEM(n = 3-4). **p*<0.05, ***p*<0.01; ****p*<0.001 vs control; One-way ANOVA with Dunnett's post-hoc analysis.

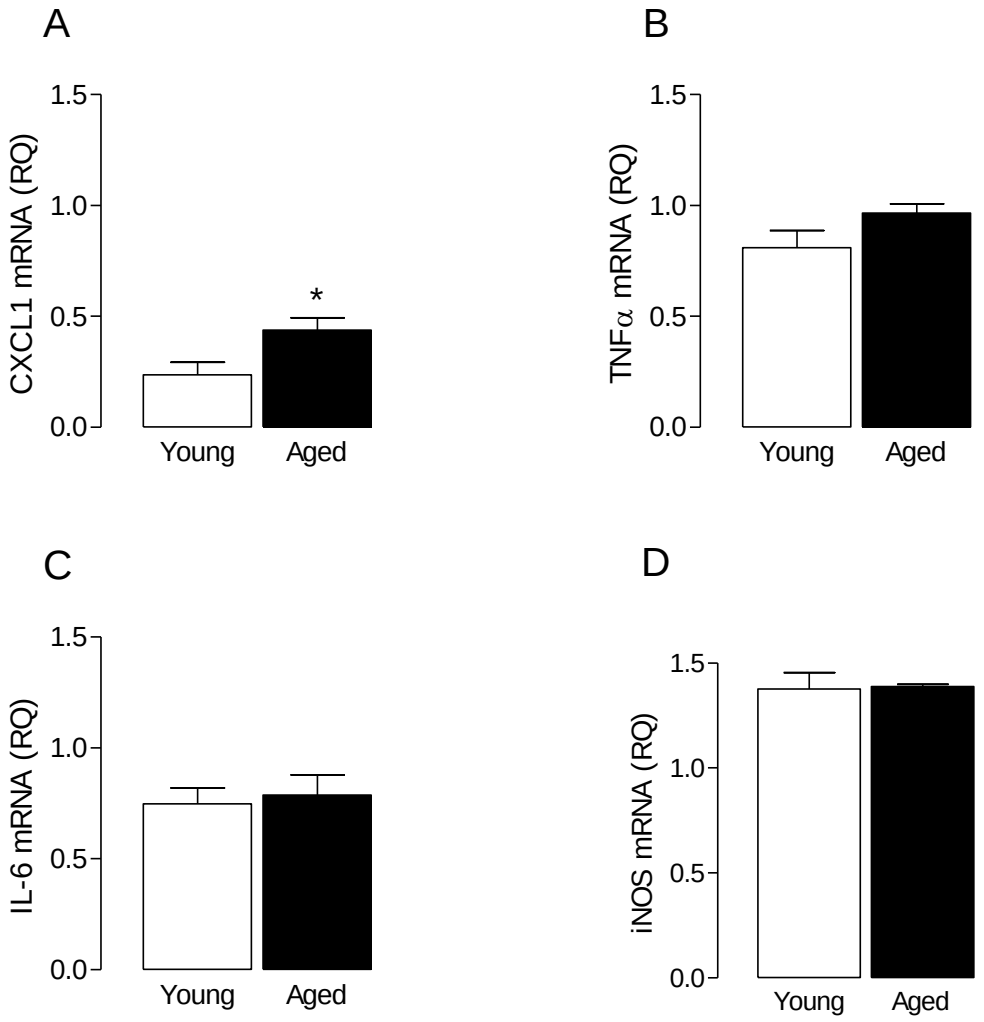

Figure 3.7 Plasma from aged mice increases CXCL1 mRNA expression in J774.2 cells.

J774.2 cells were treated with plasma from young (3m) and aged mice (18m) at a 1:5 dilution for 4 h and pro-inflammatory gene expression was examined by RT-PCR. The expression of CXCL1 mRNA was increased in cells treated with plasma from aged, compared with young, mice (A) and no changes in TNFα, IL-6 or iNOS expression was observed (B-D). Data are expressed as the mean ± SEM (n = 4-5). **p*<0.05 vs Young; Student's *t*-test for independent means.

	IQ-memory consistent	IQ-memory discrepant
N	13	13
Age (years; mean ± SD)	63.30 ± 7.16	63.23 ± 5.9
Sex (F/M)	10/3	10/3
MMSE	29.15 ± 1.14	29.15 ± 0.41
MoCA	27.54 ± 1.71	28.15 ± 1.41
Premorbid IQ z-score	0.96 ± 0.41	1.05 ± 0.22
Delayed verbal recall z-score	0.72 ± 0.54	-0.74 ± 0.31***
Cognitive asymmetry z-score	0.24 ± 0.43	1.83 ± 0.34***

Table 3.1 Demographic of IQ-memory consistent and IQ-memory discrepant participants.

Data are expressed as mean ± SD, n=13; ***p<0.001 vs. IQ-memory consistent; Student's t-test for independent means.

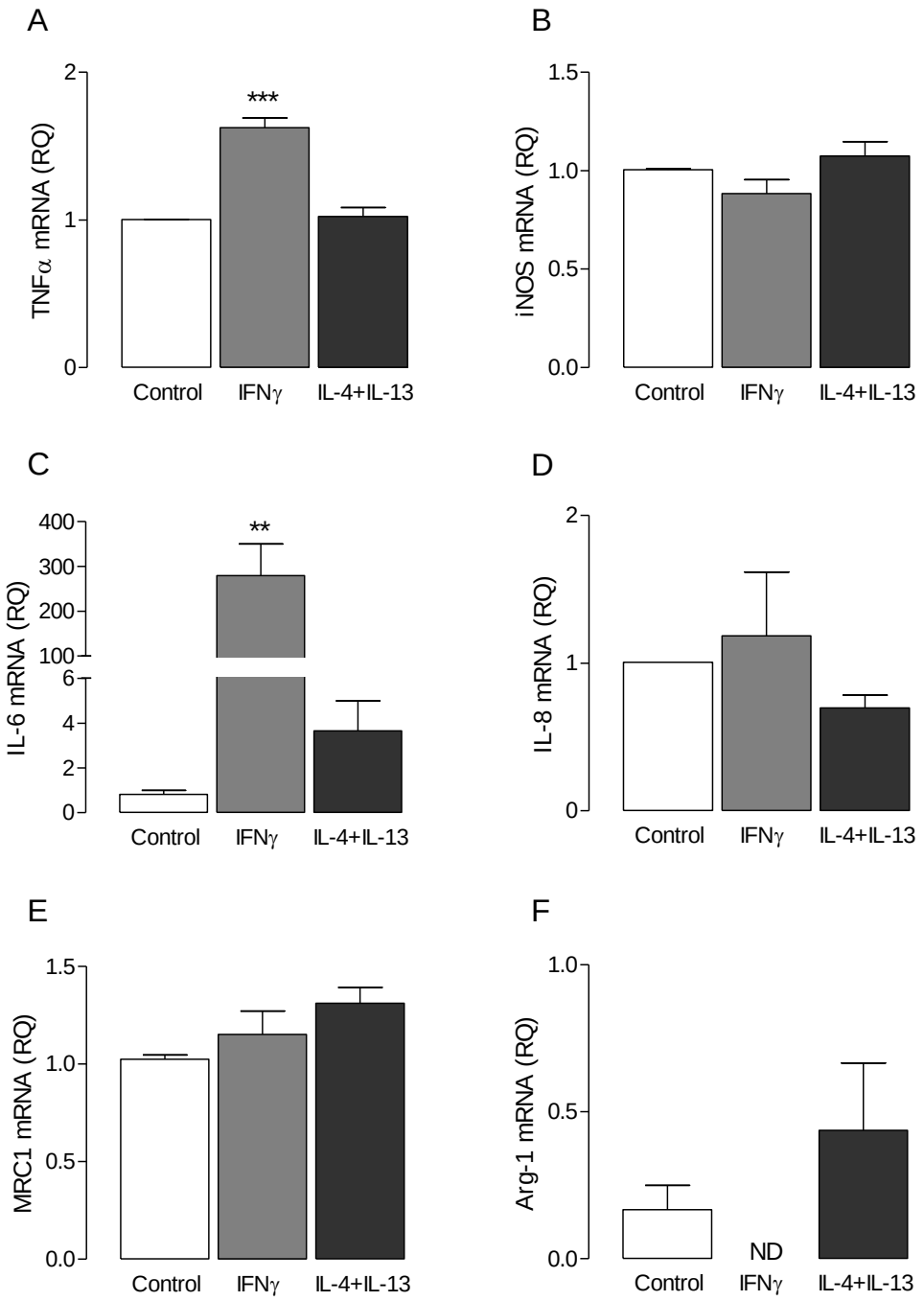

Figure 3.8 IFNγ polarises THP-1 cells to a pro-inflammatory phenotype.

THP-1 cells were incubated with or without IFNγ (20 ng/ml) or IL-4 (10 ng/ml) and IL-13 (10 ng/ml) for 4 h. Cells were harvested and mRNA expression was assessed by RT-PCR. IFNγ significantly upregulated mRNA expression of TNFα (A) and IL-6 (C). iNOS (B) and IL-8 (D) mRNA expression was unchanged in IFNγ or IL-4 + IL-13 treated cells. IL-4 did not significantly increase the expression of MRC-1 (E) and Arg-1 (F). Data are expressed as the mean ± SEM (n = 3). ***p*<0.01; ****p*<0.001 vs control; One-way ANOVA with Dunnett's post-hoc analysis. ND = not detected.

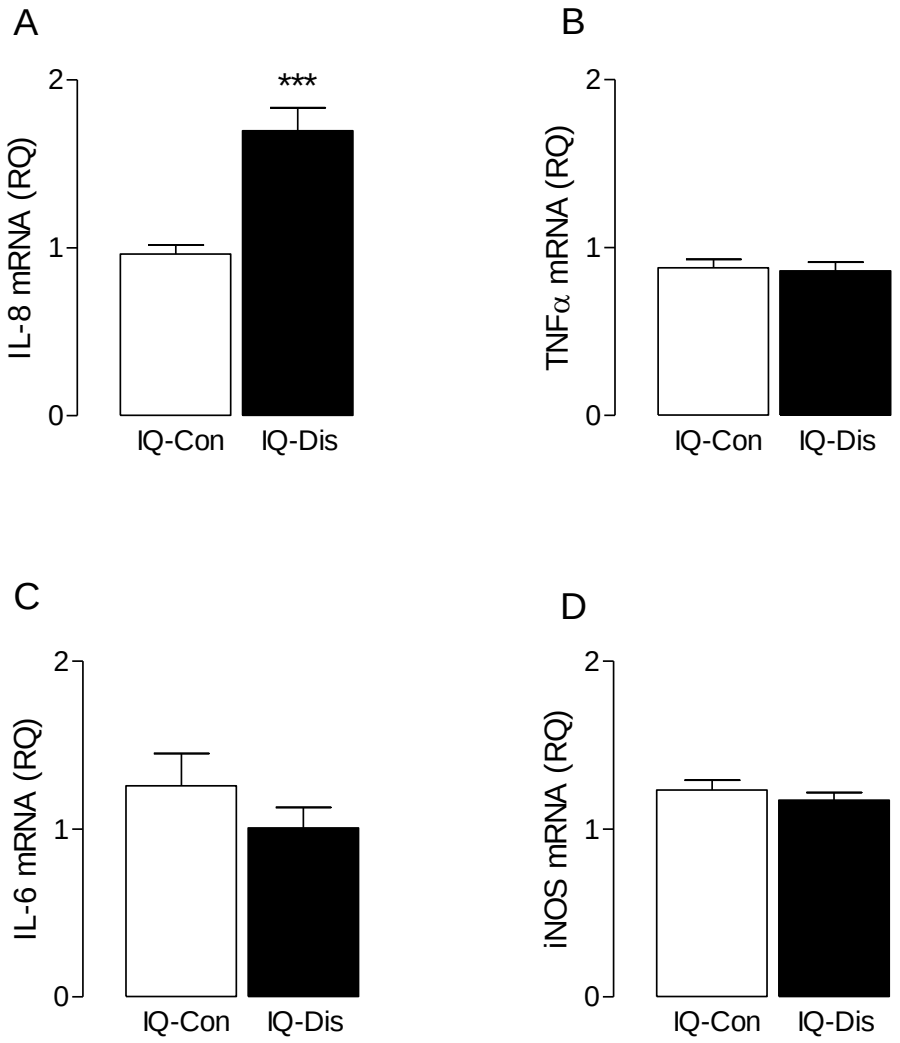

Figure 3.9 IL-8 mRNA is increased in THP-1 cells in response to plasma from IQ-discrepant individuals.

THP-1 cells were treated with or without plasma from IQ-consistent and IQ-discrepant participants (IQ-Con, IQ-Dis) at a 1:40 dilution for 4 h and analysis of pro-inflammatory marker expression performed using RT-PCR. Plasma from IQ-Dis participants significantly increased IL-8 mRNA in THP-1 cells compared with IQ-Con participants (A). No difference in TNFα (B), IL-6 (C) or iNOS (D) mRNA were observed. Data are expressed as the mean ± SEM, n = 13 for groups; ***p<0.001 vs IQ-Con; Student's t-test for independent means.

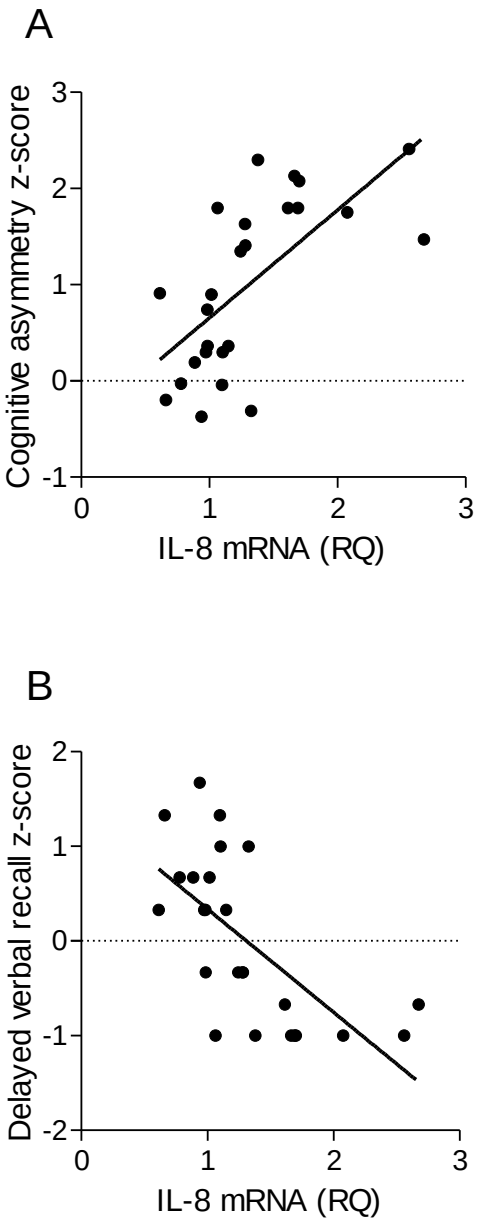

Figure 3.10 IL-8 mRNA correlates with measures of cognitive function.

Linear regression analysis of IL-8 mRNA RQ values shows a positive correlation with overall cognitive asymmetry z-score (A) when memory performance is predicted on an estimation of IQ (r=0.4335, *p*=0.0003). Linear regression analysis of IL-8 mRNA RQ values shows a negative correlation with delayed word recall on the WMS (B; r=0.4277, *p*=0.0004).

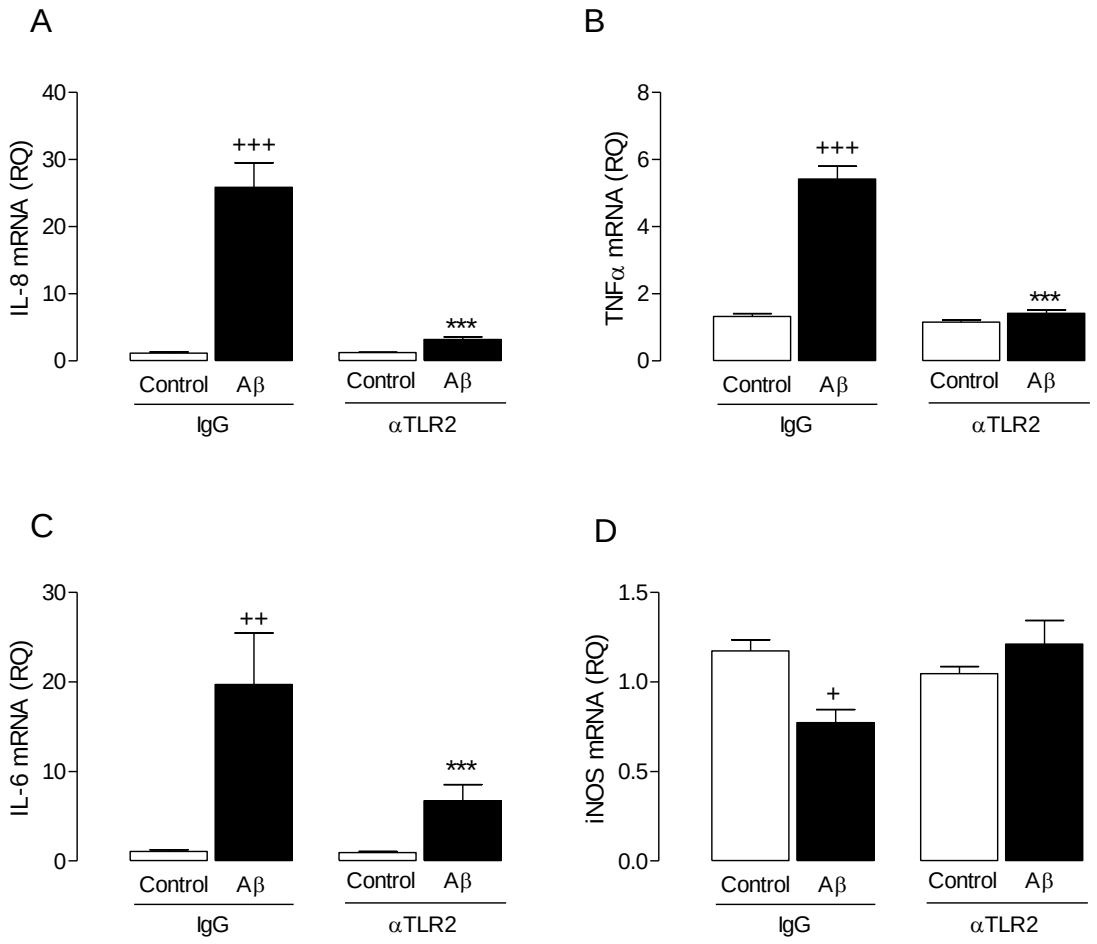

Figure 3.11 αTLR2 attenuates the Aβ induced increase in IL-8, TNFα and IL-6 mRNA expression THP-1 cells.

THP-1 cells were treated with either IgG or αTLR2 (2.5 μg/ml) for 30 min prior to incubation with Aβ (10 μM) for 4 h and pro-inflammatory marker expression examined by RT-PCR. Aβ increased the expression of IL-8, TNFα and IL-6 mRNA; this was attenuated by αTLR2 pre-treatment (A, B, C). The expression of iNOS decreased with Aβ stimulated and αTLR2 pre-treatment also attenuated this effect (D). Data are expressed as the mean ± SEM (n = 1, replicates = 8). **p*<0.05, ****p*<0.001 vs Aβ IgG; ^{+}p<0.05, ^{++}p<0.01, ^{+++}p<0.001 vs Control IgG; Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $A\beta_{effect}$ [$F_{(1,23)}$=49.02, p<0.001], $\alpha TLR2_{effect}$ [$F_{(1,23)}$=35.18, p<0.001], $interaction_{effect}$ [$F_{(1,23)}$=35.59, p<0.001]. **B:** $A\beta_{effect}$ [$F_{(1,28)}$=115.4, p<0.001], $\alpha TLR2_{effect}$ [$F_{(1,23)}$=106.4, p<0.001], $interaction_{effect}$ [$F_{(1,23)}$=89.34, p<0.001]. **C:** $A\beta_{effect}$ [$F_{(1,25)}$=17.72, p<0.001], $\alpha TLR2_{effect}$ [$F_{(1,25)}$=5.101, p<0.0329], $interaction_{effect}$ [$F_{(1,25)}$=4.856, p<0.037]. **D:** $A\beta_{effect}$ [$F_{(1,24)}$=1.639, p<0.2127], $\alpha TLR2_{effect}$ [$F_{(1,24)}$=2.821, p<0.1060], $interaction_{effect}$ [$F_{(1,24)}$=9.359, p<0.0054].

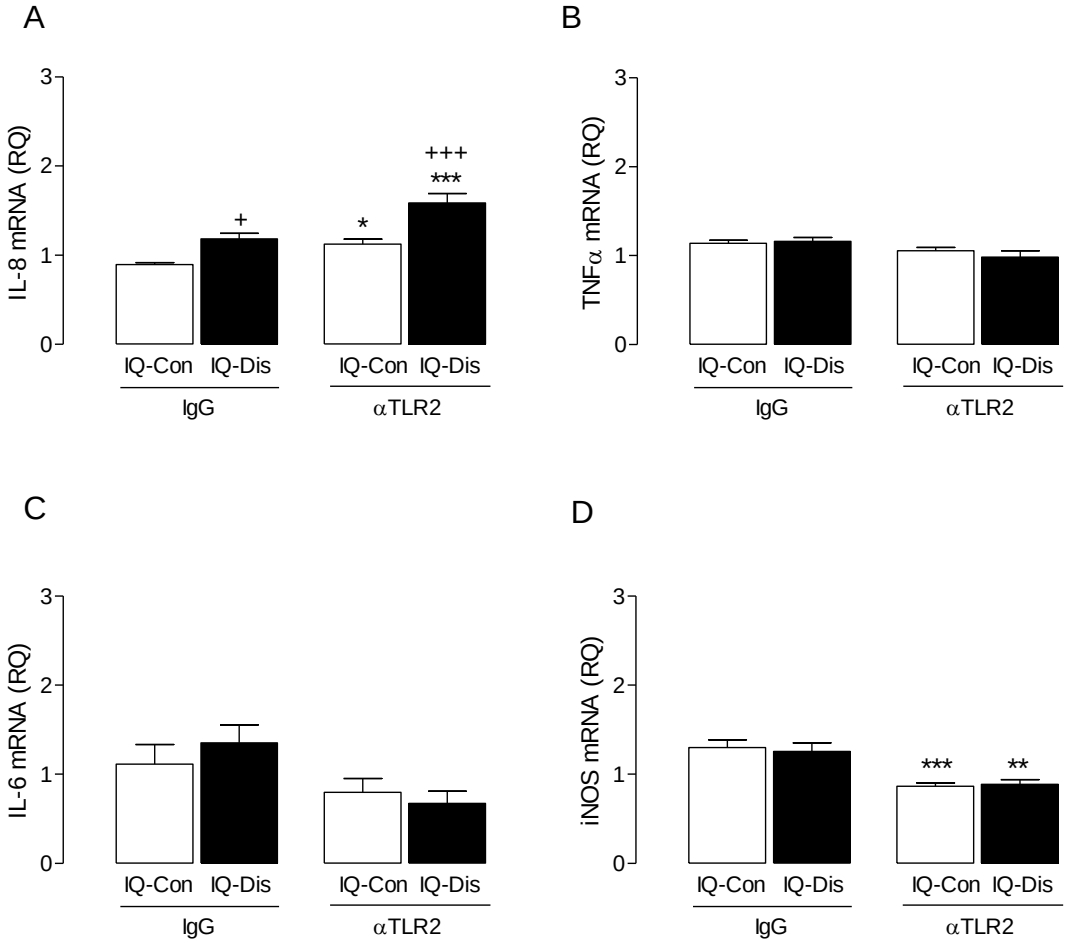

Figure 3.12 αTLR2 further increases the plasma induced increase in IL-8 mRNA expression from IQ-discrepant individuals in THP-1 cells.

THP-1 cells were treated with either IgG or αTLR2 (2.5 μg/ml) for 30 min prior to incubation with plasma (1:40) from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) subjects for 4 h and pro-inflammatory marker expression examined by RT-PCR. Plasma from IQ-Dis participants induced an increase in IL-8 mRNA compared with plasma from IQ-Con participants; this was further increased by αTLR2 pre-treatment (A). TNFα and IL-6 mRNA expression remained unchanged (B, C). The expression of iNOS was decreased with αTLR2 pre-treatment, although no differences between participant groups were observed (D). Data are expressed as the mean ± SEM (n = 13). **p*<0.05, ***p*<0.01, ****p*<0.001 vs vehicle control; ^{+}p<0.05, ^{+++}p<0.001 group control; Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,37)}$=28.43, p<0.001], $\alpha TLR2_{effect}$ [$F_{(1,37)}$=20.16, p<0.001], $interaction_{effect}$ [$F_{(1,37)}$=0.2214, p<0.001]. **B:** $Group_{effect}$ [$F_{(1,46)}$=0.2464, p<0.6220], $\alpha TLR2_{effect}$ [$F_{(1,46)}$=7.206, p<0.0101], $interaction_{effect}$ [$F_{(1,46)}$=0.8995, p<0.3479]. **C:** $Group_{effect}$ [$F_{(1,41)}$=0.1095, p<0.7424], $\alpha TLR2_{effect}$ [$F_{(1,41)}$=7.583, p<0.0087], $interaction_{effect}$ [$F_{(1,41)}$=1.023, p<0.3178]. **D:** $Group_{effect}$ [$F_{(1,43)}$=0.01973, p<0.8890], $\alpha TLR2_{effect}$ [$F_{(1,43)}$=29.85, p<0.001], $interaction_{effect}$ [$F_{(1,43)}$=0.1761, p<0.6768].

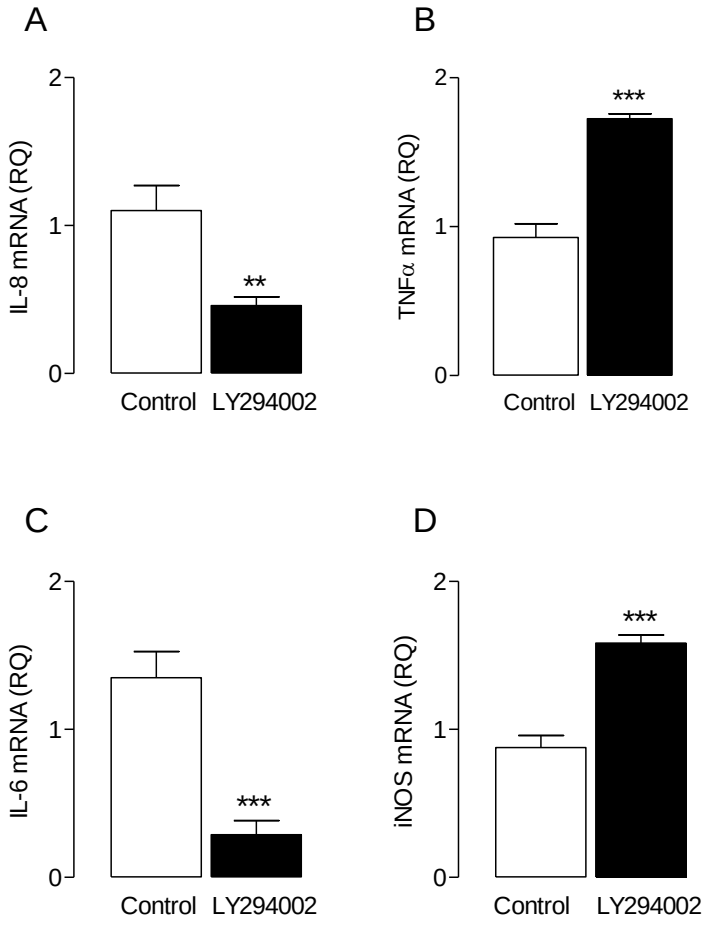

Figure 3.13 LY294002 decreases mRNA expression of IL-8 and IL-6 and increases the expression of TNFα and iNOS in THP-1 cells.

THP-1 cells were treated with or without LY294002 (10 μM) for 4 h and pro-inflammatory marker expression examined by RT-PCR. Treatment with LY294002 significantly decreased IL-8 (A) and IL-6 (C) mRNA expression, while the expression of TNFα (B) and iNOS (D) were significantly increased. Data are expressed as the mean ± SEM (n = 1, replicates = 8). ***p*<0.01, ****p*<0.001; Student's *t*-test for independent means.

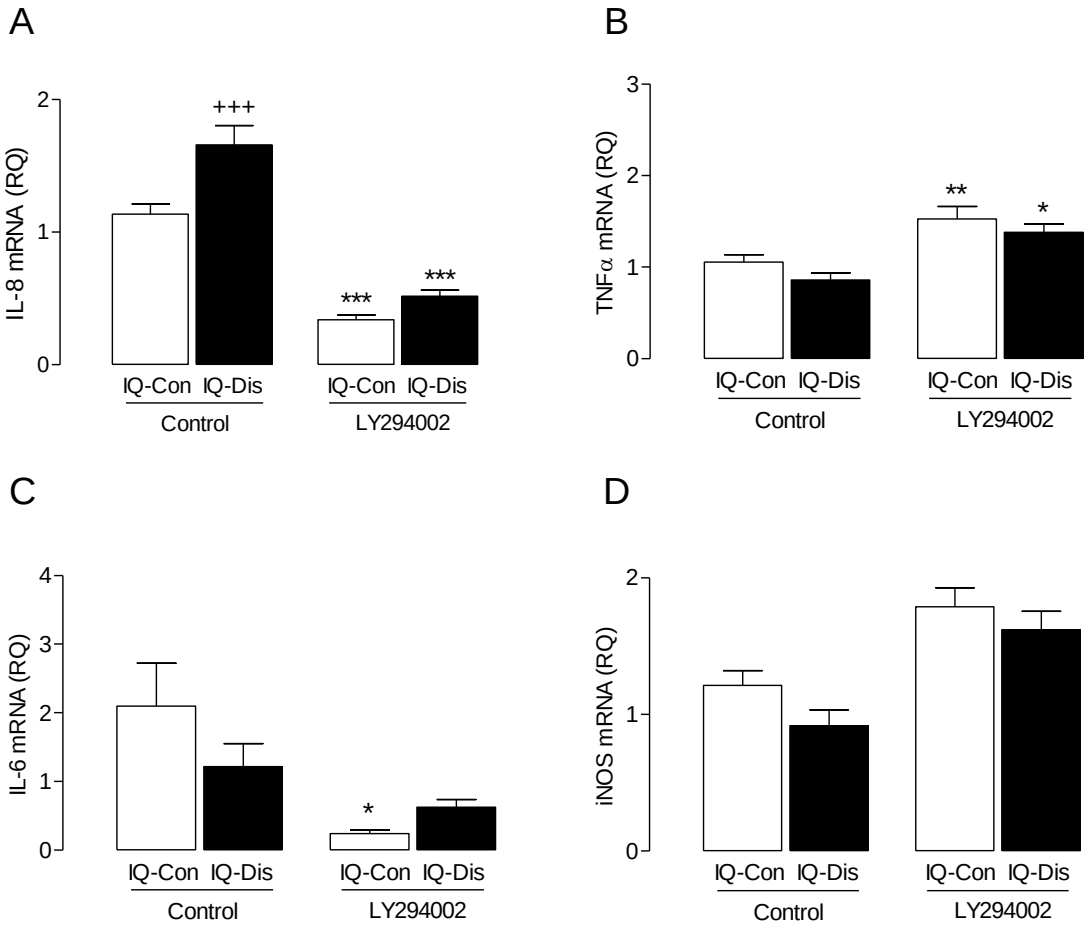

Figure 3.14 LY294002 attenuates the plasma induced increase in IL-8 mRNA expression in THP-1 cells.

THP-1 cells were treated with or without LY294002 (10 μM) for 30 min prior to incubation with plasma (1:40) from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) subjects for 4 h and pro-inflammatory marker expression examined by RT-PCR. Plasma from IQ-Dis participants induced an increase in IL-8 mRNA compared with plasma from IQ-Con participants; this was attenuated by LY294002 pre-treatment (A). LY294002 increased the expression of TNFα and decreased the expression of IL-6, although no differences between participant groups were observed (B, C). No significant differences in iNOS mRNA expression were observed (D). Data are expressed as the mean ± SEM (n = 13). **p*<0.05, ***p*<0.01, ****p*<0.001 vs vehicle control; ^{+}p<0.05, ^{+++}p<0.001 vs group control; Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,46)}$=15.28, p<0.001], $LY294002_{effect}$ [$F_{(1,46)}$=116.9, p<0.001], $interaction_{effect}$ [$F_{(1,46)}$=3.736, p<0.0594]. **B:** $Group_{effect}$ [$F_{(1,43)}$=2.993, p<0.0908], $LY294002_{effect}$ [$F_{(1,43)}$=26.22, p<0.001], $interaction_{effect}$ [$F_{(1,43)}$=0.06593, p<0.7986]. **C:** $Group_{effect}$ [$F_{(1,29)}$=0.3476, p<0.5600], $LY294002_{effect}$ [$F_{(1,29)}$=8.383, p<0.0071], $interaction_{effect}$ [$F_{(1,29)}$=2.229, p<0.1462]. **D:** $Group_{effect}$ [$F_{(1,42)}$=3.298, p<0.0765], $LY294002_{effect}$ [$F_{(1,42)}$=25.19, p<0.001], $interaction_{effect}$ [$F_{(1,42)}$=0.2405, p<0.6264].

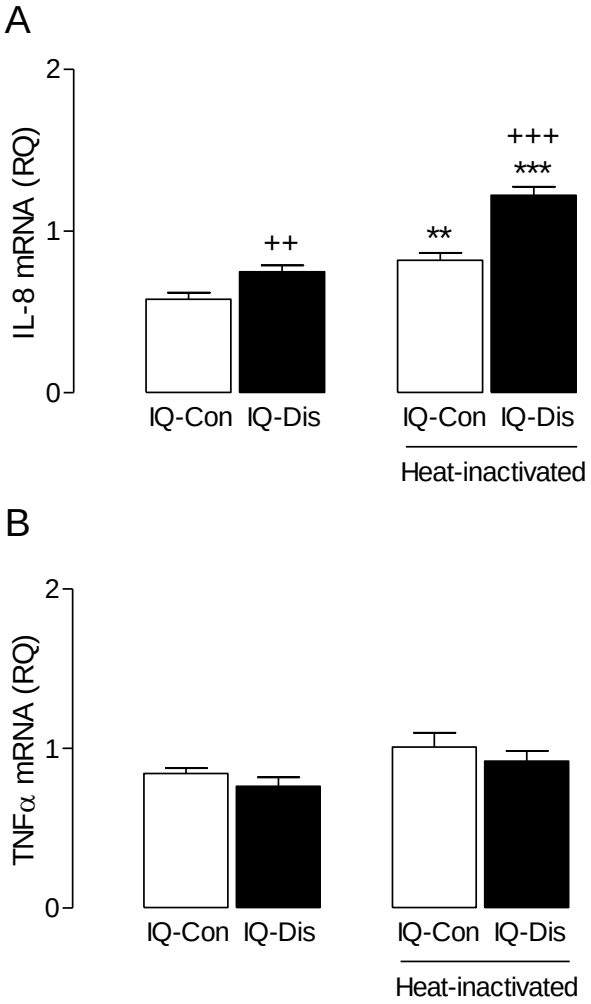

Figure 3.15 Heat-inactivation further increases the plasma induced increase in IL-8 gene expression in THP-1 cells from IQ-discrepant participants.

Plasma from IQ-discrepant participants (IQ-Dis) significantly increased IL-8 mRNA in THP-1 cells in comparison to IQ-consistent participants (IQ-Con); this was further increased with heat-inactivated plasma (A). No difference in the expression of TNFα was seen between IQ-consistent and IQ-discrepant participants in THP-1 cells in response to plasma and heat-inactivated plasma (B). Data are expressed as mean ± SEM, n = 13 for groups. **p<0.01, ***p<0.001 vs vehicle control; ^{++}p<0.01, ^{+++}p<0.001 vs group control; Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,48)}$=40.42, p<0.001], $heat\text{-}activated_{effect}$ [$F_{(1,48)}$=62.91, p<0.001], $interaction_{effect}$ [$F_{(1,48)}$=6.5, p<0.001]. **B:** $Group_{effect}$ [$F_{(1,48)}$=1.75, p<0.1921], $heat\text{-}inactivated_{effect}$ [$F_{(1,48)}$=6.52, p<0.0139], $interaction_{effect}$ [$F_{(1,48)}$=0.0078, p<0.9299].

3.4 Discussion

The data from this study show that there is an age-related neuroinflammatory phenotype, which is reflected in the periphery. Specifically, the data show that there is an increase in the expression of inflammatory cytokines in the hippocampus of aged mice; that this is associated with infiltrating immune cells; and that these changes are accompanied by an increase in circulating cytokines. Importantly the data show that incubating a monocyte cell line, J774.2 cells, with plasma from aged mice upregulates CXCL1 mRNA. Extrapolating this to a clinical situation, it was found that plasma from participants described as having subtle cognitive dysfunction increased IL-8 mRNA in monocytes compared with plasma from cognitively intact individuals. This signal in monocytes could potentially represent a marker of an underlying neuroinflammatory phenotype and cognitive decline.

Age-related changes in circulating cytokines have not been extensively documented in mice, although the response to systemic challenges and infection has repeatedly shown an increase in peripheral cytokines (Skelly et al., 2013). In the present study, age-dependent increases in the plasma concentrations of pro-inflammatory mediators CXCL1, IL-1β and TNFα, as well as the anti-inflammatory cytokine IL-10, were found in C57/BL6 mice. Circulating concentrations of TNFα have previously been found to be increased with age (Bruunsgaard et al., 1999), although other studies have found no difference (Petersen et al., 1994, Elahy et al., 2015). The data from the present study also show an increase in the level of IL-6, which ranged from 0 pg/ml in young mice to 7.8-20.6 pg/ml in aged mice, although statistical analysis could not be appropriately performed due to that fact that levels of IL-6 were undetectable in all of the young mice. Circulating concentrations of IL-6 have been consistently found to be increased with age (Franceschi et al., 2000, Forsey et al., 2003, Stowe et al., 2010, Elahy et al., 2015); however there are some reports where no change has been found (Petersen et al., 1994). Conversely, the concentration of systemic IL-10 has not previously been associated with age (Petersen et al., 1994, Forsey et al., 2003, Stowe et al., 2010).

It is now well established that breakdown of the BBB occurs during aging, most likely due to an attenuation in the expression of tight junction proteins such as claudin-5 and occludin-1 (Elahy et al., 2015). This may contribute to an infiltration of immune cells from the periphery (Blau et al., 2012), that could possibly further exacerbate the pro-inflammatory environment in the brain. Here the findings indicate a significant increase in the percentage of macrophages and neutrophils, and no difference in the percentage of microglia, was observed in aged, compared with young, mice. This is consistent with previous reports of increased infiltration of $CD45^+$ cells in the brains of aged rats (Blau et al., 2012, Barrett et al., 2015a) Significant increases in infiltrating cells are seen following trauma and in neurodegenerative disorders, with an influx of neutrophils following ischemic stroke (Moraga et al., 2015) and an increase in infiltrating bone-marrow derived microglia in AD (Simard et al., 2006). Neutrophils have also been found to be present in the brains of AD patients (Zenaro et al., 2015, Pietronigro et al., 2016). The impact of the presence of infiltrating cells has also been examined in AD mouse models. Neutrophil trafficking in the CNS has been detected in two different models of AD (5xFAD and 3xTg) and depletion or inhibition of neutrophil infiltration improved cognitive impairment (Zenaro et al., 2015). The APP/PS1 mouse model of AD has also shown an increase in infiltrating neutrophils and macrophages in comparison to WT mice (Minogue et al., 2014).

Interestingly, pro-inflammatory cytokines including TNFα and IL-1β have been shown to regulate the expression of tight junction proteins (Elahy et al., 2015). It is plausible that the observed increase in circulating IL-1β and TNFα negatively impacted on tight junction protein expression, resulting in an increase in BBB permeability and leading to the rise in macrophage and neutrophil populations within the CNS. This is consistent with the previous finding that plasma from APP/PS1 mice decreases claudin-5, occludin and zona occludens (ZO)-1 expression in a mouse endothelial cell line (Barrett et al., 2015b).

Another explanation for the marked increase in macrophage and neutrophil CNS infiltration is the increased mRNA expression of chemokines, CXCL1 and CCL2, in the hippocampus of aged mice. Chemokines are produced by cells of the brain and

periphery in order to recruit immune cells to inflammatory sites (Turner et al., 2014). CXCL1 and CCL2 are the primary chemoattractants for neutrophils and monocytes respectively. An increase in CCL2 has been previously reported in the hippocampus of aged rats (Blau et al., 2012, Barrett et al., 2015a) and has been implicated in the infiltration of macrophages into the brain parenchyma. A study by Felzien and colleagues (2001) examined mRNA expression of chemokines in young (4m) and aged (30m) C57BL6/DBA2 mice and found an increased expression of CCL3 and CCL4 mRNA in the hippocampus, cerebellum, cortex and midbrain of aged mice. CCL5 was also found to be increased in the cerebellum, but no increases in CCL2 expression were reported in any brain region. They also observed that the increase in chemokine expression occurred concurrently with an increase in microglial activation. In the present study, an increase in the expression of CCL3 and CCL5 was observed although this did not reach statistical significance. Interestingly, the expression of chemokine receptors CCR1 and CXCR2 was increased on microglia from aged mice, which are the receptors for CCL3/CCL5 and CXCL1 respectively. This could perhaps be as a result of the increase in chemokine expression in the hippocampus. The cell responsible for the upregulation in chemokine expression is unclear, as astrocytes, endothelial cells and microglia have all been shown to synthesize chemokines (Felzien et al., 2001). In a ME7 model of prion disease, production of CCL2 and CXCL1 was increased by astrocytes in response to TNFα and IL-1β injection, which occurred alongside an increase in infiltrating neutrophils (Hennessy et al., 2015). Another study reported that administration of IL-1β resulted in an influx of neutrophils in juvenile, but not adult, rats, which was attributed to differential chemokine expression including CXCL1 (Campbell et al., 2002). Here, an increase in hippocampal CXCL1 gene expression was observed in aged mice and could have therefore led to the observed increase in infiltrating neutrophils.

Normal ageing in mice is associated with significant age-dependent cognitive impairments (Shoji et al., 2016) which has been linked to increased hippocampal gliosis (Weber et al., 2015). It is possible to thoroughly explore the CNS environment in animal models, however in order to investigate this clinically it is necessary to develop a proxy measure in the periphery that is reflective of the brain environment

and cognitive state. Such biomarkers have been investigated primarily by measuring concentrations of peripheral inflammatory mediators, however studies have not yet provided a clear signature that is indicative of early cognitive dysfunction. As an alternative approach, it is possible to analyse the effect of plasma on gene expression in cell lines, which might provide a simple biomarker assay that can be carried out efficiently and reliably.

In an effort to model this in mice, the effect of plasma was assessed on J774.2 cells, a mouse monocyte cell line. In parallel, plasma from a cohort of healthy older adults exhibiting a subtle cognitive deficit was examined on the human monocyte cell line, THP-1 cells.

THP-1 cells were established from the blood of a boy with acute monocytic leukaemia (Tsuchiya et al., 1980) and J774.2 cells are derived from BALB/L mice. There are a variety of alternative monocyte cell lines including U937, HL-6p and RAW 264.7, yet THP-1 and J774.2 cells are generally chosen as they are highly plastic and sensitive to many stimuli (Aldo et al., 2013). The use of cells lines over primary cells from humans and mice has several advantages. Arguably most importantly, the degree of variability in assays is minimised due to their homogenous genetic background. They can also be stored for long periods of time without any obvious detrimental effects on cell function or viability and are easily maintained in a laboratory (Qin, 2012). However it is important to keep in mind that cell lines differ from their primary counterparts as they are derived from immortalised or transformed cells and have acquired different genetic and phenotypic differences (Andreu et al., 2017).

The delayed verbal recall assessed by the WMS measures discrete changes in episodic memory, which is a cognitive domain central to ageing and has been identified as the first, and most severely, domain affected in AD and is also an important marker for MCI (Rabin et al., 2009). In a previous study examining a separate group of healthy older adults, classified as either IQ-consistent or IQ-discrepant, LPS stimulation induced an exacerbated release of pro-inflammatory cytokines in MDMs from IQ-discrepant, compared with IQ-consistent, individuals, as

well as an increased expression of TLR2, TLR4 and CD11b at both the cell surface and mRNA level (Downer et al., 2013). The current study provides further evidence that blood-based differences are apparent in a different cohort of individuals classified in the same manner.

THP-1 and J774.2 cells were characterised in terms of their ability to adopt a pro- and anti-inflammatory-like phenotype in response to polarising cytokines, IFNγ or IL-4 (and IL-13). In agreement with the literature (Jones et al., 2015b), J774.2 cells were polarised to a pro-inflammatory phenotype by IFNγ as seen by the upregulation in TNFα, IL-6 and iNOS mRNA expression. Conversely, cells exhibited an anti-inflammatory activation state in response to IL-4 as seen by the increase in MRC-1 and Arg-1 expression. The differential metabolism of L-arginine has been one of the defining features of the original, though outdated, M1 and M2 classification of activated macrophages (Italiani and Boraschi, 2014). IL-4 promotes arginase-dependent formation of L-ornithine which leads to fibroblast proliferation and collagen production aiding tissue modelling and repair, while iNOS expression is upregulated in response to pro-inflammatory stimuli to convert L-arginine to NO to enhance pathogen clearance (Gordon, 2003). Surprisingly, the expression of CXCL1 was significantly decreased with IFNγ, an effect which contrasts with the literature; the CXCL1 gene has been reported to be induced by various other stimulants such as LPS, TNFα and IL-1β (Kim et al., 2005).

In contrast to J774.2 cells, THP-1 cells are not readily adherent and usually require a differentiating stimulus such as phorbol-12-myristate-12-acetate (PMA) to differentiate to macrophages. PMA-differentiated THP-1 cells are generally used to study macrophage function, however, THP-1 cells are frequently differentiated by excessive concentrations of PMA that result in the upregulation of genes that are expressed during the differentiation process, which can overwhelm the effect of any additional stimulus (Park et al., 2007). In light of this, the current study aimed to investigate the effect of pro- and anti-inflammatory stimuli in undifferentiated THP-1 cells. IFNγ significantly upregulated expression of pro-inflammatory markers TNFα and IL-6 while, although IL-4 and IL-13 increased the expression of MRC-1 and Arg-1, this was not statistically significant. These findings are in line with other studies,

which have shown that PMA-differentiated THP-1 cells upregulate TNFα, IL-12p40, IL-1β, IL-6 and IL-8 in response to IFNγ and LPS stimulation, as well as an increase in MRC-1 following incubation with IL-4 (Chanput et al., 2013). This was observed following 6 hours of treatment, whereas the findings presented here are after 4 hours of exposure. Similar to J774.2 cells, THP-1 cells do not exhibit any increases in IL-8 expression in response to IFNγ. Mice do not possess a direct homologue of IL-8, but CXCL1 has found to be a functional homologue of human IL-8 and shares approximately 70% sequence identity with it (Kim et al., 2005). Interestingly the expression of iNOS was not altered in response to IFNγ in THP-1 cells and the expression of Arg-1 was not well defined, demonstrating that this feature of pro/anti-inflammatory classification does not translate very well from mouse to humans.

Plasma from aged mice induced a significant upregulation of CXCL1 mRNA in J774.2 cells, while the expression of other pro-inflammatory markers was unaffected. Similarly, a specific increase in IL-8 mRNA was seen in THP-1 cells in response to plasma from IQ-discrepant, compared with IQ-consistent, individuals. The extent of IL-8 response in THP-1 cells following exposure to plasma from both IQ-consistent and IQ-discrepant individuals negatively correlated with delayed verbal recall as measured by the WMS and positively correlated with overall cognitive asymmetry score (delayed verbal recall in relation to estimated IQ). The data suggest that the upregulation of IL-8 (and CXCL1) mRNA may represent a novel biomarker assay for the detection of early cognitive dysfunction.

The analysis of the effect of plasma on cell culture model systems has been used previously to develop biomarkers and examine disease mechanisms. One such study examined the effect of plasma from patients with heart failure on angiogenesis in a human endothelial cell line (Pannella et al., 2016) and showed that plasma from patients, compared with controls, promoted sprouting angiogenesis and dysregulated Notch signalling in cells. Another study employing human cardiac myocytes found that serum from patients with septic shock induced apoptosis (Kumar et al., 2005). The current data presented indicate that there are factors present in the blood of IQ-discrepant individuals that stimulate the upregulation of

IL-8 mRNA that are not present in the blood of IQ-consistent individuals. This difference might provide an insight into disease mechanisms of early cognitive decline.

Plasma is comprised of large and small protein molecules, peptides, lipids and metabolites that emulate the physiological activity of an individual peripherally and centrally, and also reflects any existing pathology (Song et al., 2009). IL-8 is primarily a chemoattractant for neutrophils, and potent stimulators of IL-8 include TLR agonists such as LPS and Pam3CSK4, as well as Aβ and pro-inflammatory cytokines such as TNFα and IL-1β (Thornton et al., 2012). The fact that neither CXCL1 nor IL-8 mRNA was increased in IFNγ stimulated cells suggests that the response to plasma is not attributable to the presence of IFNγ in plasma. Indeed, IFNγ was below levels of detection in plasma from both young and aged animals. Higher concentrations of TNFα and IL-1β were observed in the plasma of aged animals, and could therefore be responsible for the upregulation of CXCL1 in J774.2 cells.

In order to further investigate the potential plasma-borne factor(s) stimulating IL-8 mRNA in THP-1 cells, TLR2 and PI3K were inhibited in cells prior to exposure to plasma. Stimulation of THP-1 cells with Aβ upregulated IL-8 expression and this was significantly attenuated by TLR2 inhibition, demonstrating that Aβ is partially signalling through this receptor which is consistent with previous reports (Costello et al., 2015). However, IL-8 mRNA was increased in response to plasma from both IQ-consistent and IQ-discrepant participants in cells pre-treated with αTLR2, suggesting that the plasma-borne factor(s) is not signalling through TLR2, and is also unlikely to be Aβ. IL-8 expression in THP-1 cells was decreased by PI3K inhibition in line with previous findings (Wang et al., 2015), although the response to plasma from IQ-discrepant participants was not decreased to the same extent as plasma from IQ-consistent individuals. Since heat-inactivated plasma increased IL-8 it must be concluded that the factor present in the plasma inducing IL-8 mRNA is not a proteinaceous substance.

The composition of plasma changes dramatically as we age, and the ability of the blood to regulate and impact on cognitive function and neurogenesis has been

demonstrated in a series of experiments employing heterochronic parabionts, in which the circulatory systems of young and old mice are connected. It was found that exposing a young mouse to plasma from an old mouse decreased synaptic plasticity and spatial learning and memory, which was attributed to increases in circulating chemokines including CCL11 (Villeda et al., 2011). Furthermore, exposing an old mouse to young plasma was sufficient to increase hippocampal synaptic plasticity and improve age-related cognitive impairments which was mediated partly by cyclic AMP response element binding protein (Villeda et al., 2014). β_2-microglobulin was later identified as a factor in the plasma of aged mice and young heterochronic parabionts that promotes age-related cognitive dysfunction and impairs neurogenesis (Smith et al., 2015), and plasma levels of tissue of metalloproteinases 2 (TIMP2) was also shown to confer a benefit on cognitive function in aged animals (Castellano et al., 2017). It is possible that these factors might have a role to play in the plasma induced effect on monocyte cell lines; further examination of the plasma from mice and IQ-consistent and IQ-discrepant participants is needed to determine this.

Taken together, the data presented in this study demonstrate that plasma from aged mice and IQ-discrepant participants display a specific upregulation in CXCL1/IL-8 mRNA in monocyte cell lines, which could be a valuable indicator of cognitive decline.

Chapter 4: IL-8 mRNA and glycolytic flux in plasma-treated monocytes as a biomarker of cognitive dysfunction

4.1 Introduction

Cognitive dysfunction in the elderly is a prominent feature of neurodegeneration most commonly as a result of AD and vascular dementia, but can also be due to frontotemporal dementia, Lewy body disease or PD (Sonnen et al., 2008). MCI is a heterogeneous early stage of cognitive impairment and approximately 60% of MCI patients ultimately develop AD while others progress to other conditions including Lewy body dementia, vascular dementia or PD. Pronounced deficits in cognitive function occur late in the pathogenesis of disease where significant neuronal damage has already occurred (Aisen et al., 2017). Therefore it is imperative that a biomarker indicative of early cognitive dysfunction is developed for earlier treatment intervention and monitoring of disease progression.

Numerous neuroimaging techniques have been employed in the search for a biomarker for AD with some success, such as structural MRI to detect hippocampal atrophy and PET to image amyloid burden, and these biomarkers are now used to aid a clinical diagnosis of AD. CSF has been examined extensively and overall T-tau, P-tau and Aβ42 are strongly associated with MCI and AD (Olsson et al., 2016). However the identification of a blood-based biomarker is ideal as it overcomes the issue of feasibility, accessibility and cost associated with neuroimaging and CSF sampling (Snyder et al., 2014). In an attempt to parallel CSF biomarkers, Aβ and tau concentrations in plasma have been widely examined with some degree of variability reported, however a recent meta-analysis concluded that while plasma total-tau was found to be associated with AD, the levels of Aβ40 and Aβ42 were not (Olsson et al., 2016). Plasma concentrations of cytokines, phospholipids and other proteins have also been investigated as potential biomarkers for MCI and AD but low reproducibility between studies remains a problem and no clear signature has yet emerged that is suitable for clinical use.

It is now appreciated that monocyte and macrophage metabolism is reprogrammed in response to pro- or anti-inflammatory stimuli which gives rise to polarised activated states. Pro-inflammatory or M1-like macrophages exhibit an increase in glycolysis and lactate production in response to IFNγ or TLR ligands, while anti-

inflammatory or M2-like macrophages favour oxidative phosphorylation (Zhu et al., 2015). The increase in aerobic glycolysis is a means by which the cell can rapidly generate ATP, which is necessary to quickly mount an immune response and generate microbicidal activity (Biswas and Mantovani, 2012). On the other hand, oxidative phosphorylation is a much more efficient and sustainable form of glucose metabolism, and therefore prevails during tissue remodelling and repair. The switch to glycolysis in response to pro-inflammatory stimuli such as LPS can be induced by a number of mechanisms, including upregulation of iNOS expression which generates NO to inhibit mitochondrial respiration, and induction of HIF-1α which regulates the expression of several genes involved in glycolysis (Kelly and O'Neill, 2015). HIF-1α has also been shown to target the PFKFB3 gene, which induces a switch in the PFK2 enzyme to its more active ubiquitous form, u-PFK2, which is a rate-limiting step in glycolysis as it controls intracellular levels of the glycolytic intermediate F-2,6-BP (Rodriguez-Prados et al., 2010).

This recently described metabolic switch in macrophages provides another means of functionally assessing activation phenotypes of cells in response to environmental cues. The development of new technologies including the Seahorse Extracellular Flux Analyser allow for the measurement of pH and oxygen content of media in real-time, therefore giving an indication of glycolysis and oxidative phosphorylation respectively, and has been previously used to examine metabolic reprogramming of both human and mouse macrophages in response to LPS stimulation (Haschemi et al., 2012, Izquierdo et al., 2015).

The aims of this study are:

1. To examine the effect of plasma from WT and APP/PS1 mice on gene expression and metabolic profile in J774.2 cells.
2. To investigate the effect of plasma from MCI and AD patients on gene expression and metabolic profile in THP-1 cells.

4.2 Methods

To investigate if the upregulation of CXCL1 mRNA in J774.2 cells in response to plasma can be used as a proxy measure for cognitive impairment in neurodegenerative disease, blood samples were collected from WT and APP/PS1 mice, a mouse model of AD, as described in section 2.4.2. J774.2 cells were incubated in the presence or absence of plasma from WT and APP/PS1 mice as described in section 2.5. Cells were harvested and gene expression and metabolic profile assessed and described in sections 2.15 and 2.20.

AD (n=6) and MCI (n=17) patients with a mean age of 64.56 years were recruited from the BIOMARKAPD project at St. James's and St. Vincent's Hospitals, Dublin, and Bon Secours Hospital, Tralee, Kerry. Control participants (n=20) with a mean age of 65.03 years were recruited from the Santry Sports Centre, Dublin. Cognitive function was assessed by the MoCA and blood samples were processed and plasma isolated and stored as described in section 2.6.

To investigate if the upregulation of IL-8 mRNA in THP-1 cells in response to plasma can be used as an indicator of cognitive dysfunction in distinct cohorts, plasma from MCI, AD and control cohorts were incubated with THP-1 cells as described in section 2.13. Cells were harvested and gene expression and metabolic profile assessed and described in sections 2.15 and 2.20.

4.3 Results

4.3.1 Expression of pro-inflammatory markers and glycolysis are increased in J774.2 cells in response to plasma from APP/PS1 mice

To investigate if the upregulation of CXCL1 in J774.2 cells in response to plasma is evident in an animal model of age-associated neurodegenerative disease, the effect of plasma from WT and APP/PS1, a mouse model of AD, was examined. J774.2 cells were incubated with plasma from WT and APP/PS1 mice for 4 hours and pro-inflammatory marker expression was examined by RT-PCR and metabolic function was assessed using a Seahorse XF^e24 Analyser. The data show a significant increase in the mRNA expression of CXCL1, TNFα, IL-6 and iNOS (Figure 4.1 A, B, C, D, $p<0.05$, Student's *t*-test for independent means). An increase in the extracellular acidification rate (ECAR) was observed in cells incubated with plasma from APP/PS1, compared with WT, mice (Figure 4.2 A). Plasma from APP/PS1 mice significantly increased glycolytic capacity in J774.2 cells (Figure 4.2 C, $p<0.01$), and although an increase in glycolysis was observed this did not reach statistical significance (Figure 4.2 B). The ratio of oxygen consumption rate (OCR) to ECAR indicates the cells preference for either oxidative phosphorylation or glycolysis and no changes in the ratio of OCR:ECAR (Figure 4.2 D) were observed in J774.2 cells incubated with plasma from APP/PS1, compared with WT, mice. The data suggest that there are factors in the plasma of APP/PS1 mice that upregulate pro-inflammatory gene expression and glycolytic capacity in J774.2 cells.

4.3.2 IL-8 mRNA is upregulated in THP-1 cells in response to plasma from MCI and AD patients

Having demonstrated that plasma from APP/PS1 mice alters gene expression and metabolism in J774.2 cells, the effect of plasma from MCI and AD patients was assessed in THP-1 cells. Subjects were age- and gender- matched and a significant decrease in MoCA score from MCI patients compared with controls ($p<0.01$), and from AD patients compared with MCI patients and controls ($p<0.001$), was observed (Table 4.1).

THP-1 cells were incubated with plasma from either control, MCI or AD patients for 4 hours and pro-inflammatory marker expression examined by RT-PCR. The mRNA expression of IL-8 and IL-6 was significantly upregulated in response to plasma from MCI patients compared with controls (Figure 4.3 A, C $p<0.05$, $p<0.01$, Student's *t*-test). No changes were observed in TNFα and iNOS mRNA expression in cells incubated with plasma from MCI patients (Figure 4.3 B, D). Plasma from AD patients induced a significant increase in IL-8 and TNFα mRNA in THP-1 cells compared with controls (Figure 4.4 A, B, $p<0.01$, $p<0.001$, Student's *t*-test). THP-1 cells did not exhibit any difference in the mRNA expression of IL-6 and iNOS in response to plasma from AD patients (Figure 4.4 C, D).

4.3.3 Glycolysis is increased in THP-1 cells in response to plasma from AD, but not MCI, patients

A glycolytic stress test was carried out to examine the effect of plasma from MCI and AD patients on the metabolic signature of THP-1 cells. Cells were incubated with plasma from control, MCI or AD patients for 4 hours and metabolic function assessed using a Seahorse XFe24 Analyser. THP-1 cells did not show any increase in ECAR in response to plasma from MCI patients (Figure 4.5 A). Although analysis of the mean data show a trend towards an increase in glycolysis, no significant differences were observed (Figure 4.5 B). Similarly, no changes were seen in glycolytic capacity and OCR:ECAR in cells incubated with plasma from MCI patients (Figure 4.5 C, D). An increase in ECAR was observed in THP-1 cells incubated with plasma from AD patients compared with controls (Figure 4.6 A) and the data show a significant increase in glycolysis and glycolytic capacity (Figure 4.6 B, C, $p<0.05$, Student's *t*-test). Plasma from AD patients did not alter the ratio of OCR:ECAR in THP-1 cells compared with plasma from control participants (Figure 4.6 D).

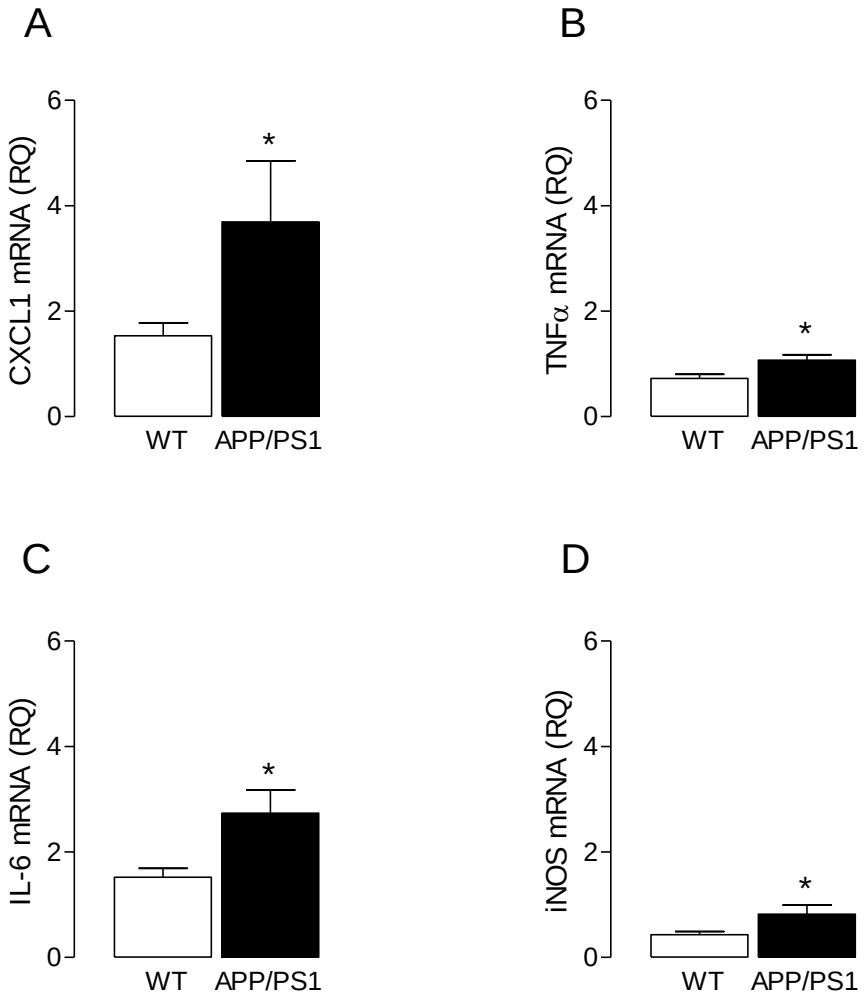

Figure 4.1 Expression of pro-inflammatory markers is increased in J774.2 cells in response to plasma from APP/PS1 mice.

J774.2 cells were incubated with plasma from either WT or APP/PS1 mice at a 1:10 dilution for 4 h and pro-inflammatory gene expression examined by RT-PCR. Plasma from APP/PS1 mice significantly upregulated CXCL1, TNFα, IL-6 and iNOS mRNA (A, B, C, D) in J774.2 cells compared with WT plasma samples. Data are expressed as the mean ± SEM, WT = 11, APP/PS1 n=9; *p<0.05 vs WT; Student's t-test for independent means.

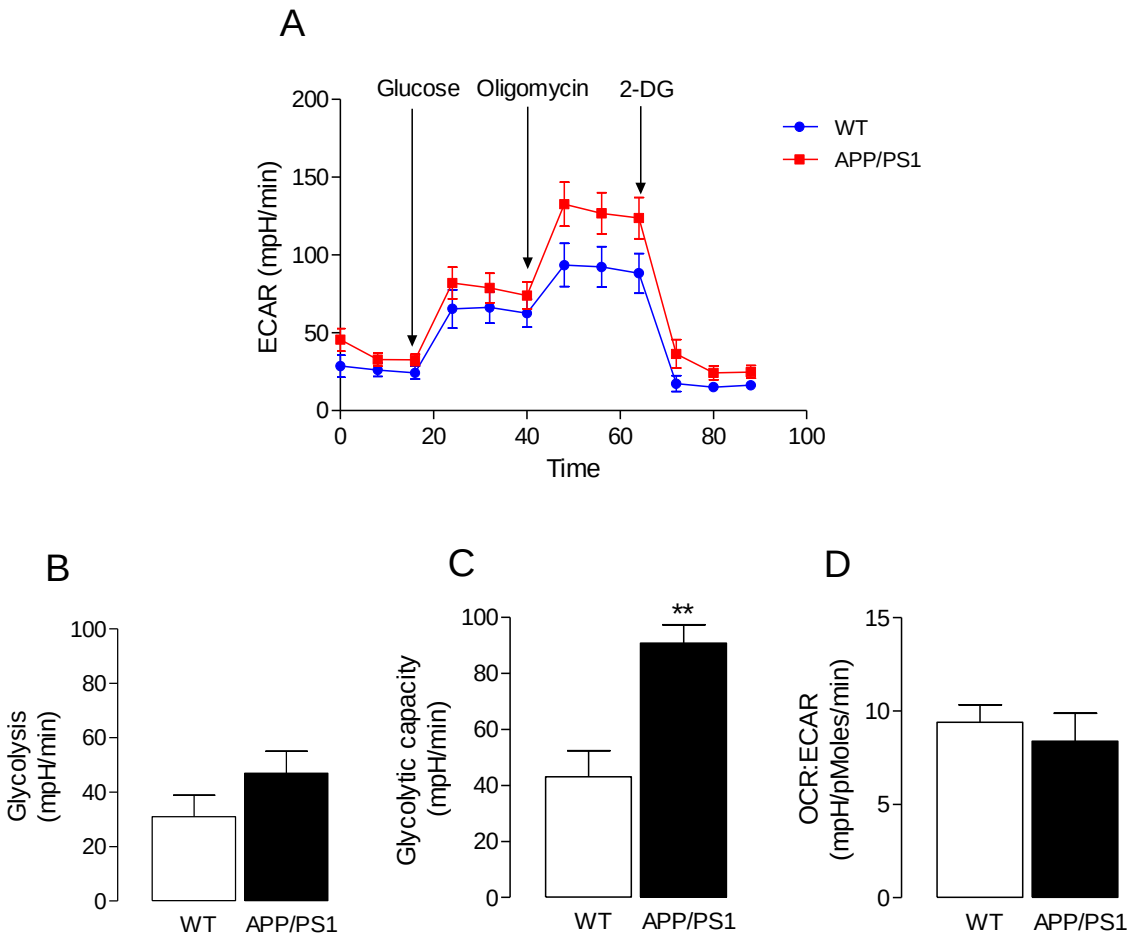

Figure 4.2 Plasma from APP/PS1 mice increases glycolysis in J774.2 cells.

J774.2 cells were incubated with plasma from WT or APP/PS1 mice at a 1:10 dilution for 4 h and a glycolytic stress test performed to examine metabolic function. The metabolic profile (A) and analysis of the mean data show a significant increase in glycolytic capacity of cells incubated with plasma from APP/PS1, compared with WT, mice (C). Glycolysis was also increased in cells in response to plasma from APP/PS1 mice; however this did not reach statistical significance (B). No change in OCR:ECAR was observed (D). Data are expressed as the mean ± SEM, WT = 10, APP/PS1 n=8; $^{**}p<0.01$ vs WT; Student's *t*-test for independent means.

	Control	MCI	AD
N	20	17	6
Age (years; mean ± SD)	65.05 ± 8.53	65.65 ± 7.03	63.50 ± 3.89
Sex (F/M)	6/14	5/12	2/4
MoCA	26.70 ± 1.62	22.00 ± 4.36**	13.25 ± 9.84***

Table 4.1 Demographic of AD and MCI patients and controls participants.

Data are presented as mean ± SD, control n = 20, MCI n = 17, AD n = 6**;** ***p*<0.01 Control vs MCI, ****p*<0.001 Control vs AD, MCI vs AD; One-way ANOVA and Student Newman-Keuls Multiple Comparison Test.

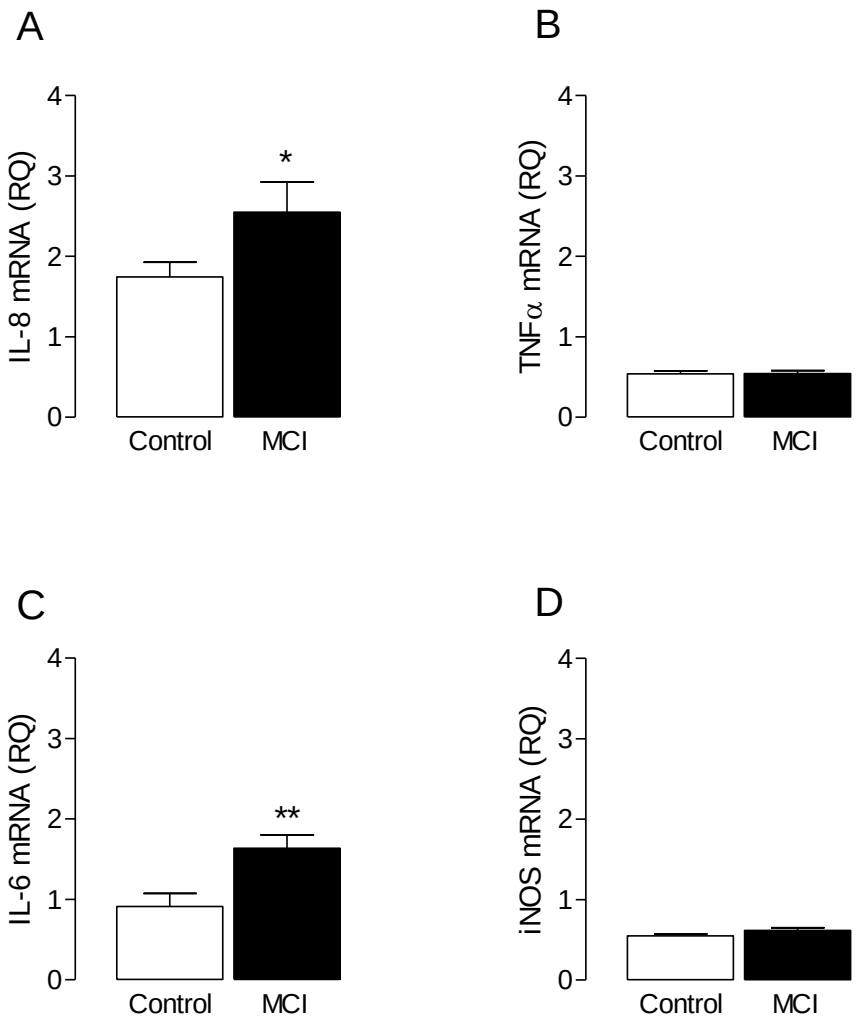

Figure 4.3 IL-8 and IL-6 mRNA is increased in THP-1 cells in response to plasma from MCI patients.

THP-1 cells were incubated with plasma from either control subjects or MCI patients at a 1:40 dilution for 4 h and pro-inflammatory gene expression examined by RT-PCR. Plasma from MCI patients significantly upregulated IL-8 (A) and IL-6 (C) mRNA in THP-1 cells compared with control plasma samples. The expression of TNFα (B) and iNOS (D) was not altered in response to plasma from MCI patients. Data are expressed as the mean ± SEM, control n = 20, MCI n = 17; **p*<0.05, ***p*<0.01 vs control; Student's *t*-test for independent means.

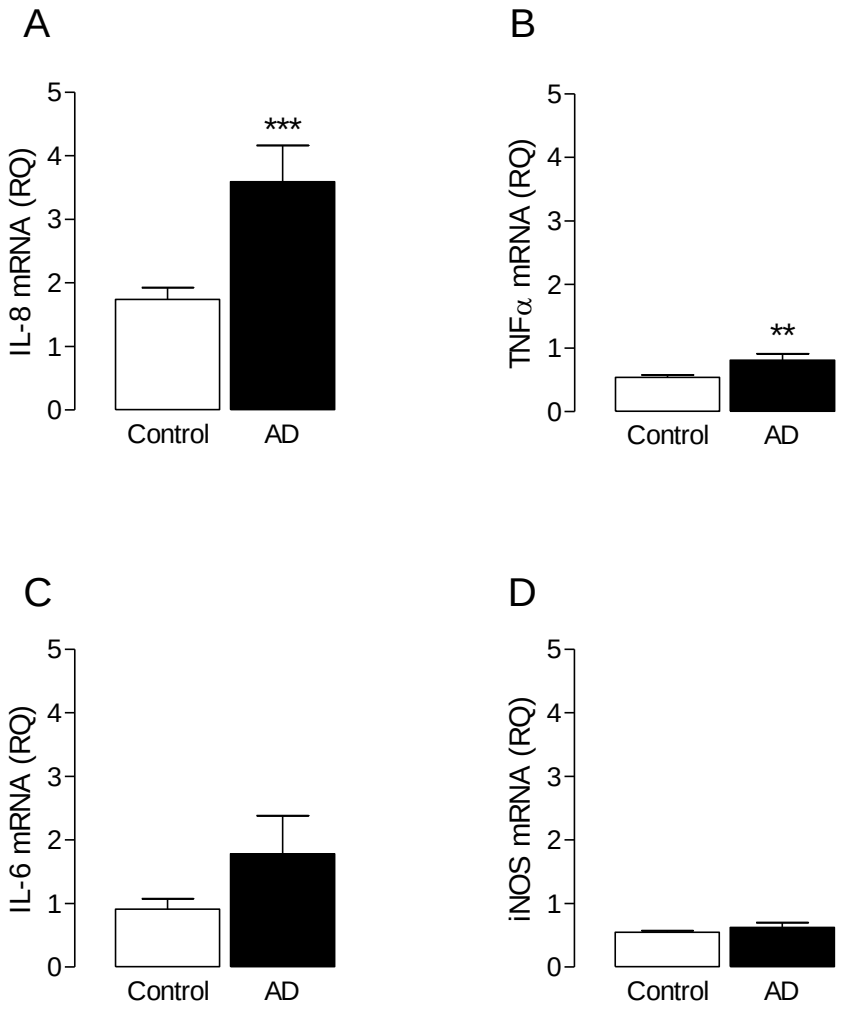

Figure 4.4 IL-8 and TNFα mRNA is increased in THP-1 cells in response to plasma from AD patients.

THP-1 cells were incubated with plasma from either control subjects or AD patients at a dilution of 1:40 for 4 h and pro-inflammatory gene expression examined by RT-PCR. Plasma from AD patients significantly upregulated IL-8 and TNFα mRNA in THP-1 cells compared with control plasma samples (A, B). The expression of IL-6 (C) and iNOS (D) was not altered in response to plasma from AD patients. Data are expressed as the mean ± SEM, control n = 20, AD n = 6, ** p<0.01; *** p<0.001 vs control; Student's t-test for independent means.

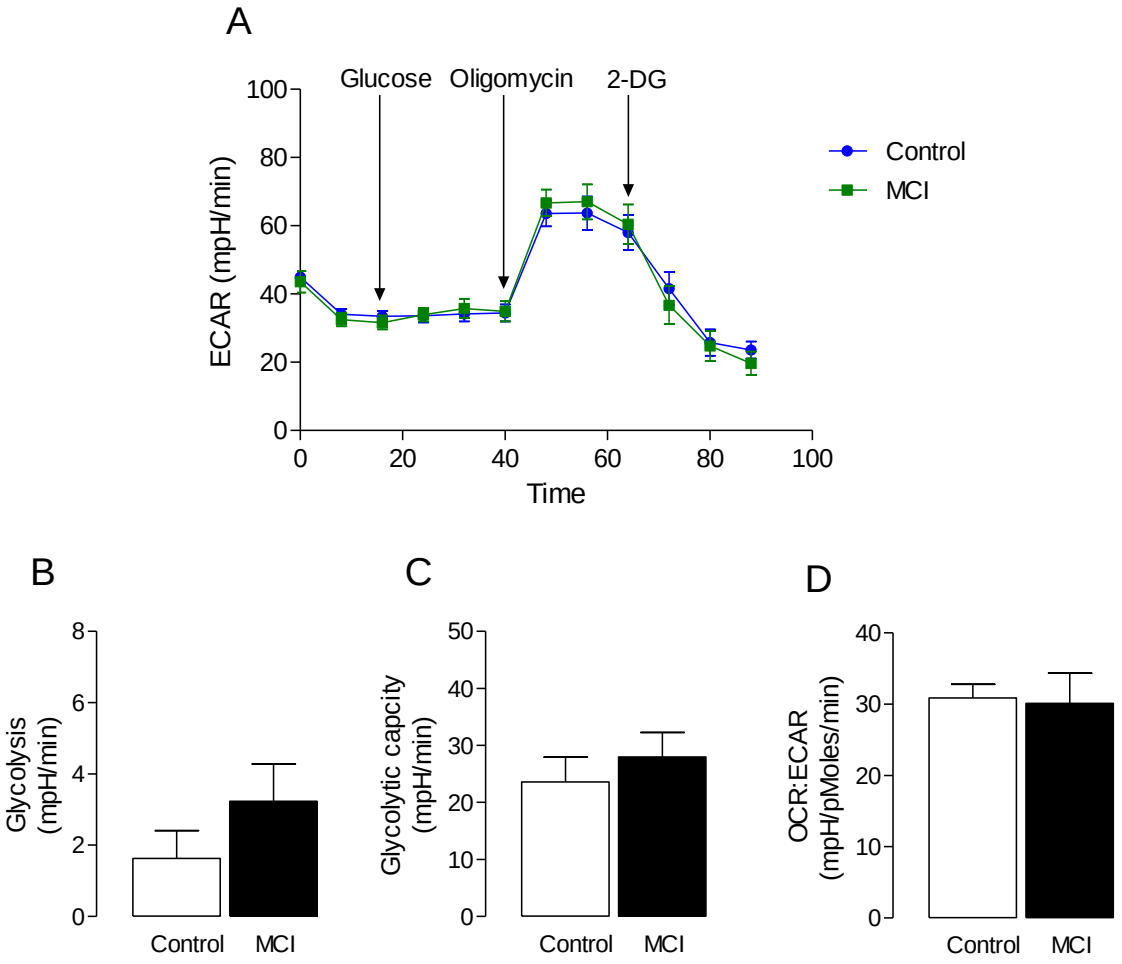

Figure 4.5 Plasma from MCI patients does not increase glycolysis in THP-1 cells.

THP-1 cells were incubated with plasma from control or MCI subjects at a 1:10 dilution for 4 h and a glycolytic stress test performed to examine metabolic function. The metabolic profile (A) shows no change in ECAR in cells incubated with plasma from MCI patients compared with control subjects. There was no significant change observed in glycolysis (B), glycolytic capacity (C) or OCR:ECAR (D). Data are expressed as the mean ± SEM, control n = 20, MCI n = 17; Student's *t*-test for independent means.

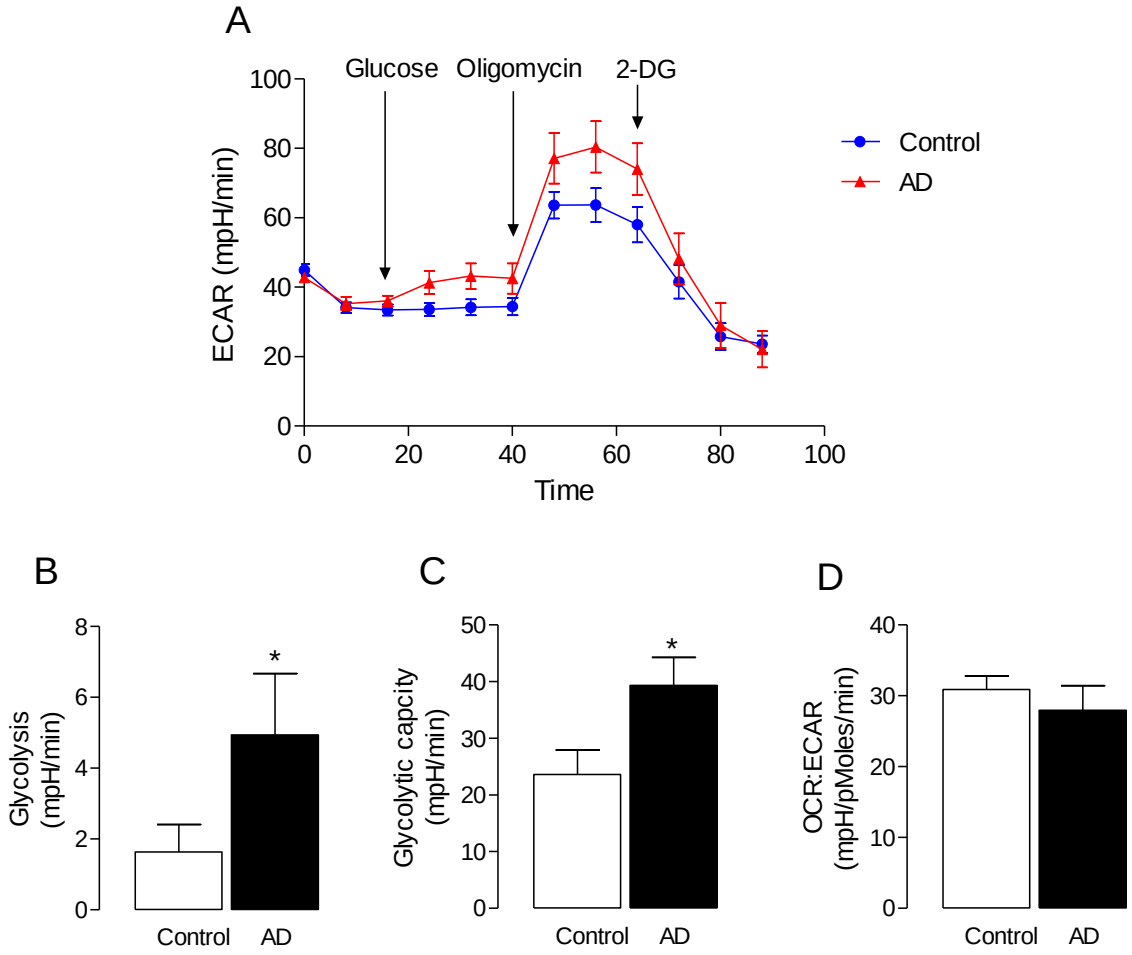

Figure 4.6 Plasma from AD patients increases glycolysis in THP-1 cells.

THP-1 cells were incubated with plasma from control or AD subjects at a 1:10 dilution for 4 h and a glycolytic stress test performed to examine metabolic function. The metabolic profile (A) shows an increase in ECAR in cells incubated with plasma from AD patients compared with control subjects. Analysis of the mean data shows a significant increase in glycolysis (B) and glycolytic capacity (C) in cells in response to AD plasma. No significant change in OCR:ECAR was observed (D). Data are expressed as the mean ± SEM, control n = 20, AD n = 6; *p<0.05 vs control; Student's t-test for independent means.

4.4 Discussion

The data presented in this chapter further investigated the use of plasma-treated monocytes as a proxy measure for cognitive dysfunction by examining the effect of plasma from MCI and AD patient cohorts. The reliability of CXCL1 and IL-8 mRNA upregulation in cultured cells in response to mouse and human plasma respectively as a potential biomarker was assessed. The metabolic profile of cells incubated with plasma was also explored as a potential biomarker. Plasma from APP/PS1 mice increased the expression of CXCL1 mRNA and other pro-inflammatory markers, as well as glycolysis, in monocytes. This was extended to a clinical cohort, and the data show that plasma from MCI and AD patients specifically upregulated IL-8 mRNA expression in monocytes. Furthermore, plasma from AD, but not MCI patients, induced an increase in glycolysis. Collectively, the data indicate that IL-8 mRNA in monocytes, and potentially an increase in glycolysis, could be a useful indicator for the detection of cognitive dysfunction related to AD.

The APP/PS1 mouse is one of the most commonly used models of AD. These mice develop Aβ plaques from the age of 4 months and significant progressive memory deficits occur from this time (Cao et al., 2007). In the present study, the mRNA expression of all pro-inflammatory markers examined (CXCL1, TNFα, IL-6 and iNOS) was upregulated in J774.2 cells in response to plasma from APP/PS1, compared with WT, mice. Although there are no comparable studies in the literature, the effect of plasma from APP/PS1 mice on an endothelial cell line has been examined. In that study, plasma from APP/PS1 mice decreased the expression of tight junction proteins which may contribute to the change in BBB permeability observed in these mice (Barrett et al., 2015b). It is possible that this effect was due to the increase in circulating IL-6, IFNγ, IL-12 and/or CXCL1 in the plasma of APP/PS1 mice (Barrett et al., 2015b).

In the previous chapter, the data revealed a specific increase in CXCL1 mRNA in J774.2 cells in response to plasma from aged C57/BL6 mice, whereas here increases in TNFα, IL-6 and iNOS mRNA were also observed. This could possibly be attributed to the difference in ages between cohorts of mice – the C57/BL6 mice assessed in

Chapter 3 were 18 months old whereas the APP/PS1 mice in this chapter were 22 months old. The development of an Alzheimer's-like pathology in APP/PS1 mice is likely to impact on the plasma composition and the concentration of inflammatory mediators could induce a differential response in cells. An increase in 12 pro-inflammatory cytokines including IL-1β, IL-3, IL-6, IL-10, TNFα and CXCL1 has been described in plasma from APP/PS1 mice aged 14 months (Sanchez-Ramos et al., 2009). However, in a study which examined plasma concentrations of 23 inflammatory cytokines in a triple transgenic mouse model of AD (3x-Tg) aged 14- and 24-months old, only IL-12p40 was found to be significantly increased in 14-month 3xTg mice, and only a decrease in all other cytokines was observed in either age group. Furthermore, only IL-3 levels were found to be significantly decreased in AD mice compared to WT in both age-groups (Yang et al., 2015). These studies demonstrate that plasma from various cohorts of mice differ substantially.

Inflammatory stimuli have been shown to induce a metabolic switch from oxidative phosphorylation to glycolysis in macrophages (Kelly and O'Neill, 2015) and, here, the metabolic signature of cells in response to plasma from WT and APP/PS1 mice was also assessed. J774.2 cells display a significant increase in glycolytic capacity when incubated with plasma from APP/PS1, compared with WT, mice. This suggests that there are pro-inflammatory factors present in the plasma of APP/PS1 mice promoting glycolytic metabolism. There was no change in the ratio of OCR:ECAR observed, indicating that this increase in glycolysis does not coincide with a decrease in oxidative phosphorylation. To my knowledge, the metabolic signature of cells in response to plasma from mice has not yet been investigated previously. This analysis could potentially represent a novel platform for biomarker development and analysis of disease mechanisms.

To determine whether there might be a parallel between data from the mouse model of AD and clinical samples, the effect of plasma from MCI and AD patients on THP-1 cells was examined. THP-1 cells showed a differential upregulation in TNFα and IL-6 mRNA in response to plasma from MCI and AD patients. However, the consistent finding between the two cohorts is an increase in IL-8 mRNA expression. Significant cognitive impairment as indicated by the MoCA was reported between

controls and MCI/AD patients, in line with the literature (Moafmashhadi and Koski, 2013). In the previous chapter, a significant increase in IL-8 mRNA was evident in cells incubated with plasma from IQ-discrepant individuals, whose MoCA scores were indicative of normal cognitive function (average MoCA score: 28.15). The data suggest that IL-8 mRNA in plasma-treated monocytes is a potential signature of early and later stage cognitive dysfunction of the AD continuum.

The findings also indicate that there are factors present in the blood of MCI and AD patients which may also be present in the plasma of IQ-discrepant individuals, as discussed in Chapter 3. Despite many studies having investigated peripheral cytokines as potential biomarkers of MCI and AD, no consistent signature has yet emerged. A meta-analysis carried out by Swardfager and colleagues (2010), which included 40 studies examining peripheral cytokines between healthy controls and AD patients, concluded that IL-6, TNFα, IL-1β, IL-12, IL-18 and TGF-β are increased in the plasma of AD patients. No significant associations were reported for IL-10, IFNγ, IL-4, C-reactive protein (CRP) and, of particular interest, IL-8. Results from studies investigating IL-8 levels in plasma are varied. An increase in plasma IL-8 has been reported to negatively correlate with cognitive function in AD patients (Alsadany et al., 2013) and higher IL-8 plasma levels were also detected in AD patients with cerebrovascular disease (Zhu et al., 2017). On the other hand, others have reported that IL-8 is unchanged in samples from patients with MCI or mild AD (Magaki et al., 2007), and some studies have shown a decrease in IL-8 in MCI and AD patients compared to controls (Kim et al., 2011, Hesse et al., 2016). Separately, IL-8 was identified in a panel of 18 plasma proteins that discriminated between controls and AD patients and that predicted conversion from MCI to AD or non-AD-related dementia (Ray et al., 2007). Two studies further examined this particular panel, and both concluded that this signature was not adequately sensitive or specific enough to act as a diagnostic biomarker (Marksteiner et al., 2011, Bjorkqvist et al., 2012). Of the original panel of 18 proteins, only EGF and CCL15 were found to be increased in plasma from AD patients in both studies. An increase in CSF IL-8 concentration has also been reported in AD patients (Galimberti et al., 2003), although other studies have found a significant decrease (Hesse et al., 2016). It is possible that

cytokines/chemokines are responsible for the upregulation in IL-8, but it is more likely to be the combined actions of a number of cytokines rather than their individual effect.

THP-1 cells exhibited an increase in glycolysis when incubated with plasma from AD, but not MCI, patients, in comparison to controls. Additionally, no significant differences were observed in the OCR:ECAR ratio. This parallels the findings in J774.2 cells in response to plasma from APP/PS1 mice. The effect of plasma from MCI and AD patients on the metabolic profile of cells has previously been investigated using a human microglial cell line, CHME-5. Pooled plasma from AD patients induced a shift towards glycolysis demonstrated by the increase in ECAR and decreased OCR (Jayasena et al., 2015), and no significant effect of pooled plasma from MCI patients was observed. Subsequent proteomic analysis of cells treated with AD plasma showed an upregulation of many enzymes involved in the glycolytic pathway, such as pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase. Plasma proteomic analysis found the levels of complement and acute-phase proteins to be increased in the plasma of MCI and AD patients, which might have induced the shift in metabolism (Jayasena et al., 2015). Plasma from AD patients has previously been speculated to contain neurotoxic factors, as exposure of rat hippocampal neurons to AD plasma upregulated markers characteristic of plaques and tangles, namely Alz-50, beta-amyloid (beta/A4), microtubule-associated protein 2 (MAP2), and ubiquitin (Brewer and Ashford, 1992). Recent metabolomics has found alterations in metabolites in both CSF and plasma from MCI and AD patients related to mitochondrial function and energy metabolism, with the TCA cycle being most affected (Trushina et al., 2013). Here, the data indicate that factors in the plasma of AD, but perhaps not MCI, patients increases glycolysis in monocytes; thus this might be a useful indicator for discrimination of AD patients but not useful in identifying earlier stages of disease progression.

The mechanism underlying the shift towards glycolysis was not assessed. However, macrophages display a shift towards glycolysis in response to cytokine stimulation such as IFNγ or TLR ligands such as LPS. It is possible that the increase in glycolysis observed here is driven through activity of PFKFB3, which maintains higher levels of

F-2,6-BP (Zhu et al., 2015). Further investigation is needed to determine the cause and mechanism of increased glycolytic flux in THP-1 cells in response to plasma from AD patients.

Taken together, the data presented shows that in plasma-treated monocytes, increased IL-8 mRNA is potentially a valuable biomarker for early cognitive decline related to AD, and that changes in glycolytic flux might also be useful for identifying later stages of cognitive dysfunction.

Chapter 5: The effect of Aβ on inflammatory markers and cytokine production in MDMs from IQ-consistent and IQ-discrepant participants

5.1 Introduction

Monocytes are primary cells of the innate immune system that originate from progenitors in the bone marrow and are recruited from the bloodstream to peripheral tissues where they differentiate into either macrophages or DCs (Shi and Pamer, 2011). They mediate the inflammatory response to invading pathogens and cancer cells but they can also contribute to disease pathogenesis. In response to various environmental cues, monocytes can differentiate into a variety of macrophage phenotypes with increased phagocytic capacity or wound healing properties.

The response of macrophages is largely mediated by a family of 13 highly conserved innate immune receptors known as TLRs, which recognise a wide range of conserved microbial products or motifs known as PAMPs. TLRs are therefore also known as PRRs and are characterised by the presence of an extracellular leucine-rich repeat domain and intracellular Toll/IL-1 receptor (TIR) domain (Vaure and Liu, 2014). The first TLR discovered, TLR4, has largely been studied by assessing the effects of its ligand LPS, a component of the cell wall of Gram-negative bacteria. The binding of LPS to TLR4 requires several accessory molecules, including CD14, an LPS-binding protein, and myeloid differentiation factor 2 (MD-2) (Vaure and Liu, 2014). On the other hand, TLR2 has been shown to recognise a variety of ligands such as peptidoglycan from Gram-positive bacteria and bacterial lipoproteins (Oliveira-Nascimento et al., 2012). The activation of TLRs, through signal transduction pathways involving NF-κB and MAPK, leads to the transcription of genes involved in the inflammatory response such as cytokines, chemokines, antimicrobial peptides and nitric oxide.

Aβ, the major constituent of plaques in the AD brain, has been reported as an endogenous ligand for both TLR2 and TLR4 (Udan et al., 2008). Aβ binds to numerous receptors on microglia (the resident immune cells of the brain) as well as TLRs, including RAGE (receptor for advanced glycation end products), LRP1 (low density lipoprotein receptor-related protein 1), CD36, Fc receptors and complement receptors, which are responsible for the recognition, internalisation and clearance of

Aβ (Doens and Fernandez, 2014). Aβ42 also interacts with CD14 on microglia and PBMCs resulting in the production of inflammatory cytokines (Fassbender et al., 2004). Aβ signalling through TLR2 and TLR4 has been linked to impairments in cognitive function, as it was found that active immunisation with Aβ42 in C57/BL6 mice induced sustained memory impairments which was not evident in TLR2/4 knockout mice (Vollmar et al., 2010). Additionally, it was revealed that immunisation with Aβ42 induced the release of TNFα and IL-6 by macrophages, an effect which was not seen in TLR2/TLR4 deficient mice.

Peripheral immune cells are suitable candidates for potential identification of biomarkers, as they are readily accessible and can be analysed by widely available techniques. The question remains whether or not specific properties/phenotypes of circulating macrophages and other immune cells may be capable of identifying an AD-like pathology, or cognitive impairment. Microglia have been shown to exhibit a decreased phagocytic capacity of Aβ in AD, and Aβ phagocytosis has also been found to be defective in PBMCs from AD patients (Avagyan et al., 2009), which might be as a result of decreased TLR expression (Fiala et al., 2007). The effects of Aβ on microglia have been widely assessed, due to the fact that the cells are exposed to Aβ when accumulation occurs in the brain. However, monocytes and macrophages are also exposed to Aβ as they are capable of trafficking to the brain in AD. In addition, Aβ is also present in the plasma, although at lower concentrations than in the CSF. Therefore it is important to examine the effect of Aβ on monocyte/macrophage function.

The aims of this study were:

1. To investigate differences in cell surface and mRNA expression of CD11b, TLR2, TLR4 and MRC-1 in MDMs from IQ-consistent and IQ-discrepant individuals and examine the effect of Aβ stimulation.
2. To investigate differences in release and mRNA expression of TNFα, IL-6 and IL-8 in MDMs from IQ-consistent and IQ-discrepant individuals and examine the effect of Aβ stimulation.

5.2 Methods

Healthy older adults (n=75) with a mean age of 65.01 years were recruited from the MRU at Trinity College Dublin. Participants were classified as either IQ-consistent (n=46) or IQ-discrepant (n=29) based on their delayed word recall in relation to their estimated IQ as described in sections 2.7.1 and 2.7.3. Analysis of MMSE and MoCA scores were assessed by colleagues in the MRU and no differences were observed between the groups. Blood samples were collected from these participants, processed and plasma and cells isolated as described in sections 2.8 and 2.9.

Plasma from participants was incubated with THP-1 cells as described in section 2.13 and harvested for analysis of gene expression by RT-PCR as described in sections 2.14 and 2.15.

MDMs from participants were assessed for cell surface marker expression by flow cytometry as described in section 2.19. Additionally, MDMs from a smaller cohort of participants were stimulated with Aβ (10 μM) as described in section 2.12 and harvested for analysis of gene expression by RT-PCR, cytokine production by ELISA and cell surface marker expression by flow cytometry as described in sections 2.14, 2.15, 2.18 and 2.19.

5.3 Results

5.3.1 IL-8 mRNA is increased in THP-1 cells in response to plasma from female IQ-discrepant participants

The effect of plasma on THP-1 cells from a cohort of 46 IQ-consistent and 29 IQ-discrepant participants was examined. Participants with a poorer than predicted performance on their delayed verbal recall in the WMS relative to their IQ estimated by the NART were described as IQ-discrepant, and participants who performed as predicted were termed IQ-consistent. The participant groups were age-matched and showed no difference in MMSE or MoCA scores. Table 5.1 shows that there was a significant difference in delayed verbal recall z-score and cognitive asymmetry z-score between the groups, the basis on which the cohorts were subdivided (Table 5.1, $p<0.001$, Student's *t*-test for independent means).

THP-1 cells were incubated with plasma from IQ-consistent and IQ-discrepant participants for 4 hours and pro-inflammatory marker expression was examined by RT-PCR. Plasma from IQ-discrepant participants induced a significant increase in IL-8 and TNFα mRNA in THP-1 cells compared with plasma from IQ-consistent participants (Figure 5.1 A, B, $p<0.05$, $p<0.01$, Student's *t*-test). No significant differences in the expression of IL-6 and iNOS mRNA were observed (Figure 5.1 C, D).

The impact of gender on the effect of pro-inflammatory marker expression exerted by plasma in THP-1 cells was also examined. The data show a significant upregulation in IL-8 mRNA in THP-1 cells in response to plasma from female IQ-discrepant participants, compared with both male IQ-discrepant and female IQ-consistent participants (Figure 5.2 A, $p<0.01$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). The expression of TNFα was higher in cells in response to plasma from female IQ-discrepant participants, and from male IQ-consistent participants, compared with female IQ-consistent participants (Figure 5.2 B, $p<0.05$, $p<0.01$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). No effect of gender on the expression of IL-6 and iNOS was observed in THP-1 cells incubated with plasma from IQ-consistent and IQ-discrepant participants (Figure 5.2 C, D).

5.3.2 Expression of CD11b, TLR2, TLR4 and CD206 in MDMs from IQ-consistent and IQ-discrepant participants

Monocytes were isolated from whole blood by density separation and magnetic sorting and cultured for 7 days *in vitro* with GM-CSF to generate MDMs and assessed for cell surface marker expression by flow cytometry. The percentage of $CD11b^+$ cells was significantly decreased in MDMs from IQ-discrepant, compared with IQ-consistent, participants (Figure 5.3 A, $p<0.05$). Despite a trend towards an increase in TLR2 and CD206 expression, no significant changes in the expression of TLR2, TLR4 and CD206 were observed on $CD11b^+$ cells (Figure 5.3 B, C, D).

5.3.3 Examining select IQ-consistent and IQ-discrepant participants with regard to IL-8 mRNA response in THP-1 cells

In order to investigate the response of MDMs from IQ-consistent and IQ-discrepant individuals to Aβ stimulation, participants with the 10 lowest IL-8 mRNA values from the IQ-consistent group, and participants with the 10 highest IL-8 mRNA values from the IQ-discrepant group, were selected for further analysis. The chosen IQ-consistent and IQ-discrepant participants for further evaluation are indicated in blue and red respectively in Figure 5.4. This smaller cohort of participants were age- and gender-matched and showed no difference in MMSE or MoCA scores. Similar to the whole cohort of participants, a significant decrease in delayed verbal recall z-score and increase in cognitive asymmetry z-score was observed in IQ-discrepant, compared with IQ-consistent, individuals (Table 5.2, $p<0.001$). The remaining analysis described in this chapter was performed on this smaller cohort of participants.

5.3.5 The effect of Aβ stimulation on MDM expression of CD11b, TLR4, TLR2 and CD206

Monocytes were isolated from whole blood by density separation and magnetic sorting and cultured for 6 days *in vitro* with GM-CSF to generate MDMs. Cells were stimulated with Aβ for 24 hours and assessed for CD11b, TLR2, TLR4 and CD206 expression using flow cytometry and RT-PCR. The percentage of $CD11b^+$ cells was significantly decreased in Aβ-stimulated MDMs from IQ-consistent, but not IQ-

discrepant, participants (Figure 5.5 A, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). Aβ significantly decreased the expression of CD11b mRNA in MDMs from both participant groups (Figure 5.5 B, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). No difference in the cell surface and mRNA expression of CD11b in unstimulated cells between IQ-consistent and IQ-discrepant participants was observed.

Irrespective of Aβ treatment, MDMs from IQ-discrepant participants displayed a significant increase in TLR2 expression on $CD11b^+$ cells, compared with IQ-consistent participants (Figure 5.6 A, $p<0.01$, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). Aβ significantly increased the cell surface expression of TLR2 on MDMs from IQ-discrepant individuals (Figure 5.6 A, $p<0.05$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). The expression of TLR2 mRNA was significantly upregulated by Aβ-stimulation, but no differences were observed between participant groups (Figure 5.6 B, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis).

Aβ induced a significant increase in TLR4 expression on $CD11b^+$ MDMs from IQ-consistent, but not IQ-discrepant, individuals (Figure 5.7 A, $p<0.01$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). No significant differences in TLR4 mRNA expression were observed between the participant groups, although a main effect of Aβ stimulation was observed (Figure 5.7 B, $p<0.01$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis).

The expression of CD206 on $CD11b^+$ cells was increased in MDMs from IQ-discrepant participants following Aβ stimulation, and the effect of Aβ on CD206 expression was greater in MDMs from IQ-discrepant, compared with IQ-consistent participants (Figure 5.8 A, $p<0.05$, $p<0.01$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). The expression of MRC-1 mRNA was significantly decreased by Aβ treatment, and no differences were observed in unstimulated and stimulated cells between the participant groups (Figure 5.8 B, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis).

5.3.6 Aβ increases IL-8, IL-6 and TNFα production and mRNA expression in MDMs

Monocytes were isolated and cultured as before to generate MDMs. Cells were stimulated with Aβ for 24 hours and assessed for release of TNFα, IL-6 and IL-8 by ELISA and TNFα, IL-6 and IL-8 mRNA expression by RT-PCR. The release and mRNA expression of TNFα was increased in response to Aβ stimulation, and the effect of Aβ on TNFα mRNA expression was significantly greater in MDMs from IQ-discrepant, compared with IQ-consistent, individuals (Figure 5.9 A, B, $p<0.05$, $p<0.01$, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). Aβ also increased the mRNA expression and production of IL-6 and IL-8 (Figure 5.10 A, B, Figure 5.11 A, B, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). However, no differences were observed between IQ-consistent and IQ-discrepant participants in the presence or absence of Aβ.

	IQ-memory consistent	IQ-memory discrepant
N	46	29
Age (years; mean ± SD)	65.78 ± 8.82	64.24 ± 7.59
Sex (F/M)	28/18	11/18
MMSE	29.52 ± 0.75	28.72 ± 0.99
MoCA	26.85 ± 3.06	26.34 ± 2.97
Premorbid IQ z-score	1.00 ± 0.39	1.26 ± 0.29
Delayed verbal recall z-score	0.85 ± 0.65	-0.51 ± 0.60***
Cognitive asymmetry z-score	0.15 ± 0.71	1.76 ± 0.62***

Table 5.1 Demographic of IQ-memory consistent and IQ-memory discrepant participants.

Data are expressed as mean ± SD, IQ-memory consistent n=46, IQ-memory discrepant n=29; ***p<0.001, IQ-memory discrepant vs. IQ-memory consistent; Student's t-test for independent means.

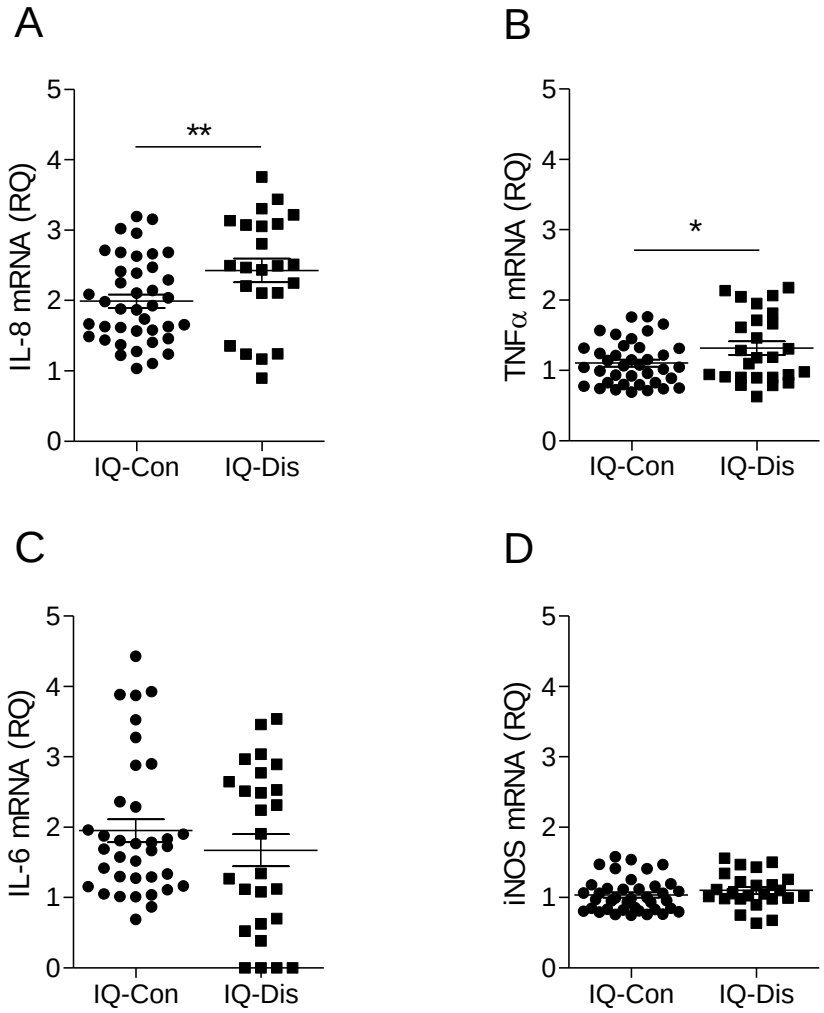

Figure 5.1 IL-8 and TNFα mRNA are increased in THP-1 cells in response to plasma from IQ-memory discrepant individuals.

THP-1 cells were treated with or without plasma from IQ-consistent and IQ-discrepant participants (IQ-Con, IQ-Dis) at a 1:40 dilution for 4 h. Cells were harvested and analysis of pro-inflammatory marker expression was performed using RT-PCR. Plasma from IQ-Dis participants significantly increased IL-8 (A) and TNFα (B) mRNA in THP-1 cells in comparison to IQ-Con participants. No differences in IL-6 (C) or iNOS (D) expression were observed. Data are expressed as the mean ± SEM, IQ-Con n=43, IQ-Dis n=28; *p<0.05, **p<0.01; Student's t-test for independent means.

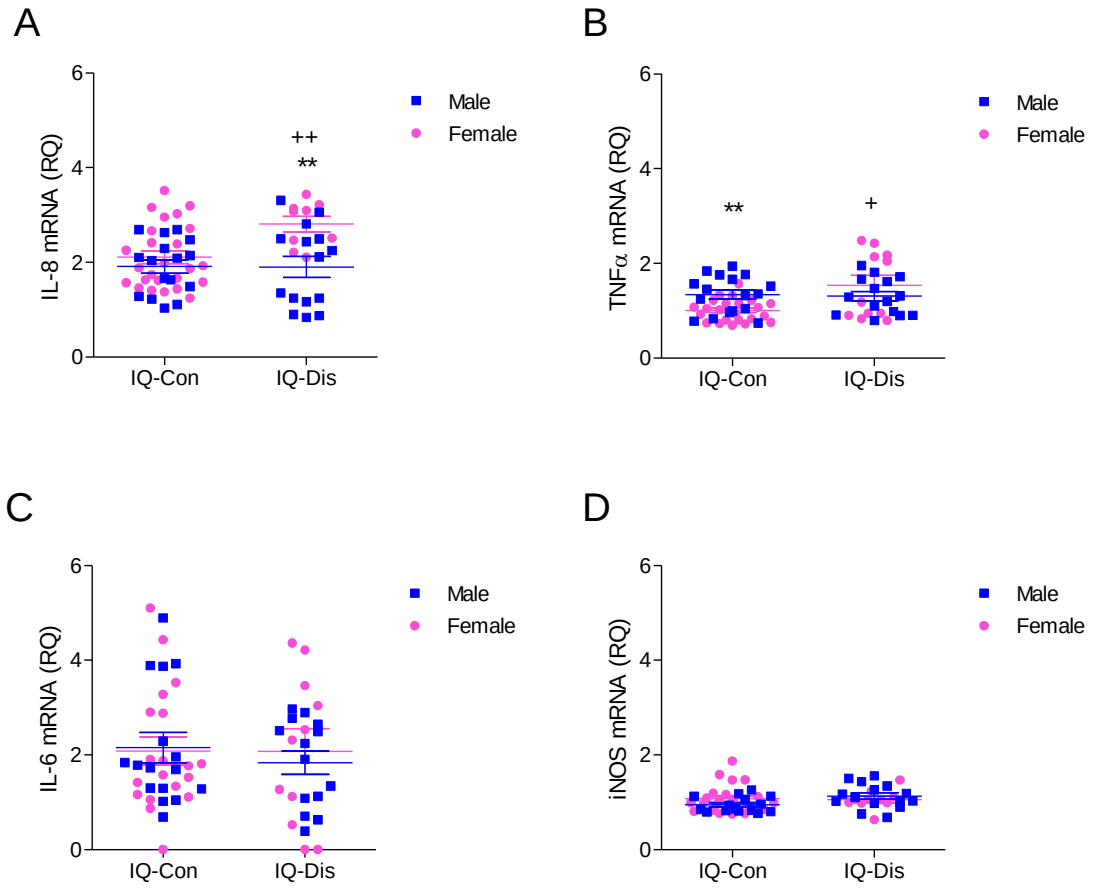

Figure 5.2 Plasma from IQ-discrepant females increases IL-8 and TNFα mRNA in THP-1 cells.

THP-1 cells were treated with or without plasma from IQ-consistent and IQ-discrepant participants (IQ-Con, IQ-Dis) at a 1:40 dilution for 4 h. Cells were harvested and analysis of pro-inflammatory marker expression was performed using RT-PCR. Plasma from female IQ-Dis participants significantly increased IL-8 (A) mRNA in THP-1 cells in comparison to male IQ-Dis participants and female IQ-Con participants. TNFα (B) mRNA was increased in female IQ-Dis and male IQ-Con participants in comparison to female IQ-Con participants. No differences in IL-6 (C) or iNOS (D) expression were observed. Data are expressed as the mean ± SEM, IQ-Con n=43, IQ-Dis n=28. ** $p<0.01$ vs group male; $^{+}p<0.05$, $^{++}p<0.01$ IQ-Dis female vs IQ-Con female. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $\text{Group}_{\text{effect}}$ [$F_{(1,58)}=6.446$, $p<0.0138$], $\text{gender}_{\text{effect}}$ [$F_{(1,58)}=7.729$, $p<0.0073$], $\text{interaction}_{\text{effect}}$ [$F_{(1,58)}=6.612$, $p<0.0127$]. **B:** $\text{Group}_{\text{effect}}$ [$F_{(1,62)}=5.502$, $p<0.0222$], $\text{gender}_{\text{effect}}$ [$F_{(1,62)}=0.2526$, $p<0.6170$], $\text{interaction}_{\text{effect}}$ [$F_{(1,62)}=6.984$, $p<0.0104$]. **C:** $\text{Group}_{\text{effect}}$ [$F_{(1,60)}=0.1293$, $p<0.7204$], $\text{gender}_{\text{effect}}$ [$F_{(1,60)}=0.01287$, $p<0.9100$], $\text{interaction}_{\text{effect}}$ [$F_{(1,60)}=0.3995$, $p<0.5297$]. **D:** $\text{Group}_{\text{effect}}$ [$F_{(1,57)}=1.544$, $p<0.2191$], $\text{gender}_{\text{effect}}$ [$F_{(1,57)}=0.2139$, $p<0.6455$], $\text{interaction}_{\text{effect}}$ [$F_{(1,57)}=2.309$, $p<0.1342$].

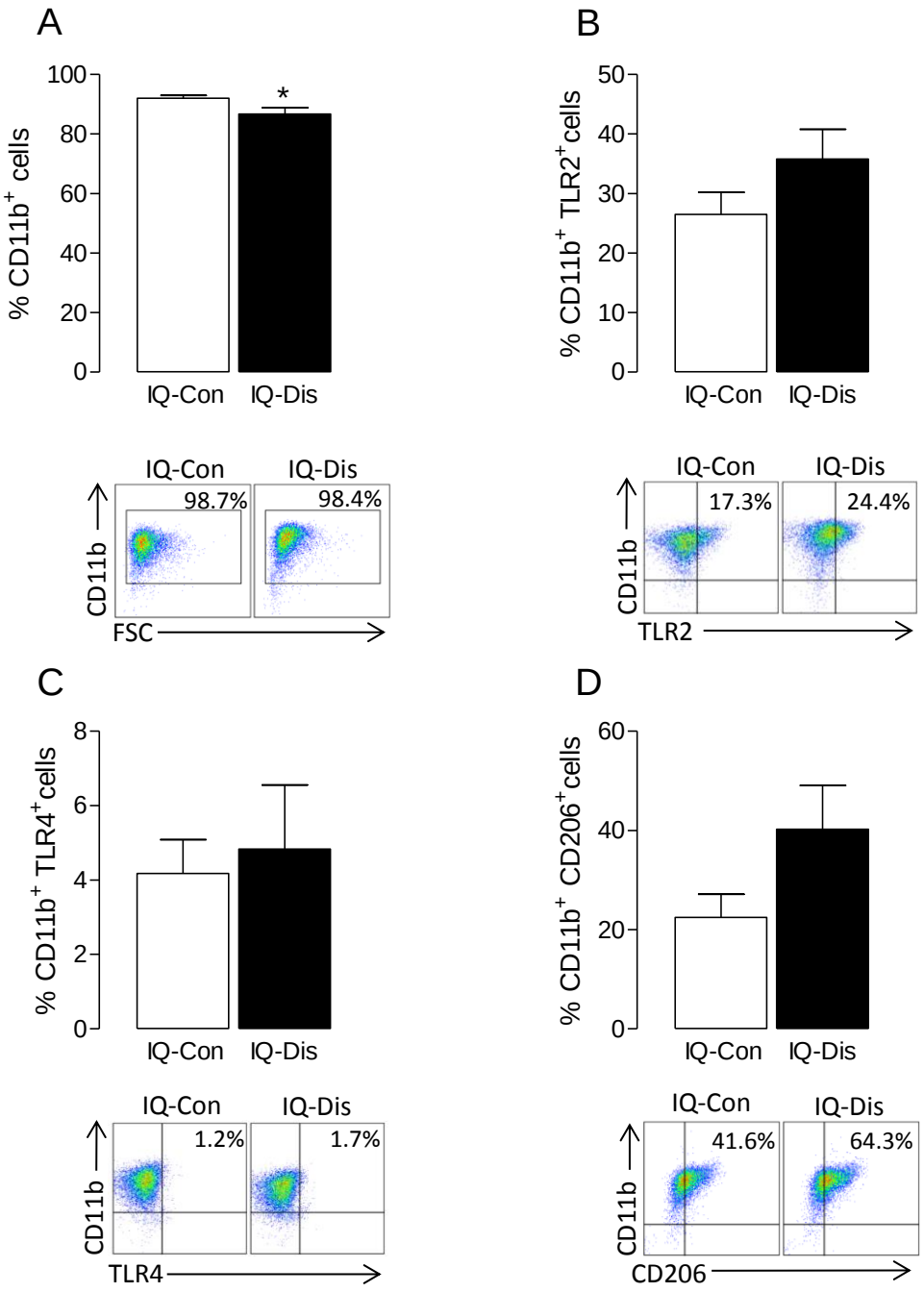

Figure 5.3 Expression of CD11b, but not TLR2, TLR4 and CD206, are altered in MDMs from IQ-consistent compared with IQ-discrepant individuals.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 7 days *in vitro* and cell surface marker expression assessed by flow cytometry. The expression of CD11b was significantly decreased in MDMs from IQ-discrepant participants (A). All further analysis was gated on this $CD11b^+$ population. No significant differences in TLR2 (B), TLR4 (C) and CD206 (D) expression were observed in MDMs from IQ-discrepant, compared with IQ-consistent, participants. Representative FACS plots are shown. Data are expressed as the mean ± SEM, IQ-Con n=43-46, IQ-Dis n=27-29. *p<0.05, Student's t-test for independent means.

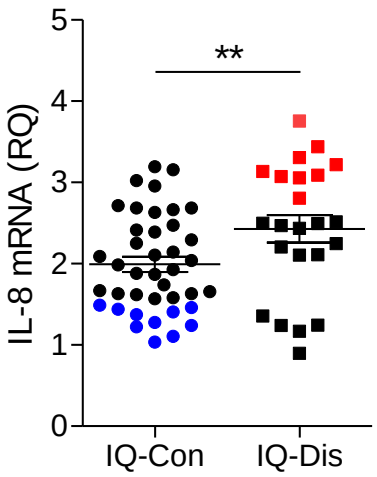

Figure 5.4 Selection of IQ-consistent and IQ-discrepant participants for further analysis based on plasma-induced IL-8 mRNA in THP-1 cells.

The data shown highlights the 10 lowest IL-8 mRNA values from IQ-consistent (IQ-Con) participants and the 10 highest IL-8 mRNA values from IQ-discrepant (IQ-Dis) participants in blue and red respectively. These participants (Table 5.2) were selected for further analysis. Data are expressed as the mean ± SEM, IQ-Con n=43, IQ-Dis n=28; ***p*<0.01; Student's *t*-test for independent means.

	IQ-memory consistent	IQ-memory discrepant
N	10	10
Age (years; mean ± SD)	64.80 ± 8.56	62.40 ± 7.69
Sex (F/M)	5/5	6/4
MMSE	29.80 ± 0.42	28.70 ± 0.95
MoCA	26.90 ± 3.38	27.10 ± 1.59
Premorbid IQ z-score	1.04 ± 0.47	1.33 ± 0.21
Delayed verbal recall z-score	0.96 ± 0.53	-0.60 ± 0.70***
Cognitive asymmetry z-score	0.07 ± 0.64	1.93 ± 0.71***

Table 5.2 Demographic of IQ-memory consistent and IQ-memory discrepant participants.

Data are expressed as mean ± SD, n=10, ****p*<0.001, IQ-memory discrepant vs. IQ-memory consistent; Student's *t*-test for independent means.

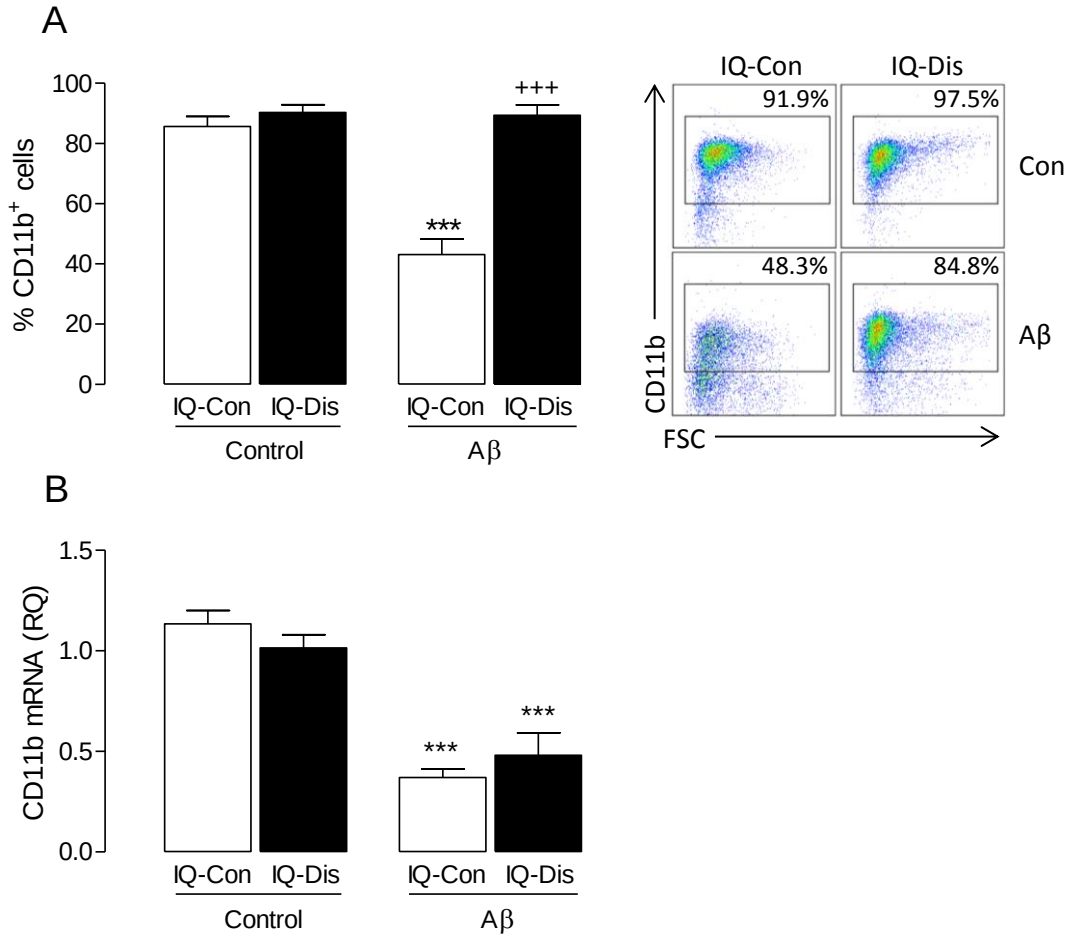

Figure 5.5 Aβ decreases CD11b expression on MDMs from IQ-consistent and IQ-discrepant individuals.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 µM) for 24 h and assessed for CD11b cell surface expression (A) by flow cytometry and mRNA (B) using RT-PCR. Aβ significantly decreased the expression of CD11b in MDMs from IQ-Con participants compared with IQ-Dis participants (A) and decreased mRNA expression in both groups (B). Representative FACS plots are shown. Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. ***p<0.001 vs vehicle control; ^{+++}p<0.001 vs IQ-Con Aβ. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $\text{Group}_{\text{effect}}$ [$F_{(1,32)}$=46.35, p<0.001], $\text{Aβ}_{\text{effect}}$ [$F_{(1,32)}$=33.63, p<0.001], $\text{interaction}_{\text{effect}}$ [$F_{(1,32)}$=31.03, p<0.001]. **B:** $\text{Group}_{\text{effect}}$ [$F_{(1,30)}$=0.0023, p<0.9613], $\text{Aβ}_{\text{effect}}$ [$F_{(1,30)}$=69.32, p<0.001], $\text{interaction}_{\text{effect}}$ [$F_{(1,30)}$=2.185, p<0.1497].

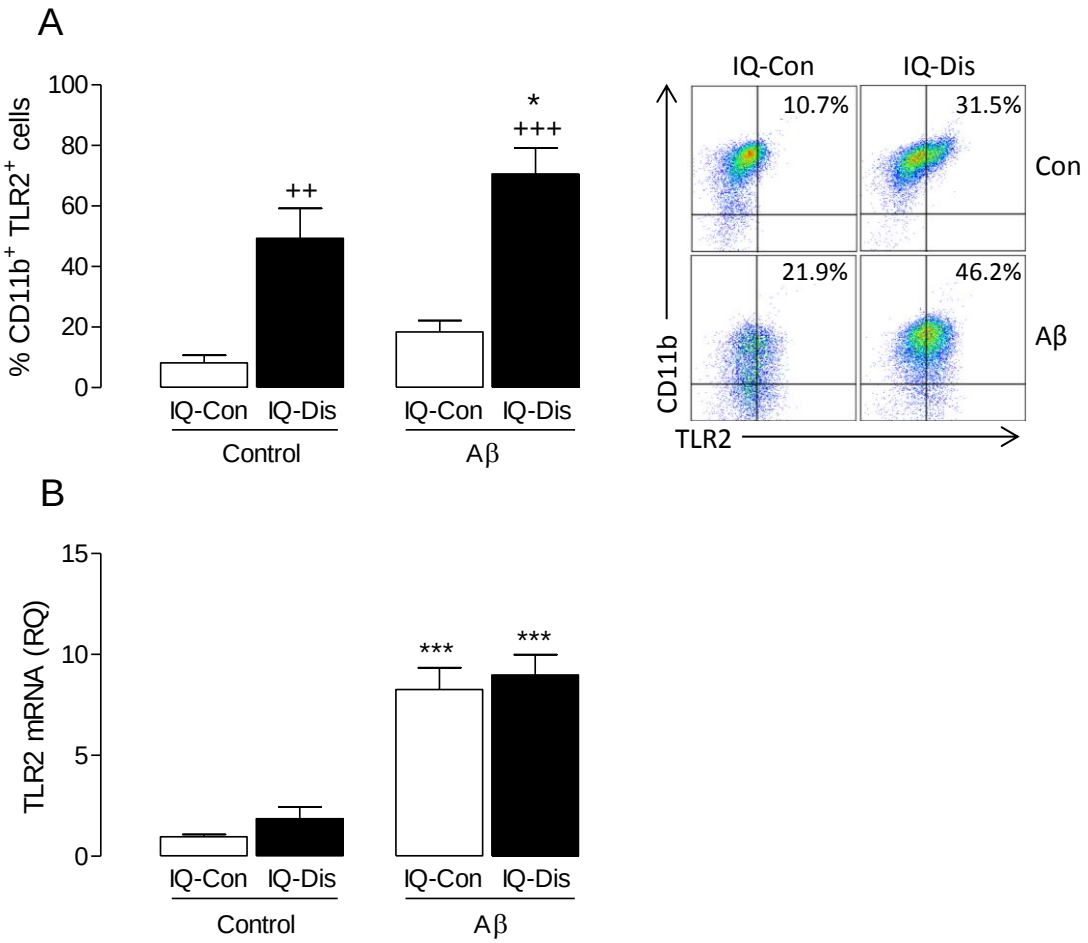

Figure 5.6 Cell surface expression of TLR2 is enhanced on MDMs from IQ-discrepant individuals and is increased by Aβ.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 µM) for 24 h and assessed for TLR2 cell surface expression (A) by flow cytometry and mRNA (B) using RT-PCR. TLR2 expression was increased in MDMs from IQ-Dis compared with IQ-Con participants; this was further exacerbated by Aβ treatment (A). Aβ significantly increased TLR2 mRNA expression and no difference was observed between the groups (B). Representative FACS plots are shown. Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. **p*<0.05, ****p*<0.001 vs vehicle control; ^{++}p<0.01, ^{+++}p<0.001 vs IQ-Con. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,31)}$=38.96, p<0.001], $Aβ_{effect}$ [$F_{(1,31)}$=4.426, p<0.0436], $interaction_{effect}$ [$F_{(1,31)}$=0.5600, p<0.4599]. **B:** $Group_{effect}$ [$F_{(1,30)}$=1.111, p<0.3002], $Aβ_{effect}$ [$F_{(1,30)}$=88.11, p<0.001], $interaction_{effect}$ [$F_{(1,30)}$=0.001, p<0.9188].

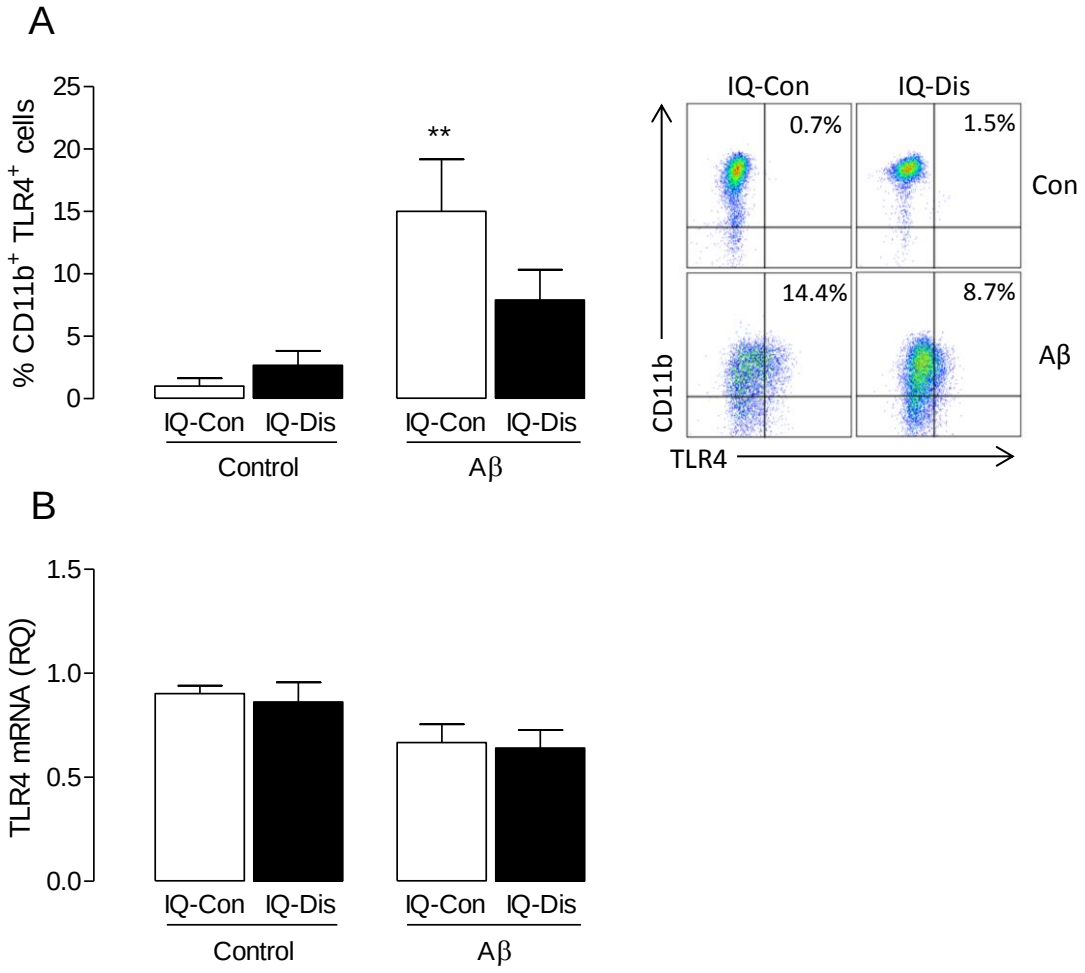

Figure 5.7 Aβ increases TLR4 expression on MDMs from IQ-consistent individuals.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 µM) for 24 h and assessed for TLR4 cell surface expression (A) by flow cytometry and mRNA (B) using RT-PCR. Aβ increased TLR4 protein expression in MDMs from IQ-Con participants and decreased TLR4 mRNA expression in both groups. Representative FACS plots are shown. Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. ***p*<0.01 vs vehicle control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,31)}$=1.164, p<0.2890], $Aβ_{effect}$ [$F_{(1,31)}$=14.58, p<0.001], $interaction_{effect}$ [$F_{(1,31)}$=3.034, p<0.0914]. **B:** $Group_{effect}$ [$F_{(1,30)}$=0.1637, p<0.6887], $Aβ_{effect}$ [$F_{(1,30)}$=7.807, p<0.009], $interaction_{effect}$ [$F_{(1,30)}$=0.0082, p<0.9283].

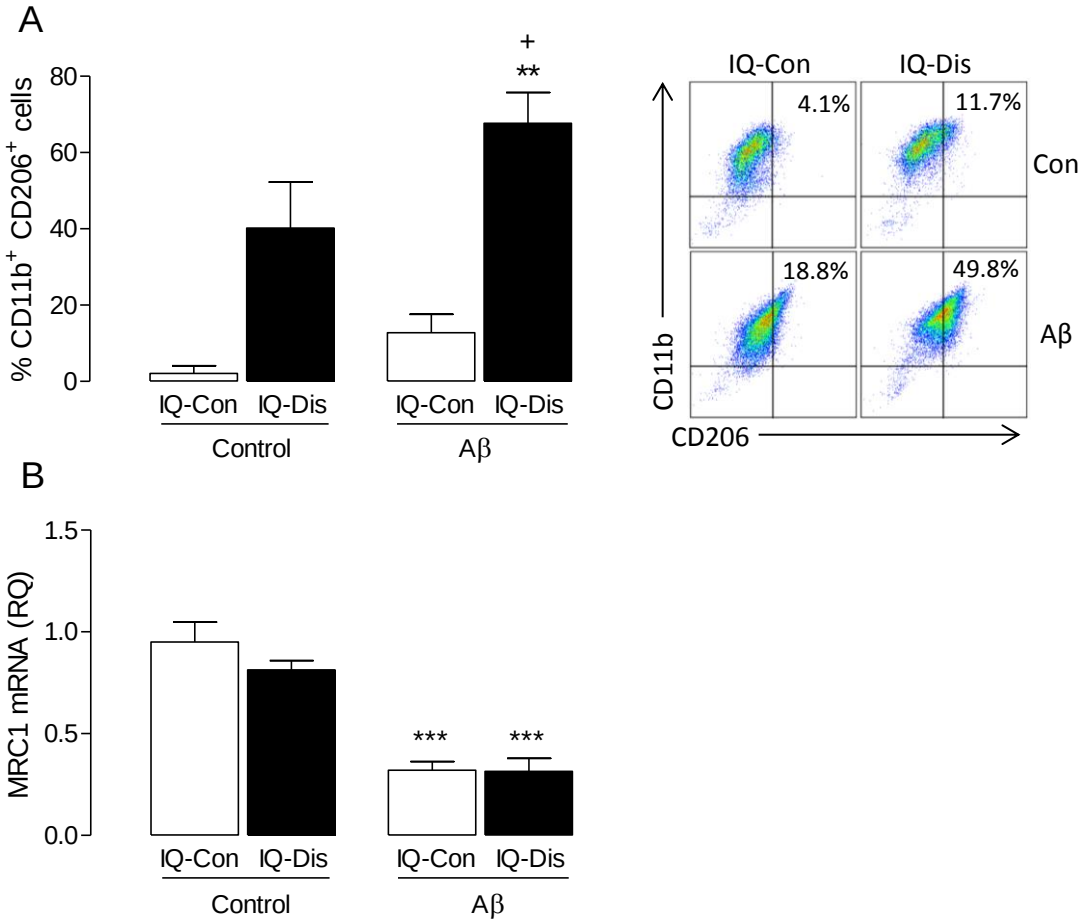

Figure 5.8 Cell surface expression of CD206 is enhanced on MDMs from IQ-discrepant individuals and is increased by Aβ.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 μM) for 24 h and assessed for CD206 cell surface expression (A) by flow cytometry and MRC1 mRNA (B) using RT-PCR. CD206 expression was significantly increased in MDMs from IQ-Dis compared with IQ-Con participants (A) and treatment with Aβ further increased CD206 expression in cells from IQ-Dis subjects. Aβ significantly decreased the mRNA expression of MRC1 (B) in both groups. Representative FACS plots are shown. Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. **p<0.01, ***p<0.001 vs vehicle control; ^{+}p<0.05 vs IQ-Con. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $\text{Group}_{\text{effect}}$ [$F_{(1,21)}$=17.02, p<0.001], $\text{A}\beta_{\text{effect}}$ [$F_{(1,21)}$=2.861, p<0.1055], $\text{interaction}_{\text{effect}}$ [$F_{(1,21)}$=0.5561, p<0.4641]. **B:** $\text{Group}_{\text{effect}}$ [$F_{(1,32)}$=1.154, p<0.2908], $\text{A}\beta_{\text{effect}}$ [$F_{(1,32)}$=72.24, p<0.001], $\text{interaction}_{\text{effect}}$ [$F_{(1,32)}$=0.9917, p<0.3268].

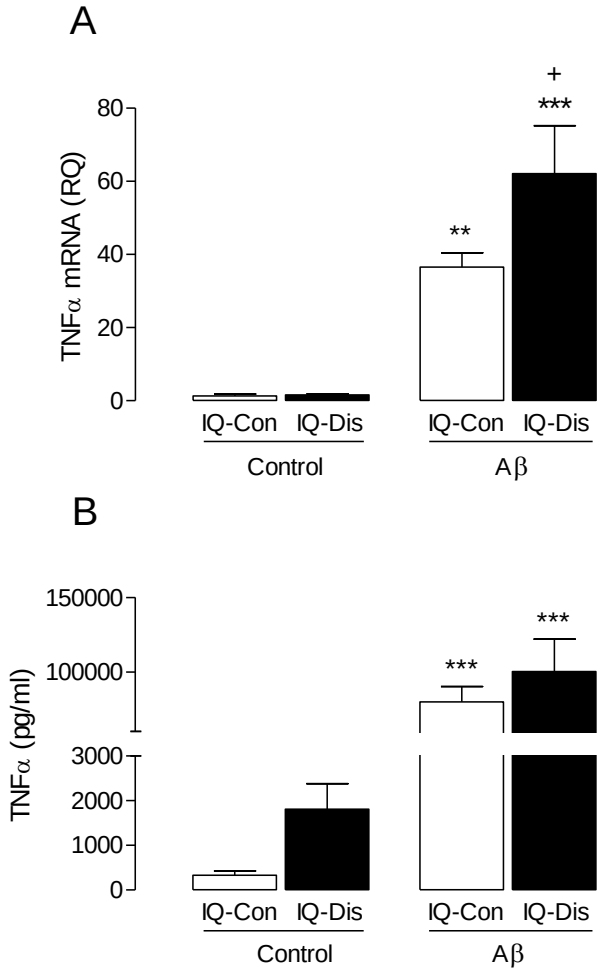

Figure 5.9 MDMs from IQ-discrepant individuals show an enhanced expression of TNFα mRNA in response to Aβ.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 μM) for 24 h and assessed for expression of TNFα mRNA (A) by RT-PCR and protein (B) by ELISA. Aβ increased TNFα mRNA and protein expression in MDMs from both groups and Aβ-stimulated MDMs from IQ-discrepant participants showed an enhanced expression of TNFα mRNA in comparison to Aβ-stimulated MDMs from IQ-consistent participants. Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. **p<0.01, ***p<0.001 vs vehicle control; ^{+}p<0.05 vs IQ-Con. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,33)}$=3.038, p<0.0906], $A\beta_{effect}$ [$F_{(1,33)}$=41.89, p<0.001], $interaction_{effect}$ [$F_{(1,33)}$=2.918, p<0.0970]. **B:** $Group_{effect}$ [$F_{(1,31)}$=0.7838, p<0.3828], $A\beta_{effect}$ [$F_{(1,31)}$=52.50, p<0.001], $interaction_{effect}$ [$F_{(1,31)}$=0.5854, p<0.4500].

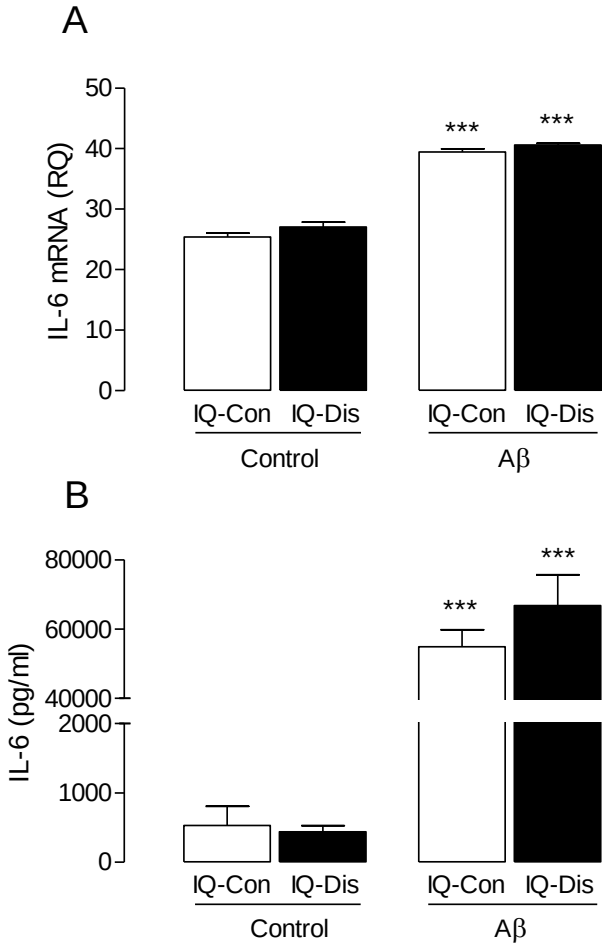

Figure 5.10 Aβ increases expression of IL-6 mRNA and protein in MDMs from both IQ-consistent and IQ-discrepant individuals.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 µM) for 24 h and assessed for expression of IL-6 mRNA (A) by RT-PCR and protein (B) by ELISA. Aβ increased IL-6 mRNA and protein expression in MDMs from both groups and no difference in expression between IQ-Con and IQ-Dis participants was observed. Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. $^{***}p<0.001$ vs vehicle control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,30)}=5.458$, $p<0.0264$], $A\beta_{effect}$ [$F_{(1,30)}=533.7$, $p<0.001$], $interaction_{effect}$ [$F_{(1,30)}=0.1473$, $p<0.7039$]. **B:** $Group_{effect}$ [$F_{(1,27)}=1.679$, $p<0.2061$], $A\beta_{effect}$ [$F_{(1,27)}=174.9$, $p<0.001$], $interaction_{effect}$ [$F_{(1,27)}=1.732$, $p<0.1992$].

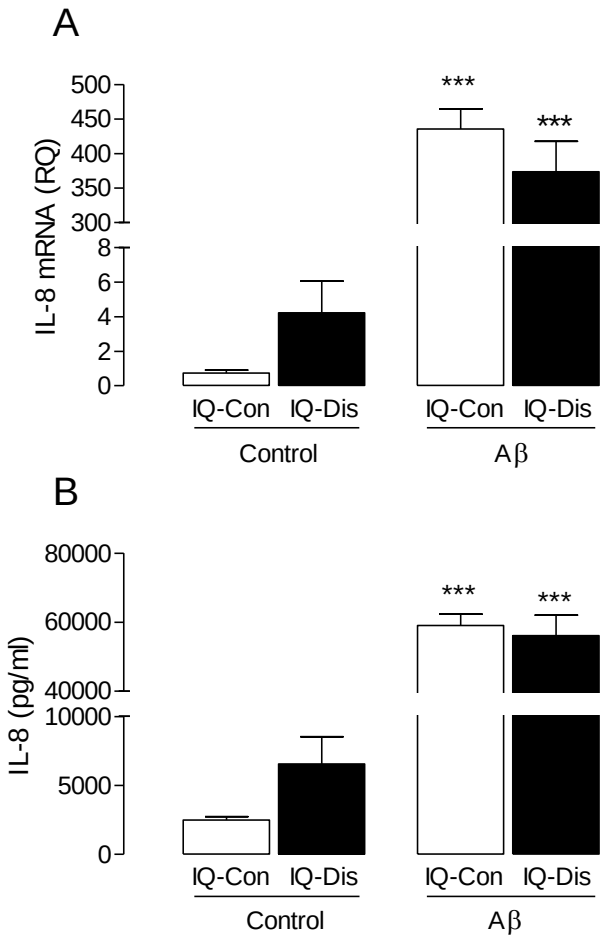

Figure 5.11 Aβ increases expression of IL-8 mRNA and protein in MDMs from both IQ-consistent and IQ-discrepant individuals.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 µM) for 24 h and assessed for expression of IL-8 mRNA (A) by RT-PCR and protein (B) by ELISA. Aβ increased IL-8 mRNA and protein expression in MDMs from both groups and no difference in expression between IQ-Con and IQ-Dis participants was observed. Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. ***p<0.001 vs vehicle control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,29)}$=1.199, p<0.2826], $Aβ_{effect}$ [$F_{(1,29)}$=225.3, p<0.001], $interaction_{effect}$ [$F_{(1,29)}$=1.501, p<0.2304]. **B:** $Group_{effect}$ [$F_{(1,31)}$=0.02567, p<0.8737], $Aβ_{effect}$ [$F_{(1,31)}$=220.3, p<0.001], $interaction_{effect}$ [$F_{(1,31)}$=0.9531, p<0.3365].

5.4 Discussion

The aim of this study was to examine inflammatory changes in individuals described as having a subtle deficit in episodic memory as a biomarker for early cognitive dysfunction. Participants were healthy older adults who were classified as either IQ-consistent or IQ-discrepant based on their performance on a delayed recall test in relation to their estimated pre-morbid IQ. The data show the robustness of IL-8 mRNA in THP-1 cells as a marker of early cognitive decline. MDMs isolated from IQ-consistent and IQ-discrepant participants display an increase in inflammatory markers and cytokine production in response to Aβ, and the findings also demonstrate an increased sensitivity of MDMs from IQ-discrepant individuals to Aβ stimulation.

In Chapter 3, the effect of plasma from IQ-consistent and IQ-discrepant individuals on the expression of pro-inflammatory markers in THP-1 cells was investigated as a potential marker for early cognitive dysfunction, and a specific increase in IL-8 mRNA was revealed in response to plasma from IQ-discrepant subjects. In this chapter, the robustness of IL-8 mRNA in THP-1 cells was investigated in a larger cohort of 46 IQ-consistent and 29 IQ-discrepant participants. Consistent with the previous finding, plasma from IQ-discrepant, compared with IQ-consistent, participants induced a significant upregulation in IL-8 mRNA in THP-1 cells. This was also accompanied by a significant increase in TNFα mRNA expression. As before, no differences in IL-6 or iNOS were observed. The data strengthen the reliability of plasma-induced IL-8 expression in THP-1 monocytes as a biomarker assay indicative of early deficits in cognitive function. Interestingly, plasma from female IQ-discrepant participants was shown to induce higher levels of IL-8 compared with male IQ-discrepant and female IQ-consistent participants. This could be due to sex-specific differences in plasma composition such as levels of estrogens and androgens, although a previous study observed that only 1.8% of plasma proteome differences are attributed to gender (Ignjatovic et al., 2011).

Monocytes positive for CD14 were isolated from whole blood samples from IQ-consistent and IQ-discrepant participants. Blood derived monocytes are commonly

differentiated into macrophages for examination of macrophage function by a range of differentiating agents including GM-CSF and macrophage CSF (M-CSF). Generally, GM-CSF polarises cells into a pro-inflammatory phenotype whereas M-CSF induces a more anti-inflammatory phenotype, and as M-CSF is present at high concentrations in the plasma under resting conditions monocytes are more likely to be pre-disposed towards an anti-inflammatory state (Neu et al., 2013). Monocytes, of which there are 3 subtypes, all express CD14 to varying degrees, therefore it is possible to generate a monocyte-enriched population on the basis of CD14 expression. Upon differentiation to macrophages, the expression of CD14 decreases and CD11b expression increases (Ammon et al., 2000, Ohradanova-Repic et al., 2016). Indeed, CD11b is highly expressed on macrophages and DCs and regulates leukocyte adhesion and migration to sites of inflammation. It can bind to a variety of ligands including complement proteins and LPS, and it was recently found that CD11b regulates TLR4-signalling induced by LPS in DCs, but not macrophages (Ling et al., 2014). Monocytes and macrophages also display a wide range of TLRs, which are innate immune receptors responsible for mediating immune activation in response to a variety of PAMPs. In particular, TLR2 and TLR4 have been implicated in AD. LPS is recognised by a receptor complex of TLR4, an LPS-binding protein and CD14 (Udan et al., 2008). CD14 also acts as an accessory receptor to TLR2, which has mostly been studied via the actions of the synthetic ligand Pam3CSK4. The expression of TLR4 is reportedly increased in glia cells associated with amyloid plaques in the brain of AD patients (Walter et al., 2007). Additionally, the mRNA and protein expression of TLR2 and TLR4 has been reported to be increased in PBMCs from AD patients (Zhang et al., 2012), and this was found to correlate negatively with MMSE scores.

The data show a decreased expression of CD11b in MDMs from IQ-discrepant, compared with IQ-consistent, participants and no significant changes in TLR2, TLR4 and CD206 expression. However, it was difficult to detect modest differences between the participant groups due to the margin of error. In a previous study, the expression of CD11b, TLR2 and TLR4 were assessed in PBMCs and MDMs from IQ-consistent and IQ-discrepant individuals and an increase in CD11b and TLR4

expression on MDMs in IQ-discrepant participants was reported (Downer et al., 2013).

In order to conduct further analysis of inflammatory differences between participants, IQ-consistent participants with the 10 lowest IL-8 mRNA values and IQ-discrepant participants with the 10 highest IL-8 mRNA values were selected. This demonstrates the use of plasma-treated monocytes as a possible screening tool for selecting participants for pilot studies and/or clinical trials. The current study aimed to expand on the previous findings by Downer and colleagues by investigating the effect of Aβ on cell surface marker expression, gene expression and cytokine release in MDMs from a separate cohort of IQ-consistent and IQ-discrepant individuals.

In the smaller cohort of participants, the data show that following Aβ stimulation, MDMs exhibit decreased cell surface expression of CD11b and increased expression of TLR2, TLR4 and CD206. The decrease in the percentage of $CD11b^{+}$ cells in the presence of Aβ was only detected in MDMs from IQ-consistent participants and may perhaps reflect a loss in cell viability and subsequent increase in cellular debris. More profound changes were observed between IQ-consistent and IQ-discrepant participants when examining the smaller cohort of selected participants. The expression of TLR2 and CD206 on $CD11b^{+}$ cells was increased in MDMs from IQ-discrepant, compared with IQ-consistent, individuals, and this was exacerbated further by Aβ stimulation.

Additionally, CD11b and MRC-1 mRNA were downregulated, while mRNA expression of TLR2 was increased, in MDMs following exposure to Aβ. However, no differences were observed in CD11b, TLR2, TLR4 or CD206 mRNA expression in the presence or absence of Aβ between MDMs from IQ-consistent and IQ-discrepant participants. This is in contrast to previous findings where a significant increase in CD11b protein expression was reported on MDMs from IQ-discrepant compared with IQ-consistent subjects (Downer et al., 2013). Furthermore, this study also revealed that MDMs from IQ-discrepant participants exhibit an increased expression of TLR2 and TLR4 mRNA, as well as an increase in protein expression of TLR4. The discrepancies between the two studies indicate that while MDMs from IQ-consistent and IQ-

discrepant participants show differential expression in protein and gene markers, it may not be the most reliable approach for the development of potential biomarkers.

While the current study does not show alterations in gene expression in MDMs from IQ-consistent and IQ-discrepant participants, the increased cell surface expression of TLR2 and CD206 in MDMs from IQ-discrepant participants suggests that these cells are more responsive to ligands for these receptors. Indeed, exposure to Aβ enhanced expression of TLR2 and CD206 in MDMs derived from IQ-discrepant subjects. Aβ has previously been reported to bind to and increase expression of TLR2 on macrophages (Udan et al., 2008) and microglia (Jana et al., 2008, Costello et al., 2015). TLR2 deficiency reduces Aβ triggered inflammation but enhances Aβ phagocytosis in both microglia and macrophages (Liu et al., 2012). Monocytes/macrophages from AD patients have been reported to display an increased expression of TLR2 and TLR4 following Aβ42 stimulation, as well as an increase in IL-6 and CCR2 (Saresella et al., 2014). This might be an explanation for the fact that the phagocytic capacity of monocytes/macrophages from AD patients is defective compared with controls (Fiala et al., 2005). CD206, or mannose-receptor, is expressed on a variety of tissue macrophage subtypes including perivascular macrophages, choroid plexus macrophages and meningeal macrophages, and plays an important role in endocytosis and phagocytosis (Galea et al., 2005). Interestingly the expression of CD206, which is regarded as an M2-like marker, was also increased by Aβ in MDMS derived from IQ-discrepant participants, which might suggest that these cells have an increased phagocytic capacity.

The study also demonstrates a discrepancy between the protein and gene expression of markers under basal conditions and following stimulation, in particular TLR2, TLR4 and MRC-1. This is likely due to the fact that data acquired via flow cytometry assesses the surface expression of receptors and does not assess internal protein levels, while PCR examines gene expression at the transcriptional level within the cell, therefore it is possible that the time point used in the experiment was not optimal for assessing both surface and mRNA expression. It has been previously reported that increases in mRNA expression occur prior to subsequent changes in protein expression, such as for pro-inflammatory cytokines IL-6, TNFα and IL-1β

(Minogue et al., 2012). The expression of TLR2 and TLR4 has also been shown to be temporally regulated. For example in a study of post-operative patients, TLR2 and TLR4 mRNA expression in PBMCs peaked at Day 0 and decreased thereafter, while cell surface expression assessed by flow cytometry peaked 24 h later on Day 1 (Jianfang et al., 2013). Furthermore in response to LPS challenge in mouse macrophages, TLR2 mRNA expression peaked at 2 h post-stimulation, while the mRNA expression of TLR4 mRNA remained constant up until 12 h and decreased 24 h after LPS stimulation (Matsuguchi et al., 2000). There is also the possibility that the discrepancies in mRNA and cell surface expression represents a feedback mechanism, or if there is an abundance of receptors already present in the cytosol, they can be recruited to the plasma membrane upon stimulation thereby forgoing transcription in the nucleus.

The effect of LPS stimulation on protein and gene expression in MDMs from IQ-consistent and IQ-discrepant subjects was also assessed but no significant changes in CD11b, TLR2, TLR4 and CD206 expression were observed in MDMs from both IQ-consistent and IQ-discrepant participants following exposure to LPS (Appendix IV, Table S1). Similar to the effects of Aβ, LPS increased TLR2 mRNA expression and decreased CD11b and MRC-1 mRNA, while the expression of TLR4 mRNA remained unchanged. The fact that MDMs derived from IQ-discrepant individuals did not show an increased sensitivity to LPS but are more responsive to Aβ stimulation, may derive from the observed increase in TLR2 expression.

The impact of Aβ treatment on the production and mRNA expression of cytokines/chemokines was also assessed on MDMs from the smaller cohort of participants. Aβ significantly increased the production of TNFα, IL-6 and IL-8 in MDMs from both groups. Similarly, LPS-treated MDMs released significantly higher levels of TNFα, IL-6 and IL-8 (Appendix IV, Table S1). Despite a trend towards an increase in cytokine production in Aβ- and LPS- stimulated MDMs from IQ-discrepant, compared with IQ-consistent, participants, this did not reach statistical significance. This is in contrast to the study conducted by Downer and colleagues (2013) which reported an exaggerated release of TNFα, IL-6, IL-12p70 and IL-10 in LPS-stimulated MDMs derived from IQ-discrepant participants. This variation

between studies might have arisen due to the differences in analytical platforms used and the number of participants included. Furthermore, due to the fact that TLR2 expression was enhanced on MDMs from IQ-discrepant participants, it is perhaps surprising that a corresponding overproduction of cytokines is not observed. The mRNA expression of TNFα, IL-6 and IL-8 was also significantly increased by Aβ (and LPS) stimulation, and here, MDMs derived from IQ-discrepant participants displayed an enhanced expression of TNFα mRNA in response to Aβ. It is possible that perhaps some markers on MDMs from IQ-discrepant participants are more sensitised to inflammatory stimuli than others, although further examination is required to assess this.

Aβ has previously been reported to induce the production of inflammatory cytokines in monocytes/macrophages, including TNFα, IL-1β, IL-6, IFNγ and IL-10 (Pellicanò et al., 2010). Although MDMs from AD patients have not been widely examined, one particular study revealed that monocytes from MCI and AD patients secreted higher levels of IL-1β, IL-6, TNFα and IL-12 under basal conditions and following LPS treatment (Guerreiro et al., 2007). However, numerous studies have examined cytokine production and the response to inflammatory stimuli in PBMCs derived from AD patients. A report by Pellicanò and colleagues (2010) revealed that PBMCs from AD patients exhibited an enhanced release of chemokines such as CCL5 and CCL4. Other studies have found that PBMCs from AD patients produce higher levels of TNFα, IL-6 and IL-1β compared with healthy controls (Reale et al., 2004), and that TNFα release from PBMCs from AD patients increases longitudinally (Francois et al., 2015). In addition, TNFα, IL-1β, IL-6 and IL-10 release from LPS-stimulated PBMCs was increased in cells derived from AD patients compared to controls (Lombardi et al., 1999), and IL-6 was increased further with the addition of Aβ42 (Kaplin et al., 2009). In response to Aβ42, PBMCs from AD patients display an enhanced production of TNFα, IL-1β, CCL2 and IL-10 compared with healthy controls (Reale et al., 2014). In contrast to these studies, a decrease in IL-10 and no difference in IFNγ or TNFα production was observed following stimulation with Aβ40 in PBMCs from AD patients compared to controls (Speciale et al., 2007). PBMCs from MCI patients have also been investigated, and it was reported that phytohemagglutinin (PHA)-

stimulated PBMCs exhibited increased levels of IL-8, IL-6 and IL-10, while LPS-stimulated PBMCs showed lower TNFα/IL-10, IL-6/IL-10 and IL-8/IL-10 ratios compared with controls (Magaki et al., 2007). Overall the findings suggest that PBMCs are dysfunctional in AD and examination of PBMCs from earlier cohorts of cognitive impairment is warranted for potential biomarkers indicative of AD.

The current study builds on previous findings reported in MDMs from a cohort of healthy older adults which exhibit a subtle deficit in episodic memory. Here, an increase in cell surface expression of TLR2 was evident in MDMs in IQ-discrepant, compared with IQ-consistent, participants. The response to Aβ was also investigated and Aβ-related effects were observed in both groups for all markers examined. In addition, the expression of TLR2 and CD206 were enhanced on Aβ-stimulated MDMs from IQ-discrepant participants, accompanied by an exaggerated increase in TNFα mRNA, suggesting a possible link between Aβ activation of TLR2/CD206 and TNFα expression. The findings indicate that inflammatory changes are evident in peripheral cells from a population with early cognitive decline, and may represent a useful platform for investigating cognitive dysfunction in neurodegenerative disease.

Chapter 6: Inflammatory and metabolic changes in PBMCs from IQ-consistent and IQ-discrepant participants in response to Aβ + LPS

6.1 Introduction

Identifying the role of metabolic pathways in regulating the functional consequences of immune cell activation and response to inflammation has gained significant interest in recent years. A shift in metabolism from oxidative phosphorylation towards glycolysis occurs in macrophages in response to pro-inflammatory stimuli including IFNγ and LPS (Kelly and O'Neill, 2015). One of the mechanisms by which this switch is thought to occur is through activation of the ubiquitous form of PFK2 (PFKFB3), and consistent with this inhibition of PFKFB3 suppresses pro-inflammatory cytokine secretion and glycolytic flux under stimulatory conditions in macrophages (Tawakol et al., 2015).

Alterations in bioenergetics and metabolism accompany the inflammatory phenotype identified in AD. Mitochondrial dysfunction has been identified as an early event in the pathogenesis of a mouse model of AD that precedes the formation of Aβ plaques (Yao et al., 2009), and decreases in the expression/activation of enzymes involved in mitochondrial bioenergetics, including cytochrome c oxidase and PDH, have been observed in the brains of AD patients *post-mortem* (Blass et al., 2000). Cerebral glucose metabolism, as measured by FDG PET, has repeatedly been shown to be reduced in AD patients, and its utility as a biomarker of early cognitive dysfunction has been demonstrated by successfully predicting conversion of MCI to AD (Cohen and Klunk, 2014). Interestingly, aerobic glycolysis in the brains of young adults has been shown to spatially correlate with Aβ deposition in later life, suggesting a link between aerobic glycolysis and the development of an Alzheimer-like pathology (Vlassenko et al., 2010).

Abnormalities in the metabolic function of peripheral cells have also been observed in AD. The expression of genes involved in the respiratory chain of oxidative phosphorylation, as detected through microarray analysis, are decreased from whole blood of MCI and AD patients (Lunnon et al., 2012). Recently, it was also reported that mitochondrial respiration was impaired in PBMCs from AD patients compared with healthy controls (Maynard et al., 2015).

The data presented in Chapter 5 indicated that some inflammatory markers were increased in Aβ-stimulated MDMs from a smaller cohort of selected IQ-discrepant participants, compared with IQ-consistent participants. Given the indications of bioenergetic dysfunction in AD and the role of inflammatory stimuli in altering the metabolic profile of cells, metabolic function of PBMCs from this cohort of participants was assessed in response to inflammatory stimuli.

The aims of this study were:

1. To compare the mRNA expression and release of inflammatory cytokines in PBMCs from IQ-consistent and IQ-discrepant individuals under resting conditions and following Aβ+LPS stimulation.
2. To compare the metabolic signature and mRNA expression of PFKFB isozymes in PBMCs from IQ-consistent and IQ-discrepant participants under resting conditions and following Aβ+LPS stimulation.

6.2 Methods

Healthy older adults were recruited from the MRU at Trinity College Dublin and classified as either IQ-consistent or IQ-discrepant as previously described in sections 2.7.1 and 2.7.3. PBMCs were isolated from whole blood samples and stored in liquid nitrogen as described in section 2.8.

PBMCs from individuals in the IQ-consistent cohort with the 10 lowest IL-8 mRNA values and individuals from the IQ-discrepant cohort with the 10 highest IL-8 mRNA values (see Figure 5.4), were selected for further analysis. These selected cohorts were age- and gender-matched and showed no difference in MMSE or MoCA scores. A significant decrease in delayed recall z-score and increase in cognitive asymmetry z-score was observed in IQ-discrepant, compared with IQ-consistent, individuals (Table 5.2, $p<0.001$).

Cryopreserved PBMCs were stimulated with a combination of Aβ (10 μM) and LPS (100 ng/ml) as described in section 2.11 and harvested for analysis of gene expression by RT-PCR, cytokine production by ELISA and metabolic function as described in sections 2.15, 2.15, 2.18 and 2.20. Subset populations of cryopreserved PBMCs were also assessed by flow cytometry as described in section 2.19.

6.3 Results

6.3.1 T cells are the predominant cell population in PBMCs

PBMCs are primarily composed of monocytes and lymphocytes (T cells, B cells, NK cells). The cell subsets in cryopreserved PBMCs in this study were assessed by flow cytometry. The majority of cells (65%) present were $CD3^+$ T cells, and the percentage of these cells was significantly higher than all other cell populations. $CD19^+$ B cells, $CD16^+$ NK cells and $CD14^+$ monocytes constituted 14%, 5% and 4% of the total population respectively (Figure 6.1, $p<0.05$, $p<0.001$, One-way ANOVA with Newman Keuls *post-hoc* analysis).

6.3.2 Pro-inflammatory cytokine production is enhanced in PBMCs stimulated with Aβ+LPS from IQ-discrepant individuals

PBMCs from IQ-consistent and IQ-discrepant participants were incubated with Aβ+LPS for 24 hours and the inflammatory phenotype was assessed by examining the mRNA expression of TNFα and iNOS by RT-PCR and release of pro-inflammatory cytokines TNFα, IL-6, IL-8 and IL-1β by ELISA. Aβ+LPS significantly increased TNFα mRNA expression, although the expression of iNOS remained unchanged (Figure 6.2 A, B, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). Although TNFα mRNA was slightly increased in resting PBMCs from IQ-discrepant subjects, no significant differences in TNFα or iNOS expression under unstimulated or stimulated conditions were observed between PBMCs from IQ-consistent and IQ-discrepant participants.

Aβ+LPS significantly increased the release of TNFα, IL-6, IL-8 and IL-1β in PBMCs from both IQ-consistent and IQ-discrepant individuals (Figure 6.3 A, B, C, D, $p<0.01$, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). The production of TNFα, IL-6, IL-8 and IL-1β was similar in unstimulated PBMCs from IQ-consistent and IQ-discrepant participants; however Aβ+LPS had a significantly greater effect on TNFα, IL-6 and IL-8 secretion in PBMCs from IQ-discrepant, compared with IQ-consistent, individuals (Figure 6.3 A, B, C, $p<0.05$, $p<0.001$, Two-

way ANOVA followed by Newman Keuls *post-hoc* analysis). This demonstrates that PBMCs from IQ-discrepant participants are more responsive to inflammatory stimuli.

6.3.3 Aβ+LPS induces a shift towards glycolysis in PBMCs which is enhanced in IQ-discrepant participants

Having established that PBMCs from IQ-consistent and IQ-discrepant subjects exhibit a differential response to Aβ+LPS, a glycolytic stress test was carried out to examine the metabolic profile of PBMCs from IQ-consistent and IQ-discrepant participants in the presence and absence of Aβ+LPS stimulation for 24 hours. The data show an increase in ECAR in PBMCs from IQ-discrepant, compared with IQ-consistent, participants, under unstimulated and stimulated conditions (Figure 6.4 A). A significant effect of Aβ+LPS treatment and a significant cohort effect in glycolysis was observed (Figure 6.4 B, $p<0.05$, $p<0.01$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis), but no significant differences were found with *post-hoc* analysis. No changes in glycolytic capacity were observed in the presence and absence of Aβ+LPS or between participant groups (Figure 6.4 C). Exposure to Aβ+LPS induced a significant decrease in OCR:ECAR in PBMCs from both participant groups, indicating a metabolic switch towards glycolysis, although no differences between IQ-consistent and IQ-discrepant individuals was observed under unstimulated and stimulated conditions (Figure 6.4 D, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). This shows that Aβ+LPS generates an increase in glycolytic flux and suggests that metabolism in PBMCs from IQ-consistent and IQ-discrepant participants may be differentially regulated.

6.3.4 PBMCs stimulated with Aβ+LPS from IQ-discrepant participants show an exaggerated increase in PFKFB3 mRNA expression and PFKFB3:PFKFB1

Due to the role that the PFKFB isozymes play in regulating glycolysis, the expression of PFKFB1 and PFKFB3 were assessed in PBMCs from IQ-consistent and IQ-discrepant participants in the presence and absence of Aβ+LPS. The expression of PFKFB1 mRNA remained unchanged in PBMCs between participant groups and no effect of Aβ+LPS stimulation was observed (Figure 6.5 A). Aβ+LPS significantly upregulated PFKFB3

mRNA expression in PBMCs from both participant groups, and while no difference in PFKFB3 mRNA was observed under resting conditions, the Aβ+LPS induced effect was significantly higher in PBMCs from IQ-discrepant, compared with IQ-consistent, individuals (Figure 6.5 B, $p<0.05$, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). The Aβ+LPS induced increase in the PFKFB3:PFKFB1 ratio was also found to be significantly enhanced in PBMCs from IQ-discrepant participants (Figure 6.5 C, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis); this was found to be significantly correlated with the rate of glycolysis (Figure 6.5 D, $p<0.0065$; linear regression analysis).

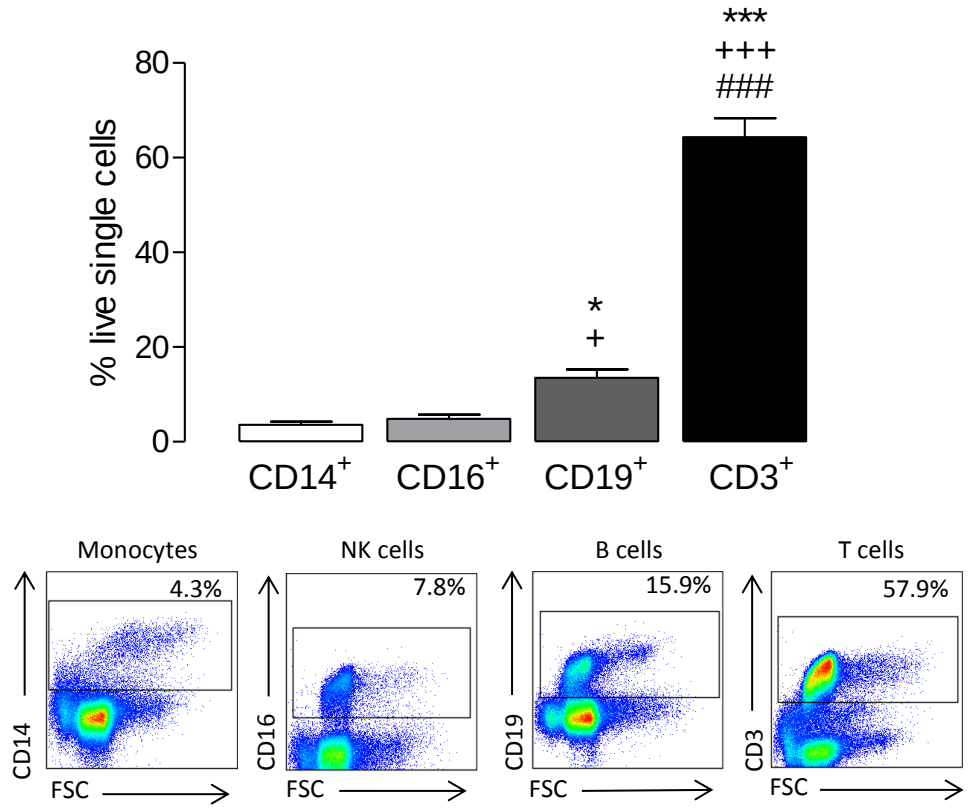

Figure 6.1 PBMCs constitute primarily T cells.

PBMCs from IQ-consistent and IQ-discrepant participants were isolated from whole blood and the percentage of monocytes and lymphocyte subset populations assessed by flow cytometry after cryopreservation. The percentage of $CD3^+$ T cells was significantly higher compared with all other cell types, and there were significantly more $CD19^+$ B cells than $CD14^+$ monocytes and $CD16^+$ NK cells. Representative FACS plots are shown. Data are expressed as the mean ± SEM, n=4. *p<0.05, ***p<0.001 vs $CD14^+$, ^+p<0.05, ^{+++}p<0.001 vs $CD16^+$, $^{\#\#\#}p$<0.001 vs $CD19^+$. One-way ANOVA followed by Newman Keuls *post-hoc* analysis.

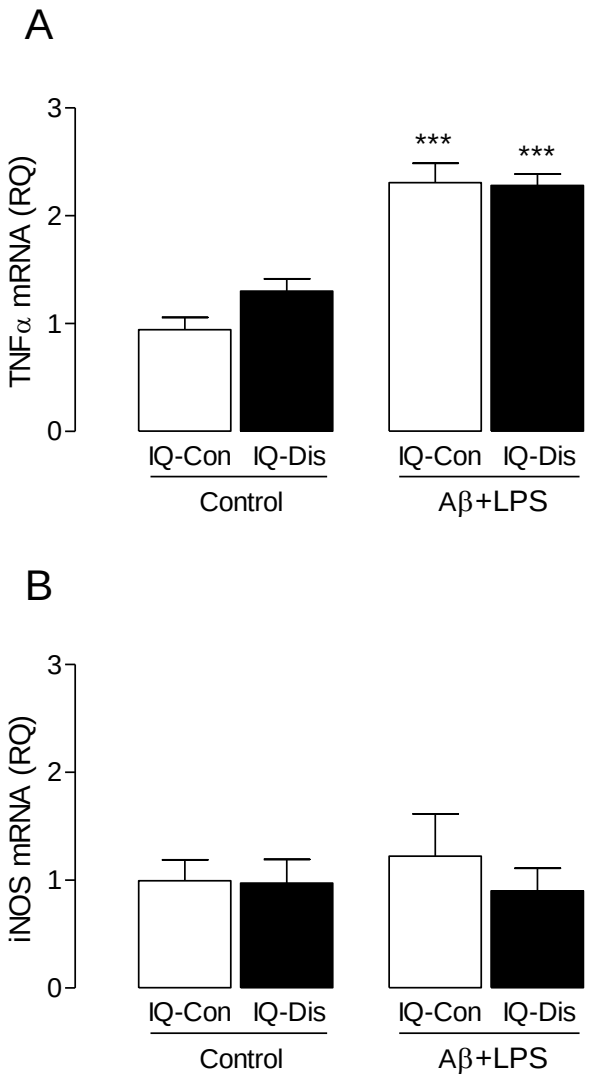

Figure 6.2 Aβ+LPS increases TNFα mRNA in PBMCs from IQ-consistent and IQ-discrepant individuals.

PBMCs from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and incubated with or without Aβ (10 μM) and LPS (100 ng/ml) for 24 h and assessed for expression of TNFα and iNOS mRNA by RT-PCR. TNFα (A), but not iNOS (B), mRNA expression was increased in response to Aβ+LPS but no differences between the participant groups were observed. Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. ***p<0.001 vs vehicle control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,28)}$=1.668, p<0.2071], $A\beta+LPS_{effect}$ [$F_{(1,28)}$=83.87, p<0.001], $interaction_{effect}$ [$F_{(1,28)}$=2.253, p<0.1446]. **B:** $Group_{effect}$ [$F_{(1,30)}$=0.4338, p<0.5151], $A\beta+LPS_{effect}$ [$F_{(1,30)}$=0.08426, p<0.7736], $interaction_{effect}$ [$F_{(1,30)}$=0.3266, p<0.5719].

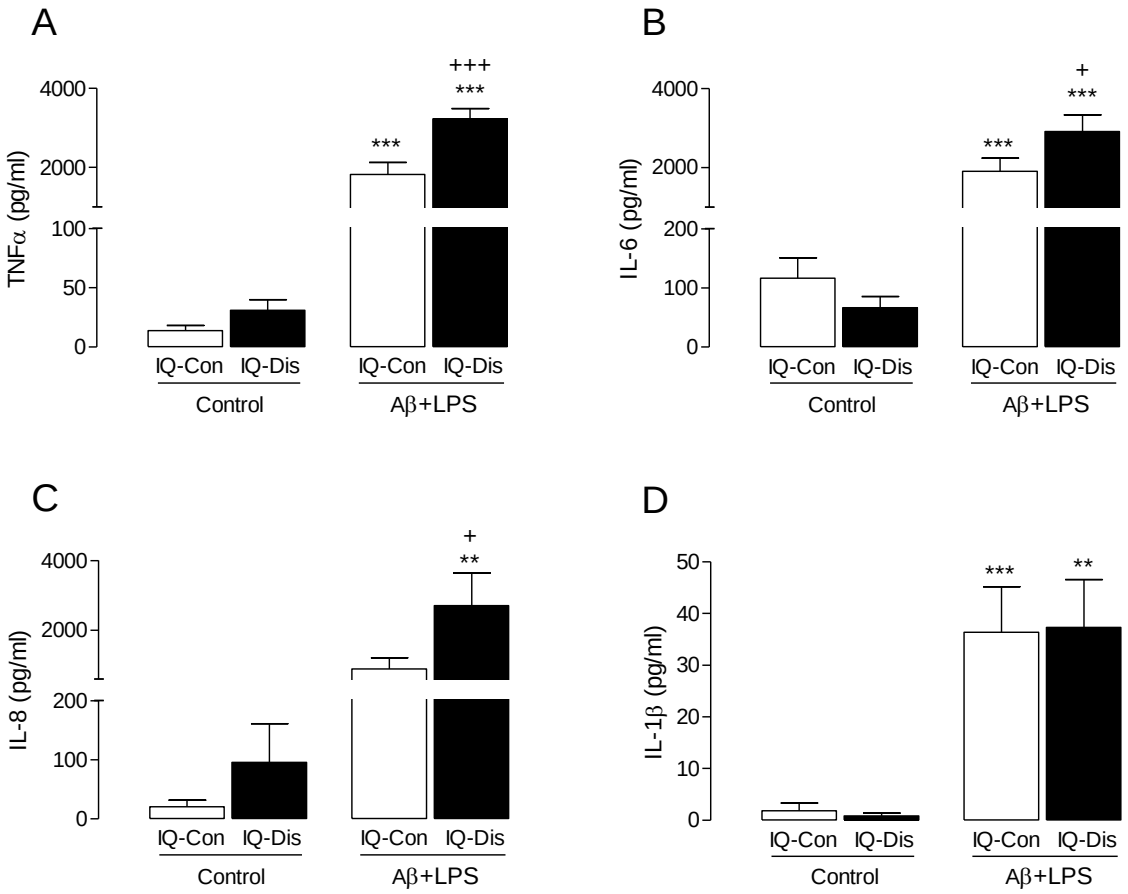

Figure 6.3 Aβ+LPS induced TNFα, IL-6 and IL-8 release is exacerbated in PBMCs from IQ-discrepant individuals.

PBMCs from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and incubated with or without Aβ (10 μM) and LPS (100 ng/ml) for 24 h and release of TNFα, IL-6, IL-8 and IL-1β was assessed by ELISA. Aβ+LPS induced the secretion of TNFα (A), IL-6 (B), IL-8 (C) and IL-1β (D) from PBMCs from both IQ-consistent and IQ-discrepant participants; and the release of TNFα, IL-6 and IL-8 was exaggerated in PBMCs from IQ-discrepant participants. Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. **p<0.01, ***p<0.001 vs vehicle control; ^{+}p<0.01, ^{+++}p<0.001 vs group control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,29)}$=12.00, p<0.0017], $Aβ+LPS_{effect}$ [$F_{(1,29)}$=147.3, p<0.001], $interaction_{effect}$ [$F_{(1,29)}$=11.43, p<0.0021]. **B:** $Group_{effect}$ [$F_{(1,32)}$=2.789, p<0.1047], $Aβ+LPS_{effect}$ [$F_{(1,32)}$=65.25, p<0.001], $interaction_{effect}$ [$F_{(1,32)}$=3.400, p<0.0745]. **C:** $Group_{effect}$ [$F_{(1,31)}$=3.421, p<0.0739], $Aβ+LPS_{effect}$ [$F_{(1,31)}$=11.57, p<0.0019], $interaction_{effect}$ [$F_{(1,31)}$=2.902, p<0.0985]. **D:** $Group_{effect}$ [$F_{(1,32)}$=0.00017, p<0.997], $Aβ+LPS_{effect}$ [$F_{(1,32)}$=30.57, p<0.001], $interaction_{effect}$ [$F_{(1,32)}$=0.0248, p<0.8777].

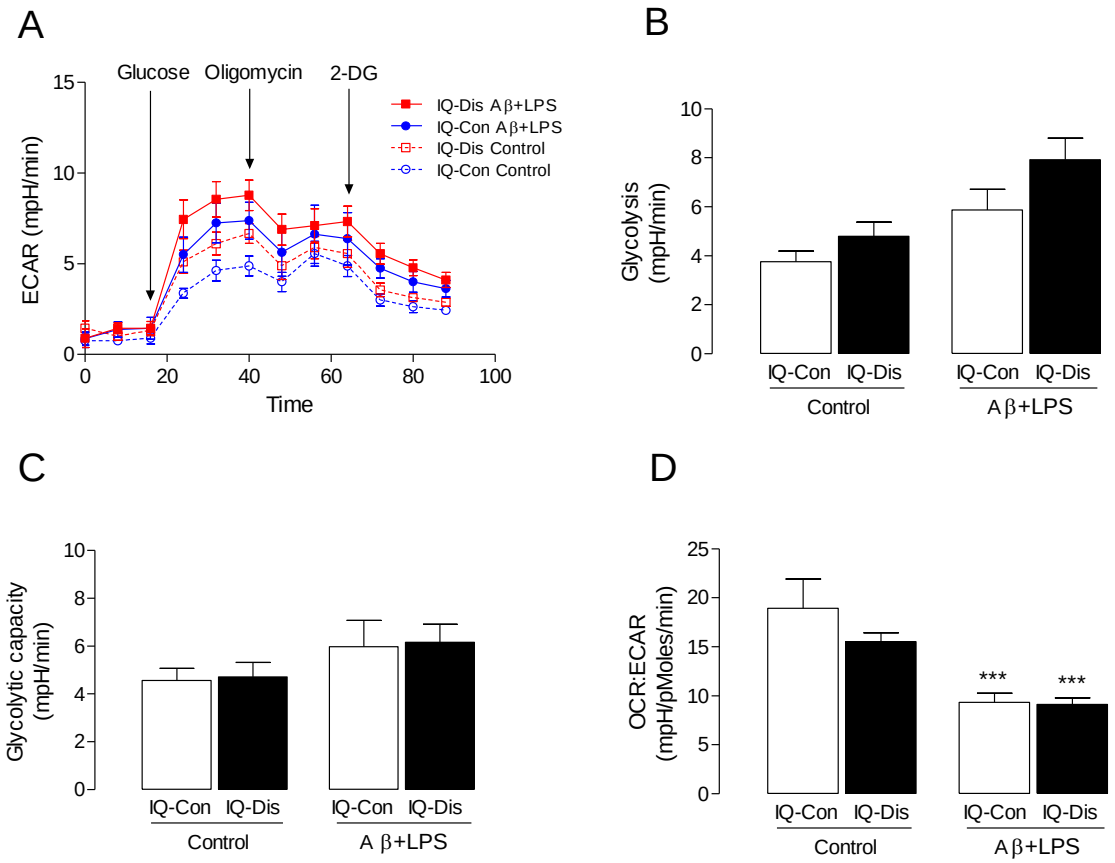

Figure 6.4 PBMCs from IQ-discrepant individuals show an increase in glycolysis which is enhanced by Aβ+LPS stimulation.

PBMCs from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and incubated with or without Aβ (10 µM) and LPS (100 ng/ml) for 24 h and a glycolytic stress test performed to examine metabolic function. The metabolic profile (A) and analysis of the mean data show a significant effect of Aβ+LPS and a significant cohort effect in glycolysis (B). No difference in glycolytic capacity (C) was observed, and Aβ+LPS significantly decreased OCR:ECAR in PBMCs from both participant groups (D). Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. ***p<0.001 vs vehicle control; Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

B: $Group_{effect}$ [$F_{(1,28)}$=4.639, p<0.0400], $A\beta+LPS_{effect}$ [$F_{(1,28)}$=13.36, p<0.0011], $interaction_{effect}$ [$F_{(1,28)}$=0.4805, p<0.4939]. **C:** $Group_{effect}$ [$F_{(1,28)}$=0.4464, p<0.8342], $A\beta+LPS_{effect}$ [$F_{(1,28)}$=3.642, p<0.8342], $interaction_{effect}$ [$F_{(1,28)}$=0.0009, p<0.9759]. **D:** $Group_{effect}$ [$F_{(1,29)}$=1.237, p<0.2751], $A\beta+LPS_{effect}$ [$F_{(1,29)}$=24.28, p<0.001], $interaction_{effect}$ [$F_{(1,29)}$=0.9774, p<0.3310].

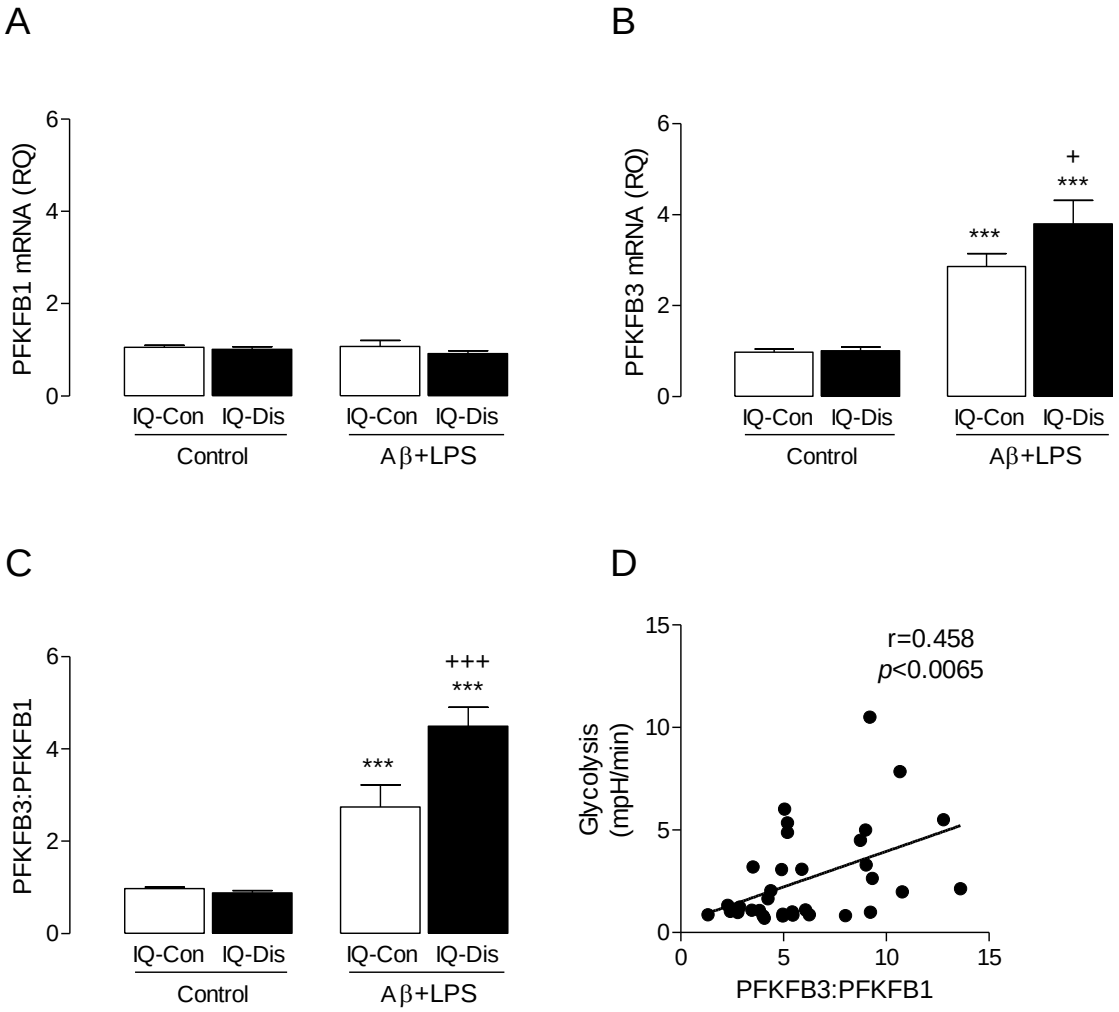

Figure 6.5 Aβ+LPS-stimulated PBMCs from IQ-discrepant individuals show an enhanced expression of PFKFB3 mRNA and an increase in PFKFB3:PFKFB1.

PBMCs from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and incubated with or without Aβ (10 µM) and LPS (100 ng/ml) for 24 h and assessed for expression of PFKFB3 and PFKFB1 mRNA. No difference in PFKFB1 mRNA expression was observed (A). PBMCs from IQ-discrepant participants show an exacerbated increase in PFKFB3 mRNA (B) and PFKFB3:PFKFB1 (C) in response to Aβ+LPS stimulation; and PFKFB3:PFKFB1 was significantly correlated with glycolysis (D). Data are expressed as the mean ± SEM, IQ-Con n=10, IQ-Dis n=10. ***p<0.001 vs vehicle control; ^{+}p<0.05, ^{+++}p<0.001 vs group control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $\text{Group}_{\text{effect}}$ [$F_{(1,25)}$=1.966, p<0.1732], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,25)}$=0.3098, p<0.5828], $\text{interaction}_{\text{effect}}$ [$F_{(1,25)}$=0.5831, p<0.4522]. **B:** $\text{Group}_{\text{effect}}$ [$F_{(1,30)}$=2.506, p<0.1239], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,30)}$=58.89, p<0.001], $\text{interaction}_{\text{effect}}$ [$F_{(1,30)}$=2.190, p<0.1493]. **C:** $\text{Group}_{\text{effect}}$ [$F_{(1,25)}$=7.461, p<0.0114], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,25)}$=78.85, p<0.001], $\text{interaction}_{\text{effect}}$ [$F_{(1,25)}$=9.214, p<0.0055].

6.4 Discussion

The present study investigated the effect of Aβ+LPS on metabolic and inflammatory markers in PBMCs from a population of healthy older adults who under-performed on a cognitive task examining episodic memory in relation to their estimated premorbid IQ. PBMCs from IQ-discrepant participants were found to have an enhanced production of inflammatory cytokines in response to Aβ+LPS stimulation, and an increase in glycolysis compared with PBMCs from IQ-consistent participants. Furthermore, the expression of PFKFB3 mRNA, and the ratio of PFKFB3:PFKFB1, was increased in Aβ+LPS-stimulated PBMCs from IQ-discrepant, compared with IQ-consistent, individuals. The rate of glycolysis was found to be significantly correlated with the PFKFB3:PFKFB1 ratio, and therefore the data suggest that Aβ+LPS stimulation of PBMCs upregulates PFKFB3 mRNA to drive glycolysis and increase the production of inflammatory cytokines. This is exacerbated in PBMCs from IQ-discrepant participants, which indicates that PBMC function is dysregulated in this cohort of individuals.

The process of density gradient centrifugation of whole blood results in the generation of a rich population of monocytes and lymphocytes (PBMCs). The composition of PBMCs varies between individuals, but it generally comprises approximately 2-10% monocytes, 6-29% NK cells, 7-23% B cells and 61-85% T cells (Reichert et al., 1991, Kasper and Randolph, 2005). Here, leukocyte populations in PBMCs after cryopreservation from 4 participants was examined and the population of cells was similar to previously reported data. Cryopreservation of PBMCs for 20 months in liquid nitrogen was not previously found to substantially affect the proportions of $CD3^+$ and $CD16^+$ cells, although in one study a slight decrease in $CD19^+$ cells was observed and the proportion of $CD14^+$ cells was highly variable (Brown et al., 1991). However, others have reported that the percentage of $CD14^+$ monocytes, as well as $CD3^+$ T cells, is not affected by cryopreservation and that the response of resuscitated cells to LPS stimulation as measured by cytokine production is also not affected (Jones et al., 2015a). The high proportion of T cells contributing to the

composition of PBMCs indicates that the responses of PBMCs to Aβ+LPS stimulation examined in this chapter are mostly T cell derived.

PBMCs from IQ-consistent and IQ-discrepant participants were stimulated with a combination of Aβ and LPS in order to induce a robust inflammatory response. As anticipated, the mRNA expression of TNFα and the production of TNFα, IL-6, IL-8 and IL-1β was significantly increased following Aβ+LPS stimulation. The expression of iNOS mRNA was not induced by Aβ+LPS stimulation and indicates that the traditional archetypal pro-inflammatory marker used in animal studies is not useful for examining the inflammatory response in human primary cells. Although no differences in TNFα mRNA were observed between IQ-consistent and IQ-discrepant participants in Aβ+LPS-stimulated cells (which contrasts to the finding that Aβ induces a greater effect in TNFα mRNA MDMs from IQ-discrepant individuals in Chapter 5), PBMCs from IQ-discrepant participants showed an exaggerated release of TNFα, IL-6 and IL-8 compared with PBMCs from IQ-consistent participants. This suggests that PBMCs from IQ-discrepant subjects are more sensitised to the effects of Aβ+LPS stimulation.

PBMCs from MCI and AD patients have been shown to exhibit higher basal levels of cytokine production and an augmented response to inflammatory stimuli, as discussed in Chapter 5. The findings described in the current chapter suggest that an enhanced response to Aβ+LPS in PBMCs may be an indicator of even earlier cognitive change. Higher secretion of TNFα and IL-1β from PBMCs is associated with an increased risk of developing AD (Tan et al., 2007), and an increased production of these cytokines has been reported in PBMCs following LPS stimulation (Jansky et al., 2003). Transcription of pro-inflammatory cytokines occurs via NF-κB signalling, which is dysregulated in PBMCs from AD patients (Ascolani et al., 2012). As the majority of PBMCs are T cells, it is likely that the findings demonstrate a T cell-specific response. This is broadly consistent with previous studies, which have showed that T cells from older adults and AD patients are more reactive to Aβ stimulation (Monsonego et al., 2003) and IL-6 release is enhanced in T cells from AD patients compared with controls (Pirker-Kees et al., 2013).

Inflammatory stimuli have been shown to alter the metabolism of immune cells, therefore the effect of Aβ+LPS on the metabolic profile of PBMCs was investigated. Aβ+LPS stimulation induced a shift towards glycolysis as demonstrated by the increase in glycolysis and decrease in the ratio of OCR:ECAR. The production of ATP via glycolysis, though an inefficient means of glucose metabolism, rapidly generates ATP to provide the energy requirements for bactericidal activity (Kelly and O'Neill, 2015). As a fall in the mitochondrial membrane potential occurs in LPS-activated macrophages, the rapid increase in ATP production is required to ensure cell survival during the host immune response (Zhu et al., 2015). An increase in glycolysis has also been shown to be required for clonal expansion of B cells, T cells and NK cells (Jones et al., 2015a). No difference in glycolytic capacity of PBMCs was observed in response to Aβ+LPS, which suggests that the cells might already be respiring at maximum capacity. The decrease in OCR:ECAR indicates a reduction in mitochondrial respiration, which is consistent with previous reports showing a decrease in OCR in LPS-activated macrophages (Haschemi et al., 2012). Microarray analysis has revealed that genes involved in glycolysis were found to be upregulated in PBMCs following LPS challenge, and the expression of mRNAs encoding genes associated with the TCA cycle are decreased (Cheng et al., 2016). Interestingly, inflammatory cytokine production in LPS-activated PBMCs is dependent on glycolysis, as inhibition with 2-DG attenuated the secretion of IL-1β, IL-6 and TNFα (Jones et al., 2015a). The findings presented here also show that an increase in glycolysis and pro-inflammatory cytokine release occur in tandem, again linking pro-inflammatory activated PBMC cytokine production with an increase in glycolytic flux.

Importantly, the data reveal that glycolysis is increased in PBMCs from IQ-discrepant compared with IQ-consistent individuals. However, no differences in OCR:ECAR were observed between participant groups suggesting that an impairment in oxidative phosphorylation does not accompany the increase in glycolysis in PBMCs from IQ-discrepant subjects. PBMCs from AD patients have previously been shown to exhibit impairments in mitochondrial respiration, as seen by a decrease in OCR (Maynard et al., 2015). Furthermore, this study also showed that ECAR was unchanged in resting PBMCs from AD patients compared with healthy controls. Another recent study has

found that proteins involved in the electron transport chain of mitochondrial respiration are decreased in PBMCs from MCI and AD patients (Delbarba et al., 2016). The data suggest that an altered metabolic profile is present in PBMCs of individuals with cognitive dysfunction and may be an indicator of early cognitive change. Impairments in mitochondrial respiration are also evident in embryonic neurons from a mouse model of AD, where a decrease in OCR and increase in ECAR was reported, indicating that defects in metabolism occur early in disease pathogenesis (Yao et al., 2009).

A significant factor in driving glycolysis is the upregulation of an isoform of PFK2, PFKFB3. PFK2 controls the intracellular levels of F-2,6-BP, which activates PFK1 to convert fructose-6-phosphate to the next intermediate of the glycolytic pathway, fructose-1,6-bisphosphate (Kelly and O'Neill, 2015). PFK2 is a bifunctional enzyme that can synthesize or degrade F-2,6-BP and can therefore regulate glycolytic flux. The L-isoform of PFK2 encoded by the PFKFB1 gene has a balanced kinase:bisphosphatase ratio and therefore maintains low levels of F-2,6-BP. However, u-PFK2 which is encoded by the PFKFB3 gene, has a significantly higher kinase:bisphosphatase ratio which results in the accumulation of the glycolytic intermediate and can therefore increase glycolytic flux (Rodriguez-Prados et al., 2010). The results from the present study reveal that while mRNA expression of PFKFB1 is not altered by Aβ+LPS stimulation in PBMCs, the expression of PFKFB3 mRNA is significantly increased following exposure to Aβ+LPS. Furthermore, PBMCs from IQ-discrepant participants display an enhanced expression of PFKFB3, and also in the ratio of PFKFB3:PFKFB1, compared with PBMCs from IQ-consistent participants. The rate of glycolysis was found to be correlated with the PFKFB3:PFKFB1 ratio, which is consistent with its ability to drive glycolysis.

PFKFB3 has been shown to be crucial for mediating T cell increases in F-2,6-BP levels, lactate production and TNFα secretion, as inhibition of PFKFB3 with a small molecule inhibitor, 3PO, attenuated these increases (Telang et al., 2012). The increase in PFKFB3 under stimulatory conditions is also necessary for cell survival, as inhibition of PFKFB3 in macrophages results in a loss in cell viability (Tawakol et al., 2015). IL-6 has been shown to increase the expression of PFKFB3 through STAT3 signalling

(Ando et al., 2010), and here, the data also show that Aβ+LPS induces an increase in IL-6 secretion, which was found to be significantly correlated with PFKFB3 mRNA expression (data not shown). PFKFB3 may also be regulated by HIF-1α, a transcription factor that promotes a switch to glycolysis under hypoxic conditions (Minchenko et al., 2002), and HIF-1α inhibition by 2-DG decreases LPS-induced production of IL-1β in mouse macrophages (Tannahill et al., 2013). Interestingly, an increase in PFKFB3 expression has been observed in the area surrounding amyloid-plaques and reactive astrocytes in a mouse model of AD, indicating a potential role for PFKFB3 in the pathogenesis of AD (Fu et al., 2015).

The results from this study demonstrate an Aβ+LPS induced upregulation in PFKFB3 expression and subsequent increase in glycolysis and production of inflammatory cytokines in PBMCs, which is enhanced in participants with an IQ-discrepant episodic memory. This indicates a role for metabolism in mediating the inflammatory response which may be a useful biomarker for cognitive dysfunction. This study employed cryopreserved PBMCs, and it has been previously shown that cryopreservation negatively impacts the bioenergetic profile of PBMCs (Jones et al., 2015a). In addition, PBMCs represent a heterogeneous population, and different cell types have been shown to exhibit differences in metabolic profiles – monocytes are energetic cells with a high level of oxidative phosphorylation and glycolysis in basal cells, while lymphocytes are more oxidative and less glycolytic under basal conditions (Chacko et al., 2013). Therefore, investigation into the bioenergetic profile of separate subsets of PBMCs in non-cryopreserved cells is necessary in future studies.

Chapter 7: Aβ+LPS-stimulated MDMs from IQ-consistent and IQ-discrepant participants: metabolism and NLRP3 inflammasome activation

7.1 Introduction

The recent expansion in the field of immunometabolism has demonstrated a close link between the immune response and bioenergetic function. Differentially-activated macrophages adopt distinct metabolic pathways to meet cellular requirements; pro-inflammatory challenged cells become more glycolytic and anti-inflammatory activated cells favour oxidative phosphorylation (Biswas and Mantovani, 2012). Metabolic dysfunction can be a cause as well as a consequence of inflammation. In pro-inflammatory macrophages, NADPH production through the PPP is increased which leads to the production of NO and ROS. This may impair mitochondrial function through inhibition of complexes in the respiratory chain and by the accumulation of mitochondrial DNA mutations (Deleidi et al., 2015). Indeed, NO-mediated inhibition of mitochondrial respiration is crucial for inducing the switch to glycolysis in LPS-activated macrophages, as inhibition of iNOS following LPS stimulation restores normal mitochondrial function (Kelly and O'Neill, 2015). The production of inflammatory cytokines such as IL-1β is dependent on the metabolic shift to glycolysis (Tannahill et al., 2013). Inhibition of complexes in the mitochondrial respiratory chain induces IL-1β production, which is reliant on the activation of the NOD-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome (Zhou et al., 2011).

The NLRP3 inflammasome is a protein complex that is an essential component of the host immune response and is responsible for the production of the inflammatory cytokines IL-1β and IL-18. It is activated by a diverse range of stimuli and typically requires two signals for activation. First, cells are primed by a TLR ligand such as LPS or pro-inflammatory cytokines which activate NF-κB to induce transcription of pro-IL-1β as well as upregulation of the NLRP3 protein (He et al., 2016b). The second signal can be provided by numerous stimuli, such as ATP, pore-forming toxins and phagocytosed material such as Aβ (Halle et al., 2008). This leads to the assembly of the inflammasome complex containing NLRP3, an adapter protein ASC (apoptosis-associated speck-like protein containing a CARD) and the active form of caspase 1, which cleaves pro-IL-1β into its mature form IL-1β (Zhou et al., 2011). Hexokinase-1 mediated glycolysis is required for NLRP3 inflammasome activation, demonstrating a

close relationship between inflammasome activity and metabolism (Moon et al., 2015).

NLRP3 activation has been implicated in the pathogenesis of AD and in animal models of AD (Heneka et al., 2013). APP/PS1 mice deficient in NLRP3 show improved cognitive function, reduced Aβ deposition and decreased cleaved caspase 1 and IL-1β in the CNS (Heneka et al., 2013). Pharmacological inhibition of the NLRP3 inflammasome also ameliorates cognitive impairments in APP/PS1 mice and decreases amyloid burden (Dempsey et al., 2017). An increase in cleaved caspase 1 has also been reported in the brains of AD patients (Heneka et al., 2013). Furthermore, monocytes from AD patients stimulated with LPS and Aβ show an increase in several inflammasome components including NLRP3, caspase 1 and IL-1β compared with healthy controls (Saresella et al., 2016). Monocytes from MCI patients also showed an increase in NLRP3, indicating that dysregulation of the inflammasome occurs in earlier stages of cognitive impairment.

In Chapter 6, PBMCs from IQ-discrepant participants showed an increase in glycolysis and an enhanced expression of PFKFB3 mRNA following incubation with Aβ+LPS compared with PBMCs from IQ-consistent participants. The current study aimed to investigate glycolysis and these markers of metabolism, as well as NLRP3 inflammasome activation, in freshly-isolated MDMs in response to Aβ+LPS stimulation.

The aims of this study are:

1. To investigate the metabolic signature of MDMs from IQ-consistent and IQ-discrepant participants under resting conditions and following Aβ+LPS stimulation.
2. To compare the mRNA expression and the release of inflammatory cytokines in MDMs from IQ-consistent and IQ-discrepant individuals under resting conditions and following Aβ+LPS stimulation.
3. To examine the expression of PFKFB isozymes and NLRP3 inflammasome activation in MDMs from IQ-consistent and IQ-discrepant participants under resting conditions and following Aβ+LPS stimulation.

7.2 Methods

Healthy older adults (n=25) with a mean age of 67.83 years recruited from the MRU at Trinity College Dublin as detailed in Chapters 4 and 5 were invited for a follow-up assessment two years after their initial assessment. Participants were classified as either IQ-consistent (n=15) or IQ-discrepant (n=10) based on their delayed word recall in relation to their estimated IQ as described in sections 2.7.1 and 2.7.4. Cognitive function was assessed by the MMSE and MoCA and no differences were observed between the groups. Blood samples were collected from these participants, processed and plasma and cells isolated as described in sections 2.8 and 2.9.

Plasma from participants was incubated with THP-1 cells as described in section 2.13 and harvested for analysis of gene expression by RT-PCR as described in sections 2.14 and 2.15.

MDMs from participants were stimulated with a combination of Aβ (10 μM) and LPS (100 ng/ml) as described in section 2.12 and harvested for analysis of gene expression by RT-PCR, protein expression by western immunoblotting, cytokine production by ELISA and metabolic profile as described in sections 2.14, 2.15, 2.16, 2.18 and 2.20.

7.3 Results

7.3.1 IL-6, but not IL-8, mRNA is increased in THP-1 cells in response to plasma from IQ-discrepant participants

As this study represents a separate group of participants, the effect of plasma on the expression of IL-8 and other pro-inflammatory markers in THP-1 cells was investigated as before to confirm data presented in Chapters 3 and 5. Individuals who performed worse than expected on a delayed recall test examining episodic memory in relation to their estimated premorbid IQ were classified as IQ-discrepant, and all other participants were classified as IQ-consistent, as previously described. The participant groups were age-matched and showed no difference in MMSE or MoCA scores. Table 7.1 shows that there was a significant difference in delayed verbal recall z-score and cognitive asymmetry z-score between the groups, the basis on which the cohorts were subdivided (Table 7.1, $p<0.001$, Student's *t*-test for independent means).

THP-1 cells were incubated with plasma for 4 hours and the mRNA expression of pro-inflammatory markers was assessed by RT-PCR. No difference in the expression of IL-8 mRNA was observed in THP-1 cells exposed to plasma from IQ-discrepant, compared with IQ-consistent, participants (Figure 7.1 A). Similarly, there were no changes in TNFα or iNOS mRNA (Figure 7.1 B, D). However, a significant increase in IL-6 mRNA was seen in THP-1 cells incubated with plasma from IQ-discrepant, compared with IQ-consistent, participants (Figure 7.1 C, $p<0.05$, Student's *t*-test). As an increase in IL-8 mRNA had previously been detected in THP-1 cells incubated with plasma from IQ-discrepant participants in two separate cohorts, the data here call into question the reliability of IL-8 mRNA as a marker of early cognitive decline.

7.3.2 Glycolysis is not altered between MDMs from IQ-consistent and IQ-discrepant participants

Monocytes were isolated from whole blood by density separation and magnetic sorting and cultured for 7 days *in vitro* with GM-CSF to generate MDMs. Cells were

harvested and a glycolytic stress test performed to examine the metabolic profile in resting MDMs between IQ-consistent and IQ-discrepant participants. The data show no significant difference in glycolysis, glycolytic capacity and OCR:ECAR in MDMs between participant groups (Figure 7.2 A, B, C, D).

7.3.3 Comparing participant cognitive profiles between initial and follow-up assessments

The participants examined in this chapter had been assessed approximately two years previously. Participant cognitive function was determined at each assessment, i.e. the WMS, MMSE and MoCA assessments were performed at both the initial and follow-up assessment, and participants were classified as either IQ-consistent/IQ-discrepant from those WMS results.

The cognitive profile of participants was compared between the two assessments. It was established that some participants who had been classified as IQ-discrepant in the initial assessment were classified as IQ-consistent in the second assessment, and vice versa. The cognitive profile of some participants remained the same over the two assessments. The change in cognitive profiles of participants at the follow-up assessment is represented in Figure 7.3.

Only participants who had remained in the same cognitive group at the second assessment were examined for further analysis and those who had changed were excluded. Delayed verbal recall z-score and cognitive asymmetry z-score between the groups is shown in Table 7.2 ($p<0.001$, Student's *t*-test). There was no difference in MMSE or MoCA scores. When the effect of plasma on THP-1 cells from only these participants was examined, a trend towards an increase in IL-8 mRNA was observed in THP-1 cells incubated with plasma from IQ-discrepant, compared with IQ-consistent, participants (Figure 7.4). All further results presented in this chapter were performed on MDMs from these participants.

7.3.4 Aβ+LPS increased mRNA expression and production of TNFα in MDMs from IQ-discrepant participants

MDMs from IQ-consistent and IQ-discrepant participants were incubated in the presence and absence of Aβ+LPS for 24 hours and the inflammatory phenotype was assessed by examining pro-inflammatory marker expression by RT-PCR, release of pro-inflammatory cytokines by ELISA and protein levels of IL-1β by western immunoblotting. Aβ+LPS significantly increased TNFα mRNA expression and secretion in MDMs from both participant groups, however the effect of Aβ+LPS stimulation was greater in MDMs from IQ-discrepant participants (Figure 7.5 A, B, $p<0.01$, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis).

IL-6 production and mRNA expression were significantly increased following Aβ+LPS stimulation in MDMs from both participant groups, however MDMs from IQ-discrepant participants exhibited a significantly reduced response compared with MDMs from IQ-consistent participants (Figure 7.6 A, B, $p<0.01$, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). Similarly, Aβ+LPS induced a significant increase in IL-8 mRNA expression and release in MDMs, but the effect on IL-8 mRNA was diminished in MDMs from IQ-discrepant participants and no difference in IL-8 secretion between the groups was observed (Figure 7.7 A, B, $p<0.01$, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis).

The mRNA expression and secretion of IL-1β, as well as protein levels of pro-IL-1β, were also increased in MDMs derived from both IQ-consistent and IQ-discrepant participants following exposure to Aβ+LPS, and a significantly reduced effect was observed in IL-1β mRNA and protein expression in MDMs from IQ-discrepant participants (Figure 7.8 A, B, $p<0.05$, $p<0.01$, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). The mature form of IL-1β could not be detected by western immunoblotting. The data show a differential expression of pro-inflammatory cytokines in MDMs from IQ-consistent and IQ-discrepant participants following stimulation with Aβ+LPS.

7.3.5 The expression of proteins involved in the NLRP3 inflammasome are not altered in MDMS following Aβ+LPS stimulation

Aβ and LPS activate the NLRP3 inflammasome and, having demonstrated that IL-1β secretion was increased in response to Aβ+LPS, the expression of pro-caspase 1, ASC and NLRP3 in MDMs were investigated. No significant changes in any of the proteins examined were observed following exposure to Aβ+LPS, or between MDMs from IQ-consistent and IQ-discrepant individuals (Figure 7.9, A, B, C). The cleaved form of caspase 1 could not be detected.

7.3.6 Aβ+LPS significantly increased expression of TLR2 mRNA, but decreased TLR4 mRNA, in MDMs

As Aβ and LPS bind to TLR2 and TLR4 to induce an inflammatory response, the effect of Aβ+LPS stimulation on TLR2 and TLR4 mRNA expression in MDMs from IQ-consistent and IQ-discrepant participants was investigated. Treatment with Aβ+LPS significantly increased TLR2 and decreased TLR4 mRNA in MDMs, and this effect was significantly less in MDMS from IQ-discrepant participants for TLR2 mRNA expression (Figure 7.10 A, B, $p<0.05$, $p<0.01$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). MDMs from IQ-discrepant participants showed a decrease in TLR2 and TLR4 mRNA expression under resting conditions but no significance was observed following *post-hoc* analysis.

7.3.7 MDMs from IQ-discrepant participants exhibit a shift towards glycolysis

The metabolic profile of MDMs from IQ-consistent and IQ-discrepant participants was examined following incubation with Aβ+LPS for 24 hours. The data show an increase in ECAR in unstimulated MDMs from IQ-discrepant compared with IQ-consistent participants and MDMs exhibit an increase in ECAR following Aβ+LPS stimulation (Figure 7.11 A). Analysis of the mean data show a significant increase in glycolysis and glycolytic capacity and decrease in OCR:ECAR in MDMs treated with Aβ+LPS (Figure 7.11 B, C, D, $p<0.05$, $p<0.01$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). A significant increase in glycolytic capacity and decrease in OCR:ECAR was observed in resting MDMs from IQ-discrepant compared with IQ-

consistent participants, demonstrating an altered metabolic phenotype between subject groups (Figure 7.11 B, C, $p<0.05$, $p<0.001$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis).

7.3.8 Expression of PFKFB1, but not PFKFB3, mRNA is increased in MDMs from IQ-discrepant participants

As the differential expression of PFKFB isozymes had been associated with the glycolytic response of PBMCs to Aβ+LPS stimulation in Chapter 6, the mRNA and protein expression of PFKFB1 and PFKFB3 were assessed in MDMs in the presence and absence of Aβ+LPS from IQ-consistent and IQ-discrepant participants. MDMs from IQ-discrepant participants showed an enhanced expression of PFKFB1 mRNA compared with IQ-consistent participants, and Aβ+LPS significantly decreased the mRNA expression of PFKFB1 in MDMs from both participant groups (Figure 7.12 A, $p<0.05$, $p<0.01$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). No significant differences were observed in PFKFB3 mRNA in response to Aβ+LPS stimulation or between participant groups (Figure 7.12 B). An increase in PFKFB3:PFKFB1 was observed in Aβ+LPS-stimulated MDMs from IQ-consistent individuals; this was significantly diminished in MDMs from IQ-discrepant individuals (Figure 7.12 C, $p<0.05$, Two-way ANOVA followed by Newman Keuls *post-hoc* analysis). The protein expression of PFKFB1, PFKFB3 and the ratio of PFKFB3:PFKFB1 remained unchanged under resting and stimulated conditions and no difference was observed between participant groups (Figure 7.13 A, B, C).

	IQ-memory consistent	IQ-memory discrepant
N	15	10
Age (years; mean ± SD)	70.27 ± 7.14	65.40 ± 7.66
Sex (F/M)	5/10	8/2
MMSE	28.93 ± 1.03	28.50 ± 1.65
MoCA	27.27 ± 2.40	27.40 ± 2.22
Premorbid IQ z-score	1.22 ± 0.46	1.22 ± 0.19
Delayed verbal recall z-score	1.09 ± 0.60	0.03 ± 0.25***
Cognitive asymmetry z-score	0.13 ± 0.70	1.18 ± 0.18***

Table 7.1 Demographic of IQ-memory consistent and IQ-memory discrepant participants.

Data are expressed as mean ± SD, IQ-memory consistent n=15, IQ-memory discrepant n=10; ***p<0.001 vs. IQ-consistent; Student's t-test for independent means.

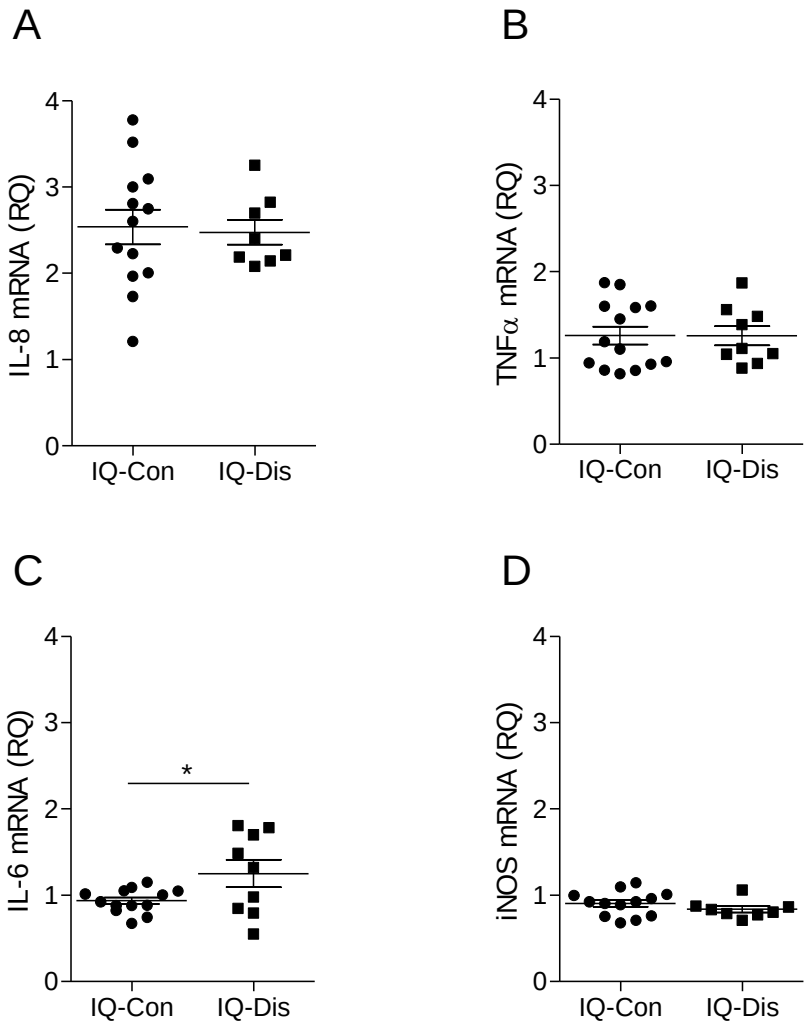

Figure 7.1 IL-6 mRNA is increased in THP-1 cells in response to plasma from IQ-discrepant individuals.

THP-1 cells were treated with or without plasma from IQ-consistent and IQ-discrepant participants (IQ-Con, IQ-Dis) at a 1:40 dilution for 4 h. Cells were harvested and analysis of pro-inflammatory marker expression was performed using RT-PCR. IL-6 (C) mRNA is increased in THP-1 cells in response to plasma from IQ-Dis participants in comparison to IQ-Con participants. No significant differences in IL-8 (A), TNFα (B) or iNOS (D) were observed. Data are expressed as the mean ± SEM, IQ-Con n=15, IQ-Dis n=10; *p<0.05; Student's t-test for independent means.

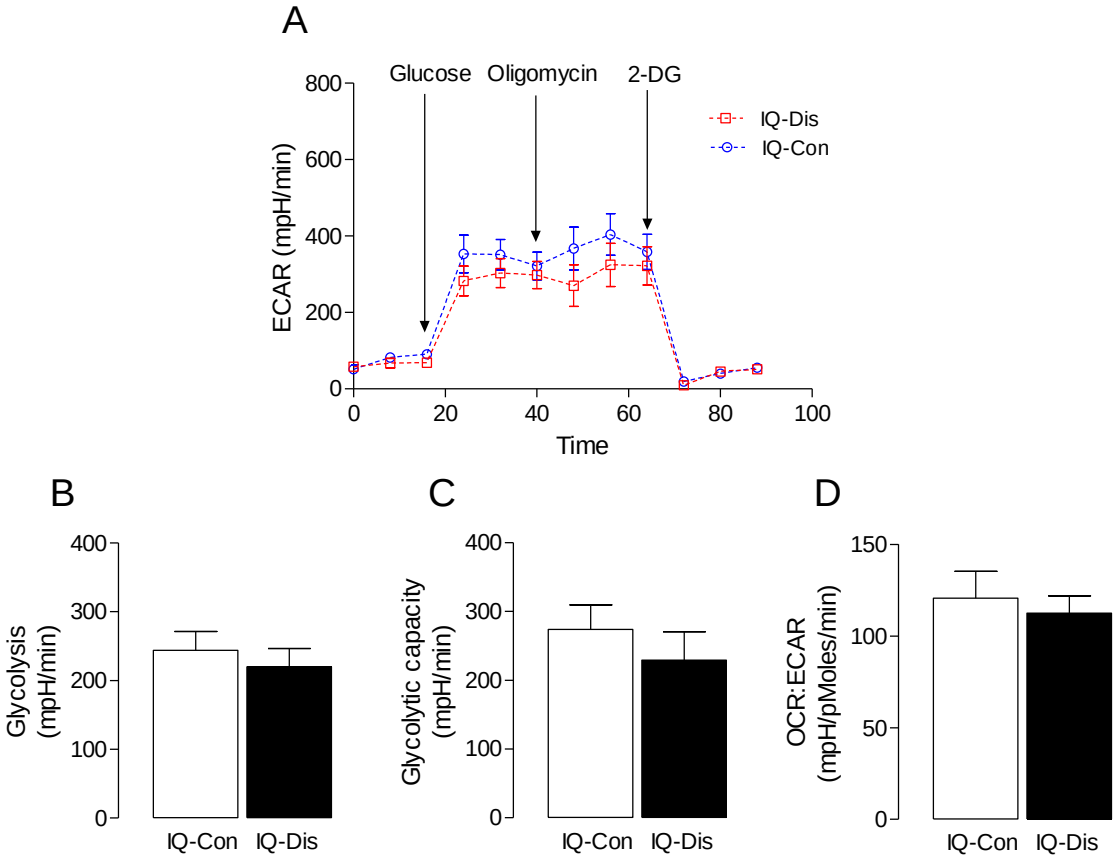

Figure 7.2 Glycolysis is not altered between MDMs from IQ-consistent and IQ-discrepant participants.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated to MDMs with GM-CSF (10 ng/ml) for 7 days *in vitro* and a glycolytic stress test performed to examine metabolic function. The metabolic profile (A) was assessed using the Seahorse XF24 Analyser and analysis of the mean data show no difference in glycolysis (B), glycolytic capacity (C) or OCR:ECAR (D) in between MDMs from IQ-consistent and IQ-discrepant participants. Data are expressed as the mean ± SEM IQ-Con n=14, IQ-Dis n=8. Student's *t*-test for independent means.

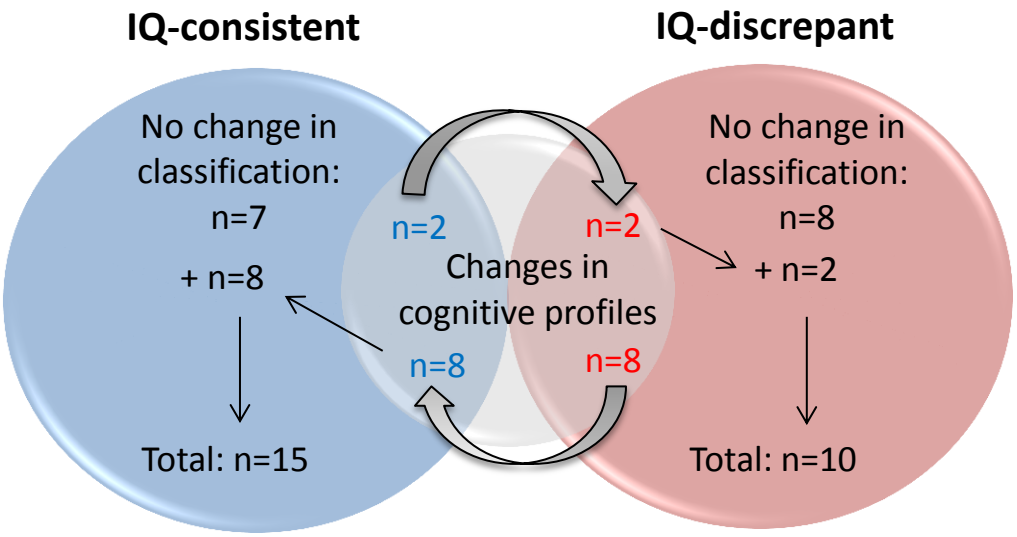

Figure 7.3 Schematic representation of the change in participant cognitive profiles at follow-up assessment.

Approximately two years following the initial assessment, participant's episodic memory was examined using the WMS and individuals were again classified as IQ-consistent or IQ-discrepant based on their performance in relation to their premorbid IQ estimated by the NART. Some participants remained in the same cognitive group as their initial classification (IQ-consistent n=7, IQ=discrepant n=8), however some participants previously identified as IQ-consistent had changed to IQ-discrepant (n=2), and another group of participants identified as IQ-discrepant had now changed to IQ-consistent (n=8). The total number of individuals in each group at follow-up assessment was therefore 15 for IQ-consistent and 10 for IQ-discrepant.

	IQ-memory consistent	IQ-memory discrepant
N	7	8
Age (years; mean ± SD)	70.71 ± 7.06	66.75 ± 8.00
Sex (F/M)	3/4	6/2
MMSE	29.14 ± 0.69	28.25 ± 1.75
MoCA	27.42 ± 2.82	26.87 ± 2.17
Premorbid IQ z-score	1.14 ± 0.57	1.24 ± 0.21
Delayed verbal recall z-score	1.42 ± 0.54	0.04 ± 0.28***
Cognitive asymmetry z-score	-0.289 ± 0.81	1.20 ± 0.21***

Table 7.2 Demographic of IQ-memory consistent and IQ-memory discrepant participants.

Data are expressed as mean ± SD, IQ-memory consistent n=7, IQ-memory discrepant n=8; ***p<0.001 vs. IQ-memory consistent; Student's t-test for independent means.

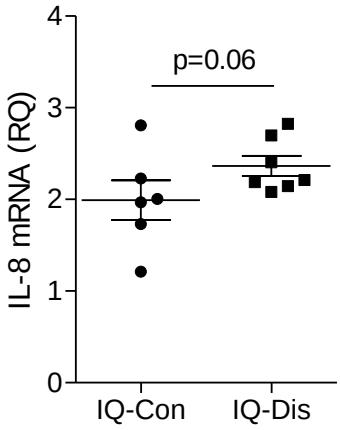

Figure 7.4 Plasma from IQ-discrepant participants induces a trend towards an increase in IL-8 mRNA in THP-1 cells.

THP-1 cells were treated with or without plasma from IQ-consistent and IQ-discrepant participants (IQ-Con, IQ-Dis) at a 1:40 dilution for 4 h. Cells were harvested and analysis of pro-inflammatory marker expression was performed using RT-PCR. A trend towards an increase in IL-8 mRNA was observed in THP-1 cells incubated with plasma from IQ-discrepant compared with IQ-consistent participants, although this did not reach statistical significance. Data are expressed as the mean ± SEM, IQ-Con n=7, IQ-Dis n=8; Student's t-test for independent means.

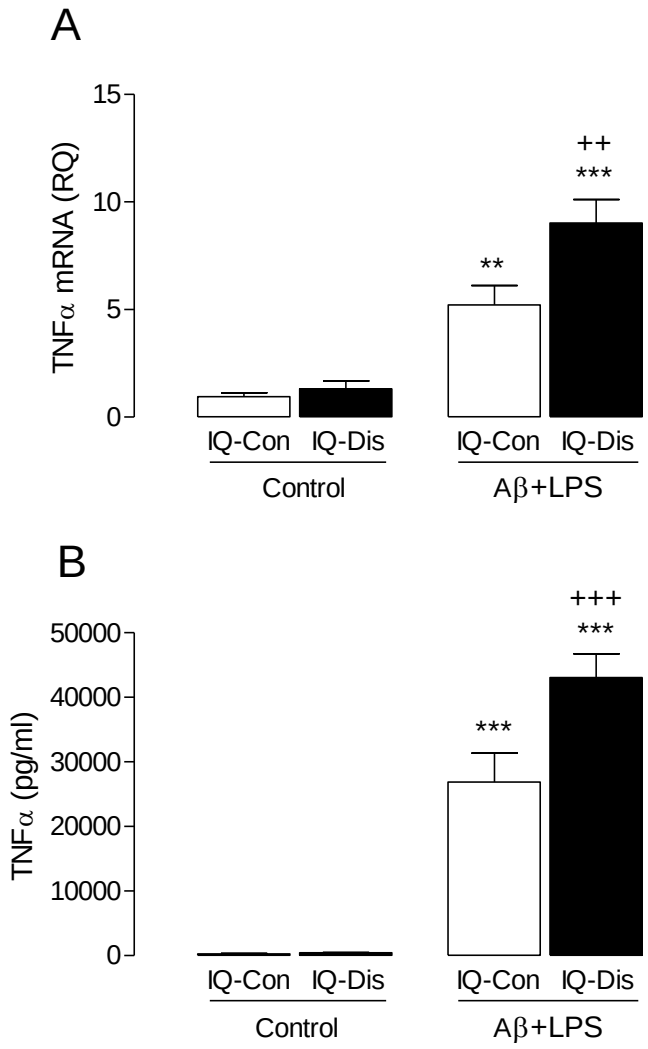

Figure 7.5 MDMs from IQ-discrepant individuals show an enhanced expression of TNFα mRNA and release in response to Aβ+LPS.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 μM) and LPS (100 ng/ml) for 24 h and assessed for expression of TNFα mRNA (A) by RT-PCR and protein (B) by ELISA. Aβ+LPS increased TNFα mRNA and protein expression in MDMs from both participants groups and the effect of Aβ+LPS was greater in MDMs from IQ-discrepant, compared with IQ-consistent, participants. Data are expressed as the mean ± SEM, IQ-Con n=7, IQ-Dis n=6. $^{**}p<0.01$, $^{***}p<0.001$ vs vehicle control; $^{++}p<0.01$, $^{+++}p<0.001$ vs group control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $\text{Group}_{\text{effect}}$ [$F_{(1,20)}=7.856$, $p<0.0110$], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,20)}=64.70$, $p<0.001$], $\text{interaction}_{\text{effect}}$ [$F_{(1,20)}=5.386$, $p<0.0310$]. **B:** $\text{Group}_{\text{effect}}$ [$F_{(1,19)}=8.309$, $p<0.00095$], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,19)}=148.4$, $p<0.001$], $\text{interaction}_{\text{effect}}$ [$F_{(1,19)}=7.992$, $p<0.0108$].

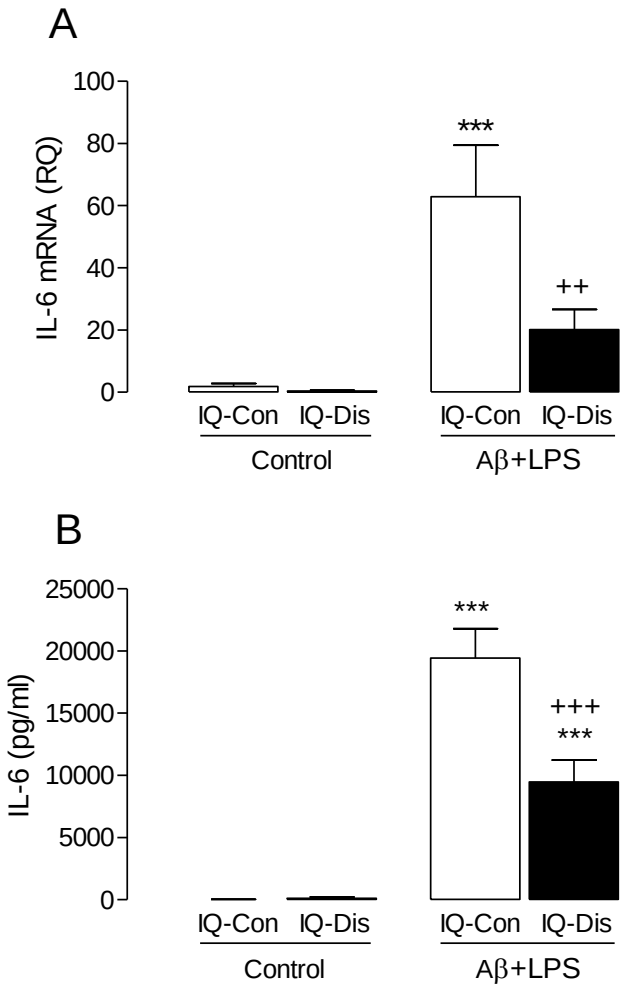

Figure 7.6 Aβ+LPS-induced IL-6 mRNA and release are attenuated in MDMs from IQ-discrepant individuals.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 μM) and LPS (100 ng/ml) for 24 h and assessed for expression of IL-6 mRNA (A) by RT-PCR and protein (B) by ELISA. Aβ+LPS increased IL-6 mRNA and protein expression in MDMs from both participant groups; however MDMs from IQ-discrepant participants exhibited a diminished response to Aβ+LPS stimulation compared with IQ-consistent participants. Data are expressed as the mean ± SEM, IQ-Con n=7, IQ-Dis n=6. ***p<0.001 vs vehicle control; ^{++}p<0.01, ^{+++}p<0.001 vs group control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $\text{Group}_{\text{effect}}$ [$F_{(1,19)}$=5.928, p<0.0249], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,19)}$=19.62, p<0.001], $\text{interaction}_{\text{effect}}$ [$F_{(1,19)}$=5.145, p<0.0352]. **B:** $\text{Group}_{\text{effect}}$ [$F_{(1,18)}$=11.30, p<0.0035], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,18)}$=96.53, p<0.001], $\text{interaction}_{\text{effect}}$ [$F_{(1,18)}$=11.86, p<0.0029].

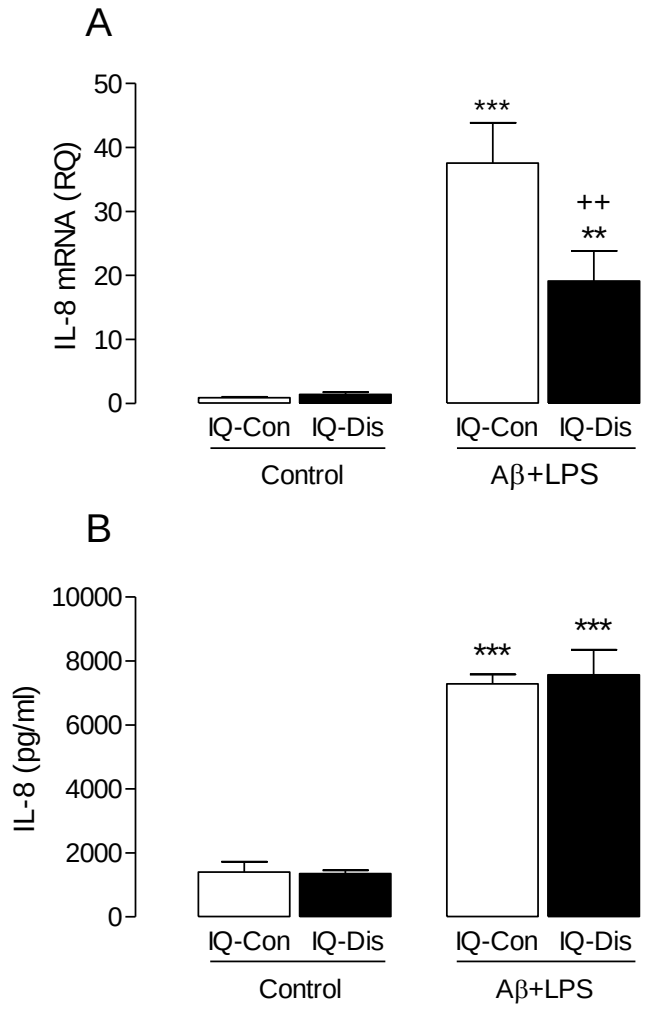

Figure 7.7 Aβ+LPS-induced IL-8 mRNA, but not IL-8 release, is attenuated in MDMs from IQ-discrepant individuals.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 μM) and LPS (100 ng/ml) for 24 h and assessed for expression of IL-8 mRNA (A) by RT-PCR and protein (B) by ELISA. Aβ+LPS increased IL-8 mRNA and protein expression in MDMs from both participant groups; however MDMs from IQ-discrepant participants exhibited a diminished response in IL-8 mRNA to Aβ+LPS stimulation compared with IQ-consistent participants. Data are expressed as the mean ± SEM, IQ-Con n=7, IQ-Dis n=6. $^{**}p<0.01$, $^{***}p<0.001$ vs vehicle control; $^{++}p<0.01$ vs group control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $\text{Group}_{\text{effect}}$ [$F_{(1,20)}=5.249$, $p<0.0329$], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,20)}=48.00$, $p<0.001$], $\text{interaction}_{\text{effect}}$ [$F_{(1,20)}=5.811$, $p<0.0.0257$]. **B:** $\text{Group}_{\text{effect}}$ [$F_{(1,18)}=0.06609$, $p<0.800$], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,18)}=194.7$, $p<0.001$], $\text{interaction}_{\text{effect}}$ [$F_{(1,18)}=0.1374$, $p<0.7512$].

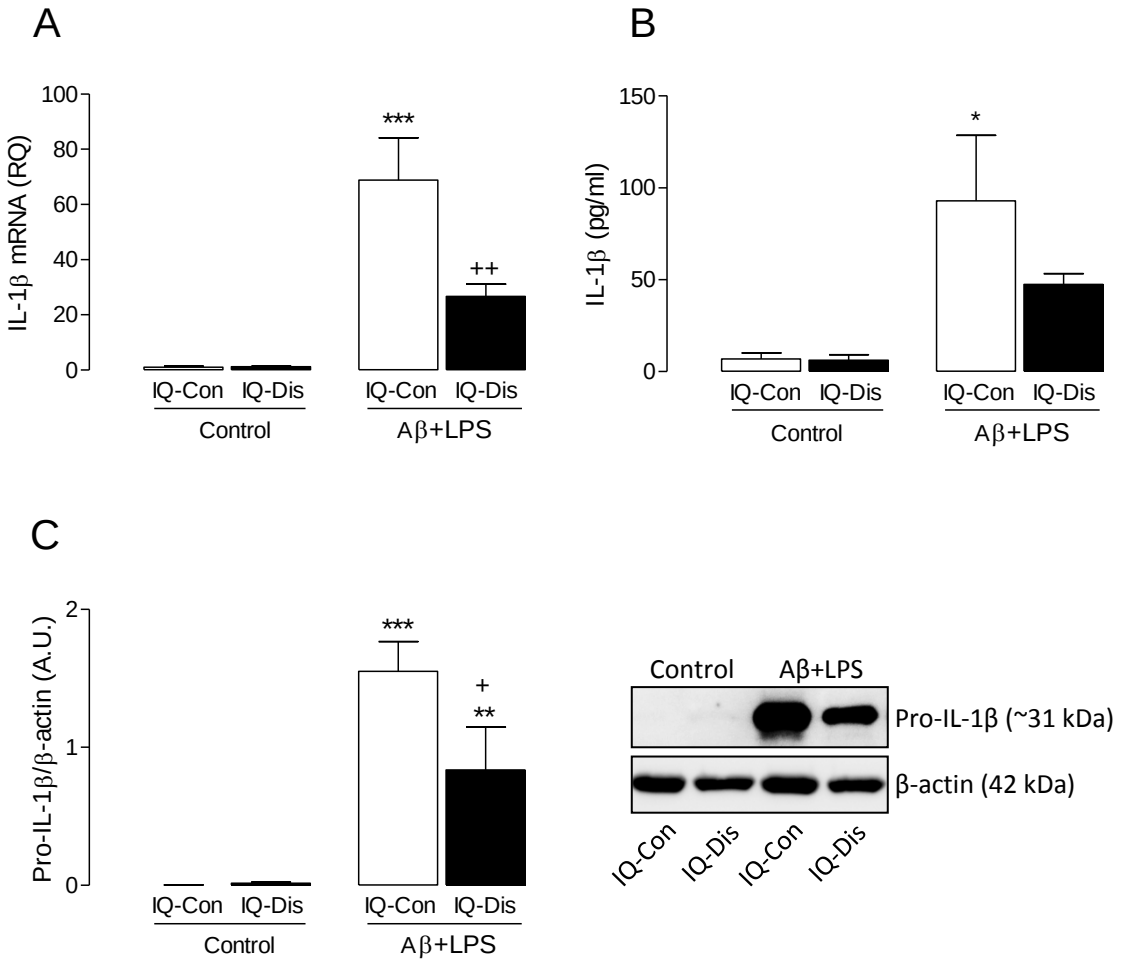

Figure 7.8 Aβ+LPS-induced IL-1β expression is attenuated in MDMs from IQ-discrepant individuals.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 μM) and LPS (100 ng/ml) for 24 h and assessed for expression of IL-1β mRNA (A) by RT-PCR and protein by ELISA (B) and western immunoblotting (C). Aβ+LPS increased IL-1β mRNA, secretion and pro-IL-1β protein expression in MDMs; however MDMs from IQ-discrepant participants exhibited a diminished response to Aβ+LPS stimulation compared with IQ-consistent participants. A representative immunoblot is shown. Data are expressed as the mean ± SEM, IQ-Con n=6-7, IQ-Dis n=5-6. **p*<0.05, ***p*<0.01, ****p*<0.001 vs vehicle control; ^{+}p<0.05 ^{++}p<0.01 vs group control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $\text{Group}_{\text{effect}}$ [$F_{(1,19)}$=6.186, p<0.0223], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,19)}$=30.64, p<0.001], $\text{interaction}_{\text{effect}}$ [$F_{(1,19)}$=6.272, p<0.0251]. **B:** $\text{Group}_{\text{effect}}$ [$F_{(1,19)}$=1.454 p<0.2426], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,19)}$=11.04, p<0.0036], $\text{interaction}_{\text{effect}}$ [$F_{(1,19)}$=1.364, p<0.2573]. **C:** $\text{Group}_{\text{effect}}$ [$F_{(1,15)}$=4.160, p<0.0594], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,15)}$=47.20, p<0.001], $\text{interaction}_{\text{effect}}$ [$F_{(1,15)}$=4.444, p<0.0523].

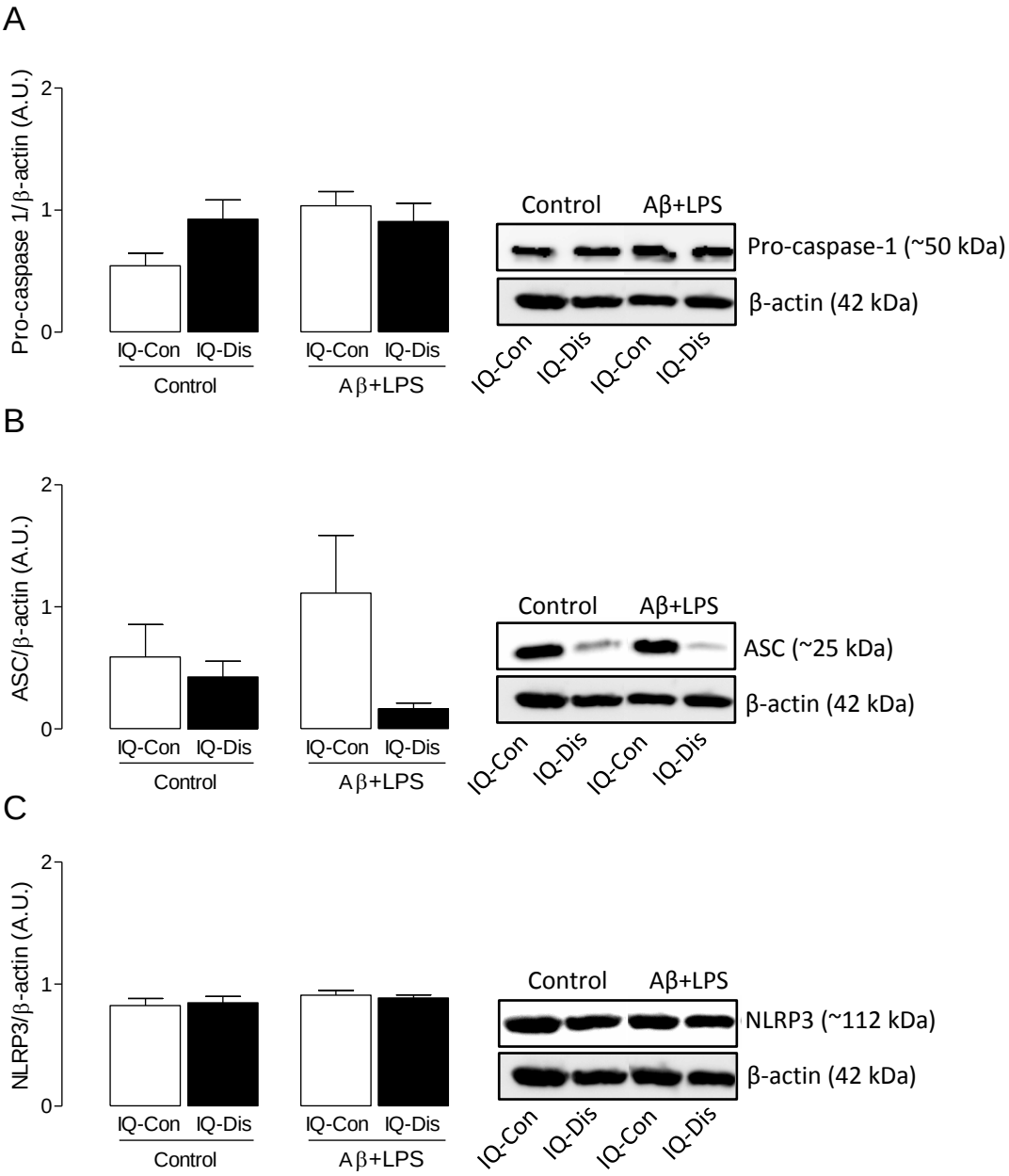

Figure 7.9 Aβ+LPS did not alter pro-caspase 1, ASC or NLRP3 protein in MDMs from IQ-consistent and IQ-discrepant individuals.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 μM) and LPS (100 ng/ml) for 24 h and expression of proteins involved in the NLRP3 inflammasome assessed by western immunoblotting. The levels of pro-caspase 1 (A), ASC (B) or NLRP3 (C) were not altered in MDMs by Aβ+LPS stimulation or between participant groups. Data are expressed as the mean ± SEM IQ-Con n=6, IQ-Dis n=5. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,15)}$=0.8999, p<0.3579], $Aβ+LPS_{effect}$ [$F_{(1,15)}$=3.148, p<0.0963], $interaction_{effect}$ [$F_{(1,15)}$=3.703, p<0.0963]. **B:** $Group_{effect}$ [$F_{(1,15)}$=2.577, p<0.1292], $Aβ+LPS_{effect}$ [$F_{(1,15)}$=0.1450, p<0.7087], $interaction_{effect}$ [$F_{(1,15)}$=1.275, p<0.2765]. **C:** $Group_{effect}$ [$F_{(1,15)}$=0.00005, p<0.9945], $Aβ+LPS_{effect}$ [$F_{(1,15)}$=2.055, p<0.1722], $interaction_{effect}$ [$F_{(1,15)}$=0.2751, p<0.6076].

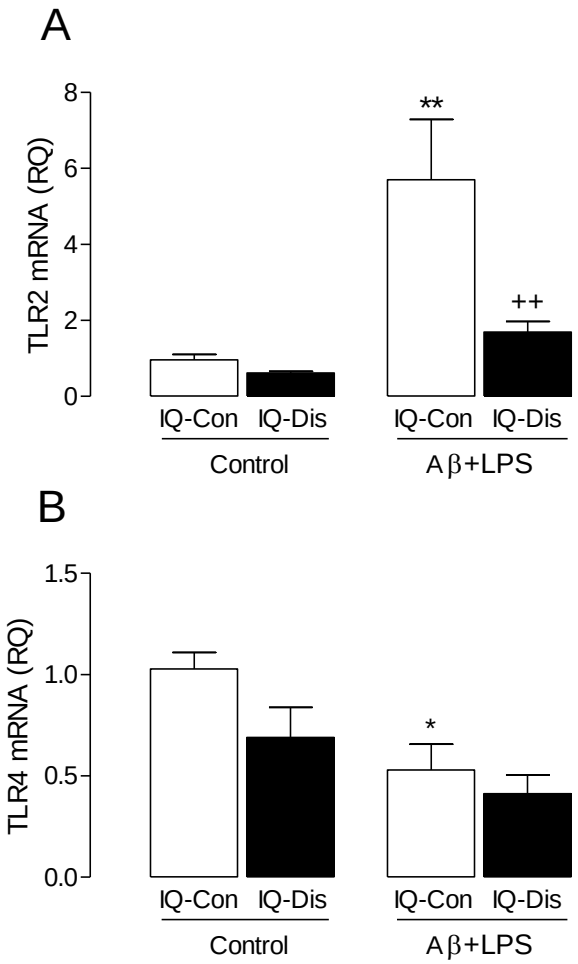

Figure 7.10 TLR2 and TLR4 mRNA are differentially regulated by Aβ+LPS stimulation in MDMs.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 μM) and LPS (100 ng/ml) for 24 h and assessed for expression of TLR2 and TLR4 mRNA RT-PCR. Aβ+LPS increased TLR2 mRNA (A) and decreased TLR4 mRNA (B) and the expression of TLR2 mRNA in MDMs from IQ-discrepant participants was significantly less compared to MDMs from IQ-consistent. Data are expressed as the mean ± SEM, IQ-Con n=7, IQ-Dis n=6. **p*<0.05, ***p*<0.01 vs vehicle control; ^{+}p<0.05 ^{++}p<0.01 vs group control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $\text{Group}_{\text{effect}}$ [$F_{(1,21)}$=5.631, p<0.0273], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,21)}$=10.09, p<0.0045], $\text{interaction}_{\text{effect}}$ [$F_{(1,21)}$=3.999, p<0.0586]. **B:** $\text{Group}_{\text{effect}}$ [$F_{(1,19)}$=3.687, p<0.0700], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,19)}$=10.70, p<0.004], $\text{interaction}_{\text{effect}}$ [$F_{(1,19)}$=0.8744, p<0.3615].

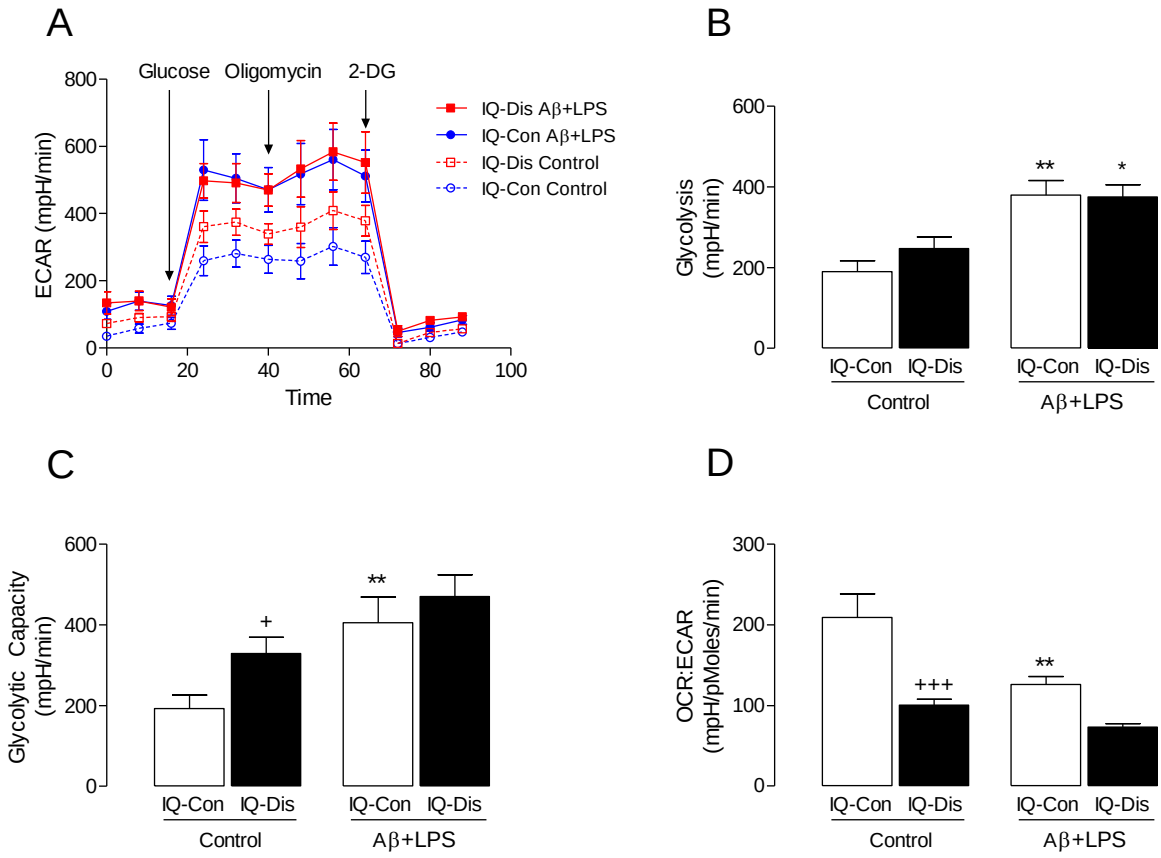

Figure 7.11 MDMs from IQ-discrepant participants display a shift towards glycolysis.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated to MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro*. Cells were incubated with or without Aβ (10 µM) and LPS (100 ng/ml) for 24 h and a glycolytic stress test performed to examine metabolic function. The metabolic profile (A) was assessed using the Seahorse XF24 Analyser and analysis of the mean data show a significant increase in glycolysis (B) and glycolytic capacity (C) and decrease in OCR:ECAR (D) in MDMs stimulated with Aβ+LPS. An increase in glycolytic capacity (C) and decrease in OCR:ECAR (D) is observed in resting MDMs from IQ-discrepant, compared with IQ-consistent, participants. Data are expressed as the mean ± SEM IQ-Con n=7, IQ-Dis n=6. **p*<0.05, ***p*<0.01 vs vehicle control; $^{+}p<0.05$ $^{+++}p<0.001$ vs group control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

B: $Group_{effect}$ [$F_{(1,17)}$=0.7530, p<0.3976], $A\beta+LPS_{effect}$ [$F_{(1,17)}$=27.26, p<0.001], $interaction_{effect}$ [$F_{(1,17)}$=1.032, p<0.3240]. **C:** $Group_{effect}$ [$F_{(1,19)}$=3.735, p<0.0683], $A\beta+LPS_{effect}$ [$F_{(1,19)}$=11.63, p<0.0029], $interaction_{effect}$ [$F_{(1,19)}$=0.4667, p<0.5028]. **D:** $Group_{effect}$ [$F_{(1,18)}$=21.32, p<0.001], $A\beta+LPS_{effect}$ [$F_{(1,18)}$=10.03, p<0.0053], $interaction_{effect}$ [$F_{(1,18)}$=2.559, p<0.1271].

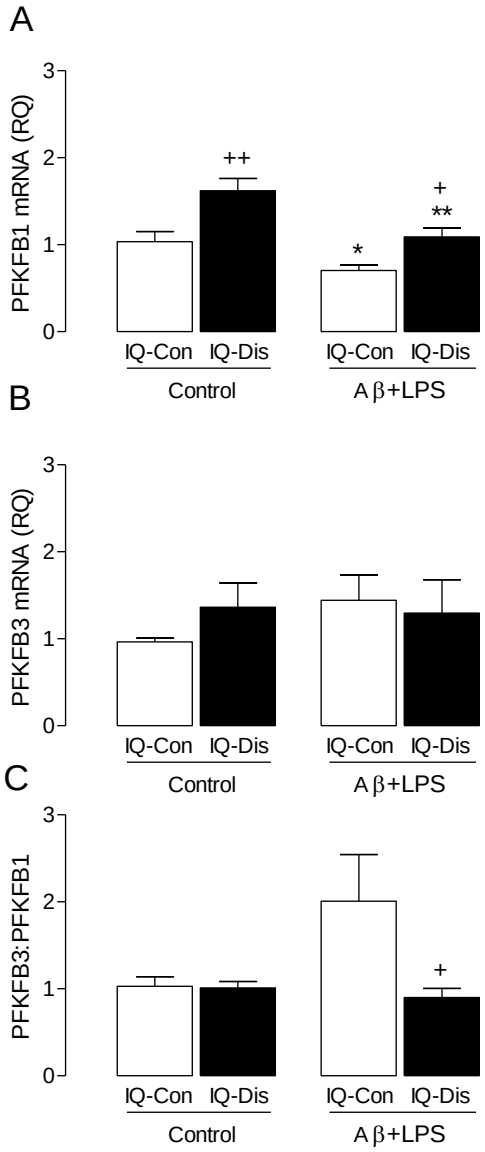

Figure 7.12 PFKFB1 mRNA expression is increased in MDMs from IQ-discrepant individuals.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 µM) and LPS (100 ng/ml) for 24 h and assessed for mRNA expression of PFKFB1 and PFKFB3 by RT-PCR. The expression of PFKFB1 was significantly upregulated in MDMs from IQ-discrepant participants (A). No change was observed in PFKFB3 mRNA (B), and MDMs from IQ-discrepant participants showed a decrease in the PFKFB3:PFKFB1 ratio following Aβ+LPS treatment (C). Data are expressed as the mean ± SEM IQ-Con n=7, IQ-Dis n=6. $^{+}p<0.05$ $^{++}p<0.01$ vs group control. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $\text{Group}_{\text{effect}}$ [$F_{(1,19)}$=20.57, p<0.0002], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,19)}$=16.22, p<0.0007], $\text{interaction}_{\text{effect}}$ [$F_{(1,19)}$=0.8433, p<0.3700]. **B:** $\text{Group}_{\text{effect}}$ [$F_{(1,19)}$=0.1903, p<0.6676], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,19)}$=0.4921, p<0.4915], $\text{interaction}_{\text{effect}}$ [$F_{(1,19)}$=0.8689, p<0.3630]. **C:** $\text{Group}_{\text{effect}}$ [$F_{(1,18)}$=3.337, p<0.0844], $\text{Aβ+LPS}_{\text{effect}}$ [$F_{(1,18)}$=1.984, p<0.1760], $\text{interaction}_{\text{effect}}$ [$F_{(1,18)}$=3.131, p<0.0938].

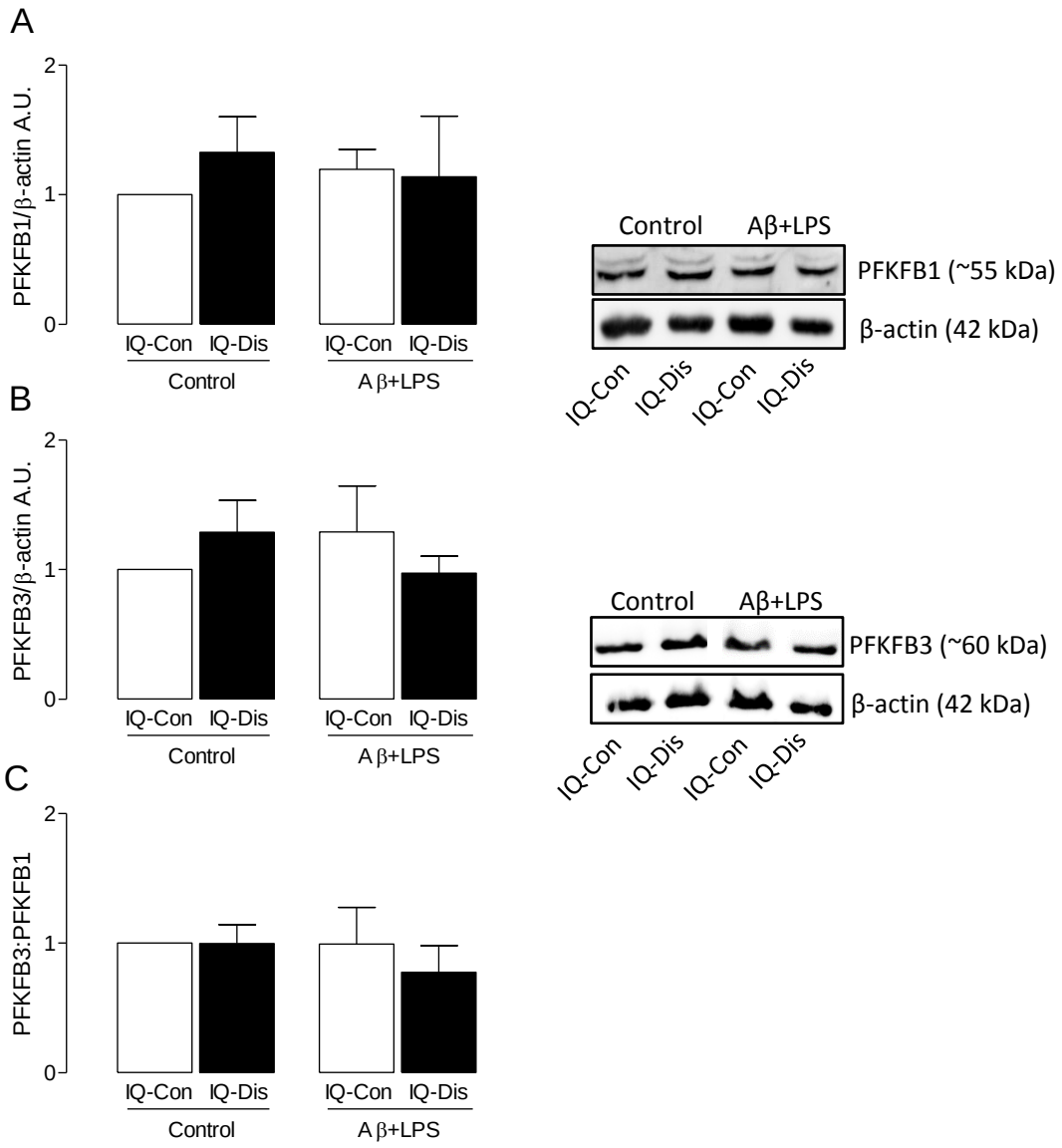

Figure 7.13 Protein expression of PFKFB1 and PFKFB3 are not altered in MDMs from IQ-consistent and IQ-discrepant participants or in response to Aβ+LPS.

Monocytes from IQ-consistent (IQ-Con) and IQ-discrepant (IQ-Dis) participants were isolated from whole blood and differentiated into MDMs with GM-CSF (10 ng/ml) for 6 days *in vitro.* Cells were incubated with or without Aβ (10 μM) and LPS (100 ng/ml) for 24 h and assessed for proteins involved in the NLRP3 inflammasome by western immunoblotting. The levels of PFKFB1 (A), PFKFB3 (B) or PFKFB3:PFKFB1 (C) were not altered in MDMs by Aβ+LPS stimulation or between participant groups. Data are expressed as the mean ± SEM IQ-Con n=6, IQ-Dis n=5. Two-way ANOVA followed by Newman Keuls *post-hoc* analysis.

A: $Group_{effect}$ [$F_{(1,15)}$=0.3116, p<0.5849], $A\beta+LPS_{effect}$ [$F_{(1,15)}$=0.0003, p<0.9851], $interaction_{effect}$ [$F_{(1,15)}$=0.6344, p<0.4382]. **B:** $Group_{effect}$ [$F_{(1,16)}$=0.005, p<0.9436], $A\beta+LPS_{effect}$ [$F_{(1,16)}$=0.002, p<0.9617], $interaction_{effect}$ [$F_{(1,16)}$=1.831, p<0.1948]. **C:** $Group_{effect}$ [$F_{(1,15)}$=0.3572, p<0.5590], $A\beta+LPS_{effect}$ [$F_{(1,15)}$=0.3824, p<0.5456], $interaction_{effect}$ [$F_{(1,15)}$=0.3391, p<0.5690].

7.4 Discussion

The aim of this study was to examine changes in metabolism and inflammation in MDMs stimulated with Aβ+LPS from individuals classified as IQ-consistent and IQ-discrepant. MDMs derived from IQ-discrepant participants showed differential expression of pro-inflammatory cytokine mRNA expression and production following Aβ+LPS treatment compared with MDMs from IQ-consistent participants. This was accompanied by a metabolic shift towards glycolysis, but was not associated with an increase in PFKFB3 mRNA or protein expression. Aβ+LPS-induced IL-1β production in MDMs was reduced from IQ-discrepant, compared with IQ-consistent, subjects and suggests that the NLRP3 inflammasome is differentially activated between participant groups, although no change in the expression of other components was detected. The data indicate that MDMs from IQ-consistent and IQ-discrepant participants differ in their metabolic and inflammatory responses to Aβ+LPS and that this may be a useful indicator of cognitive dysfunction.

An important observation highlighted in this chapter is that some participants described as either IQ-consistent or IQ-discrepant in their initial assessment had changed classification upon re-assessment approximately two years later. Therefore this classification is not fixed and fluctuates over time. However, when pro-inflammatory gene expression in THP-1 cells was examined following exposure to plasma from all participants collected at the additional assessment, no increase in IL-8 mRNA was identified between IQ-discrepant and IQ-consistent participants, although there was a significant increase in IL-6 mRNA expression. The lack of change in IL-8 mRNA is perhaps surprising, as a robust increase in IL-8 expression had been observed in THP-1 cells with plasma from two separate cohorts of IQ-discrepant participants (Chapters 3 and 5). Additionally, there was no change in measures of glycolysis in resting MDMs between IQ-consistent and IQ-discrepant participants. Interestingly, when only participants whose IQ-consistent/IQ-discrepant classification had not changed between assessments were examined, a trend towards an increase in IL-8 mRNA was observed in response to plasma from IQ-discrepant participants. This suggests that the classification of some participants was unreliable, possibly due to variations in testing sessions and the robustness of

neuropsychological assessments. In this study, in order to more accurately examine the inflammatory and metabolic phenotype in MDMs between subject groups, only participants whose cognitive description had not changed between assessments were further evaluated.

The data showed a greater effect of Aβ+LPS stimulation on TNFα mRNA and release in MDMs from IQ-discrepant, compared with IQ-consistent participants. This is consistent with previous observations in Aβ-stimulated MDMs described in Chapter 5 and Aβ+LPS-stimulated PBMCs in Chapter 6, and also from reports from Downer and colleagues who investigated LPS-stimulated MDMs from a similar cohort of participants (Downer et al., 2013). However, the Aβ+LPS effect on IL-6, IL-8 and IL-1β expression was significantly attenuated in MDMs from IQ-discrepant, compared with IQ-consistent, subjects which is in contrast with the results presented in Chapter 5 and 6 as well as the study conducted by Downer and colleagues. No significant changes in cytokine levels from resting MDMs were observed between participant groups. Pro-inflammatory cytokine production in monocytes and PBMCs from MCI and AD patients and the response to Aβ and/or LPS stimulation has been discussed in Chapters 5 and 6. While overall the literature suggests that there is an increase in basal levels of inflammatory mediators and an exaggerated production in response to inflammatory stimuli in cells from patients (Guerreiro et al., 2007, Reale et al., 2004, Lombardi et al., 1999), variations between the studies might be due to differences in inflammatory stimuli used and stages of cognitive impairment of patients.

The differential expression of TNFα from other pro-inflammatory cytokines in response to Aβ+LPS suggests that these cytokines are regulated differently in MDMs from IQ-consistent and IQ-discrepant participants. A common pathway for pro-inflammatory cytokine production through TLR signalling is via NK-κB activation, which is dysfunctional in PBMCs from AD patients (Ascolani et al., 2012). It is possible that disrupted NF-κB signalling also occurs in earlier stages of cognitive decline. TNFα production is also regulated by MAPK, cyclic AMP and AP-1 transcription factors, and is secreted following Aβ stimulation through JNK and ERK MAPK and NF-κB signalling, but not through p38 MAPK, in mouse bone marrow-derived macrophages (BMDMs)

(O'Halloran et al., 2014). However, Aβ (unlike LPS) has not been reported to induce NF-κB activation in human macrophages, indicating that TNFα production under these conditions occurs via a different intracellular signalling pathway (Smits et al., 2001). For example, TNFα production in THP-1 cells induced by Aβ is dependent on protein kinase C signalling pathways (Klegeris, 1997). The current study employed both Aβ and LPS as an inflammatory stimulus and therefore it is possible that a number of intracellular signalling pathways were activated which could have resulted in different patterns of cytokine/chemokine production in MDMs from IQ-consistent and IQ-discrepant participants.

Another possibility for these differences might be due to alterations in TLR2 and TLR4 receptor expression. Aβ+LPS upregulated TLR2 mRNA although a significant attenuation was observed in MDMs from IQ-discrepant, compared with IQ-consistent participants, similar to the pattern of IL-6, IL-8 and IL-1β secretion. In contrast, the expression of TLR4 mRNA was decreased following exposure to Aβ+LPS and, although not significant, there was a small decrease in MDMs from IQ-discrepant, compared with IQ-consistent, individuals. This is consistent with previous findings where an increase in TLR2, but not TLR4, mRNA is observed in LPS-stimulated mouse macrophages (Matsuguchi et al., 2000). However as mRNA levels do not necessarily correlate with protein expression as discussed in Chapter 5, it is not possible to conclusively determine if differences in cytokine production are as a result of differential TLR expression.

The secretion and mRNA expression of IL-1β was increased following Aβ+LPS stimulation in MDMs from both participant groups, consistent with previous reports in human monocytes (Lorton et al., 1996, Saresella et al., 2016). The observed reduction in IL-1β in MDMs from IQ-discrepant participants suggests that the NLRP3 inflammasome is not activated to the same extent by Aβ+LPS as MDMs from IQ-consistent participants. The data indicate that the NLRP3 inflammasome was successfully 'primed' as pro-IL-1β levels showed a similar pattern to IL-1β mRNA and release, however mature IL-1β could not be detected by western immunoblotting. Similarly, while the pro-form of caspase 1 was observed in MDMs, the active form of caspase 1 was not detected. It is possible that MDMs were over-exposed to LPS, as it

has been previously reported that active caspase 1 could not be detected in chronically-activated BMDMs which exhibited a reduction in NLRP3 activity (Gurung et al., 2015). Aβ+LPS did not significantly alter the levels of pro-caspase 1, ASC or NLRP3. Pro-caspase 1 and ASC are present at an adequate concentration in the cell and therefore may not necessarily be upregulated upon inflammasome activation (Sutterwala et al., 2014) and indeed no increase in ASC is observed following LPS stimulation in macrophages (Coll et al., 2015). It is perhaps surprising that no change in NLRP3 expression was observed, as LPS has been previously shown to increase its expression (Coll et al., 2015) and upregulation of NLRP3 transcription is required during the priming stage of inflammasome activation.

The expression of pro-caspase 1, ASC or NLRP3 were not altered in resting or stimulated MDMs from IQ-consistent and IQ-discrepant participants. An increase in NLRP3 expression has been reported in Aβ+LPS stimulated monocytes from MCI and AD patients compared with controls (Saresella et al., 2016). This study also revealed an increase in caspase 1 positive monocytes co-expressing NLRP3 from AD patients compared with controls, and an exaggerated release of IL-1β following Aβ+LPS stimulation in monocytes from AD, but not MCI, patients. This suggests that perhaps inflammasome activation is not an early event in cognitive dysfunction and occurs later during disease progression. Polymorphisms in the NLRP3 gene are reported to confer an increased risk of late-onset AD (Tan et al., 2013), implicating NLRP3 in disease pathogenesis. Inhibition of the NLRP3 inflammasome has demonstrated beneficial effects on cognitive function and reduced Aβ plaque burden and inflammation in AD models (Daniels et al., 2016, Dempsey et al., 2017, Yin et al., 2017). Further investigation of NLRP3 inflammasome activity in earlier stages of cognitive decline is necessary to determine if this might be a suitable target for preventing subsequent deterioration.

An important finding is that Aβ+LPS increased glycolysis in MDMs and that MDMs from IQ-discrepant participants showed an increased glycolytic capacity and a cellular preference for glycolysis over oxidative phosphorylation, compared with MDMs from IQ-consistent participants. Following Aβ+LPS stimulation, this distinction in glycolytic capacity between participant groups was not evident, however a similar

trend was observed in the OCR:ECAR ratio although this did not reach statistical significance. This suggests that mitochondrial respiration is decreased or dysregulated to a greater extent in MDMs from IQ-discrepant participants, and that they are more sensitised to the effects of Aβ+LPS. This is in contrast to the finding described in Chapter 6 where PBMCs from IQ-consistent and IQ-discrepant participants showed no difference in OCR:ECAR, although a similar decrease in response to Aβ+LPS was observed. This discrepancy is possibly due to the response of different cell types, as PBMCs comprise mainly of T cells and variations between the bioenergetic profiles of immune cells exist (Chacko et al., 2013). A study conducted by Maynard and colleagues (2015) revealed a decrease in OCR but no change in ECAR in PBMCs from AD patients compared with controls, as previously discussed in Chapter 6. The current findings indicate that discrete changes in glycolysis are perhaps more evident in MDMs, and that this could provide the basis of a biomarker for early cognitive dysfunction.

The rate of glycolysis can be controlled through the expression of PFK2 isoforms (Rodriguez-Prados et al., 2010). Aβ+LPS-treated PBMCs from IQ-discrepant participants displayed an enhanced expression of PFKFB3, but no difference in PFKFB1 mRNA, and the PFKFB3:PFKFB1 was significantly correlated with the rate of glycolysis indicating a role of the differential expression of these enzymes in driving glycolysis. Therefore, the mRNA expression and protein levels of PFKFB1 and PFKFB3 in MDMs were also investigated. MDMs from IQ-discrepant, compared with consistent, participants exhibited an increased expression of PFKFB1 mRNA, and the expression of PFKFB1 mRNA was decreased following Aβ+LPS stimulation in MDMs from both participant groups. Surprisingly, no significant increase in the mRNA and protein expression of PFKFB3 was observed in MDMs from IQ-discrepant participants or in the presence of Aβ+LPS in MDMs from both subject groups. This is in contrast to previous reports, where LPS increased PFKFB3 mRNA and protein levels and did not alter the mRNA expression of PFKFB1 (Rodriguez-Prados et al., 2010). It is also an unexpected finding, as Aβ+LPS induced a clear switch to glycolysis in MDMs from both subject groups.

The ability of LPS to alter metabolism in macrophages has been demonstrated through a number of mechanisms, therefore it is plausible that glycolytic flux in MDMs is increased via an alternative pathway. One such mechanism could be via increased expression of HIF-1α through mammalian target of rapamycin (mTOR) activation, resulting in the increased expression of glycolytic and inflammatory genes (Kelly and O'Neill, 2015). HIF-1α is stabilised by increases in an intermediate of the TCA cycle, succinate, and can increase the production of IL-1β (Tannahill et al., 2013). Glycolysis is also controlled by pyruvate dehydrogenase kinase 1 (PDK1), which regulates the PDH complex responsible for converting pyruvate into acetyl-CoA, and can therefore limit the amount of acetyl-CoA that enters the TCA cycle. Knockdown of PDK1 decreases glycolysis and enhances oxidative phosphorylation in LPS-treated macrophages and is required for LPS- and Pam3CSK4-induced inflammatory cytokine production (Tan et al., 2015b). Furthermore, LPS decreases activation of AMPK, which regulates the cellular AMP/ATP ratio, and has antagonising actions on mTOR and NF-κB thereby influencing HIF-1α signalling and pro-inflammatory cytokine production (Sag et al., 2008). Interestingly, the expression of PFKFB3 and another key rate-limiting enzyme in glycolysis, hexokinase II, are decreased by knockdown of the monocarboxylate transporter 4 (MCT4) in LPS-activated macrophages. MCT4 is responsible for transporting intracellular lactate to the extracellular environment (Tan et al., 2015a), and provides another mechanism by which PFKFB3 expression is regulated.

It is possible that the shift to glycolysis exhibited by MDMs from IQ-discrepant participants is linked with TNFα production, as an exaggerated increase is seen in response to Aβ+LPS which is not observed with other pro-inflammatory cytokines. Indeed, the data show a significant correlation between TNFα mRNA and secretion with the OCR:ECAR ratio, and a significant correlation of TNFα release with glycolysis and glycolytic capacity (data not shown). TNFα has been shown to increase glycolysis and decrease oxidative phosphorylation in prostate epithelial cells (Vaughan et al., 2013) and can impair the activity of complexes involved in the electron transport chain of mitochondrial respiration (Morris and Berk, 2015). As well as an increase in glycolytic flux resulting in TNFα upregulation, it is therefore also possible that

Aβ+LPS-induced production of TNFα impaired mitochondrial respiration thereby increasing glycolysis in MDMs. Further examination is required to determine the precise relationship between TNFα production and glycolysis in MDMs between IQ-consistent and IQ-discrepant participants.

A link between metabolism and NLRP3 inflammasome activation has been previously demonstrated in macrophages, as inhibition of hexokinase-1 suppresses caspase-1 activation and IL-1β production in macrophages (Moon et al., 2015). Inhibition of pyruvate kinase M2 (PKM2), which is the final step in the glycolytic pathway resulting in the production of pyruvate, also decreases NLRP3 activity (Xie et al., 2016). While an increase in IL-1β production and glycolysis following Aβ+LPS stimulation was revealed, the current findings do not provide sufficient evidence for a link between metabolism and NLRP3 inflammasome activation due to the absence of mature IL-1β and caspase 1. The effect of pharmacological intervention on regulators of the glycolytic pathway and subsequent result on pro-inflammatory cytokine production and NLRP3 inflammasome components is required to examine this association further.

Overall, the data from this study show that Aβ+LPS increased pro-inflammatory cytokine expression and production in MDMs, and exerted a greater effect on TNFα alone in cells from IQ-discrepant participants. A shift in metabolism to glycolysis was evident in MDMs from IQ-discrepant participants, although this was not associated with NLRP3 inflammasome activation. In conclusion, Aβ+LPS stimulation of MDMs and examination of TNFα production and glycolytic activity may be a useful biomarker for the detection of early cognitive decline.

Chapter 8: General Discussion

This study aimed to investigate blood-based biomarkers for early cognitive decline using a two-fold approach. First, the response of monocyte cell lines to plasma was assessed. Secondly, markers in peripheral immune cells from a cohort of older adults described as having an IQ-discrepant memory were examined following incubation with inflammatory stimuli. The main findings of this study were that IL-8 mRNA is increased in THP-1 cells incubated with plasma from individuals with cognitive dysfunction (IQ-discrepant participants, MCI and AD patients). In addition, glycolysis in THP-1 cells was increased following incubation with plasma from AD patients. Importantly, inflammatory stimuli exerted a greater effect on cells derived from IQ-discrepant participants assessed by gene and protein expression, cytokine production and metabolic profile. Overall, the findings suggest that plasma-treated monocytes and pro-inflammatory-stimulated peripheral immune cells could be useful as potential blood-based biomarkers indicative of early cognitive decline.

A specific upregulation in IL-8 mRNA was observed in THP-1 cells incubated with plasma from IQ-discrepant participants and MCI and AD patients. This distinct change has also been detected in a separate group of MCI patients who later progressed to AD and also in another independent cohort of AD patients (McDonald et al, unpublished data). Changes in TNFα and IL-6 mRNA expression were also observed, however these were sporadic and not as persistent as the change in IL-8 expression. A summary of significant increases for each of the markers for all cohorts examined in the study is shown in Table 8.1.

IL-8 is a widely studied pro-inflammatory chemokine and was originally described as a chemoattractant for neutrophils to sites of infection/damage, although it can also act as a chemotactic factor for other immune cells including monocytes, lymphocytes and other granulocytes (Turner et al., 2014). It is released by a large variety of cells including monocytes/macrophages, T cells and endothelial cells in response to a wide range of stimuli and is regulated by a complex network of signal transduction pathways (Baggiolini and Clark-Lewis, 1992, Hoffmann et al., 2002). IL-8 is a critical regulator of inflammation and although neutrophil recruitment is its main function it also has a role in wound healing, angiogenesis and metastasis (Bosch et al., 2002). Its expression is reportedly increased in the brain tissue of AD patients

post-mortem (Sokolova et al., 2009). Many studies have examined the plasma and serum concentrations of IL-8 as a potential biomarker for MCI and AD, however findings are extremely variable across studies with some reporting increases (Alsadany et al., 2013, Malashenkova et al., 2017, Ray et al., 2007), decreases (Kim et al., 2011, Hesse et al., 2016), or finding no changes (Magaki et al., 2007, Swardfager et al., 2010), in IL-8 concentrations compared with controls.

	Significant changes in mRNA expression			
Cohort	**IL-8**	**TNFα**	**IL-6**	**iNOS**
AD (n=6) vs control (n=20)	↑	↑	—	—
MCI (n=17) vs control (n=20)	↑	—	↑	—
IQ-discrepant (n=13) vs IQ-consistent (n=13) Group 1	↑	—	—	—
IQ-discrepant (n=28) vs IQ-consistent (n=43) Group 2	↑	↑	—	—
IQ-discrepant (n=8) vs IQ-consistent (n=7) Group 3	—	—	—	—

Table 8.1 Changes in inflammatory markers in THP-1 cells in response to plasma from all participant and patient cohorts.

Overall, it can be concluded that plasma-induced IL-8 mRNA in THP-1 cells is a reliable, consistent marker of cognitive dysfunction, as it is observed across 3 separate cohorts (AD, MCI and IQ-discrepant). From the 3 groups of IQ-consistent and IQ-discrepant participants, a robust increase in IL-8 is evident in 2 of the groups, with the lack of significance in the third group most likely attributed to a relatively small number of individuals in the cohorts.

The identification of preclinical stages of AD is crucial for early intervention of disease-modifying therapies, and the benefit of validated blood-based biomarkers for this stage would be indispensable. The classification of healthy older adults as either IQ-consistent or IQ-discrepant in this study describes a subtle deficit in episodic memory that might occur before clinically significant deficits in cognitive

function arise such as in MCI and AD. However it has not yet been determined whether cognitive decline progresses with time in individuals classified as IQ-discrepant.

Episodic memory can be broadly described as the ability to remember past experiences, and deficits in episodic memory have been detected from 3-6 years before the diagnosis of AD (Bäckman et al., 2001). The delayed recall subset of the WMS is a commonly-used measure of episodic memory and has been used in longitudinal studies to identify those with a higher risk of developing AD (Elias et al., 2000). In the present study, the degree of discrepancy between the participant's performance on the WMS and NART, which estimates premorbid intellectual function, provides a sensitive neuropsychological index of intra-individual asymmetry between cognitive performances in two domains (one which is resilient to neurodegeneration and one with greater susceptibility to cognitive impairment). This approach has previously been used to detect subtle cognitive deficits relative to premorbid function (Jacobson et al., 2009), and it has also been reported that an asymmetric cognitive profile may infer a greater risk for progression to probable AD (Jacobson et al., 2002). Interestingly, the presence of an APOE ε4 allele is associated with an asymmetric cognitive profile (Jacobson et al., 2005).

Taking this into account, the discrimination of participants as IQ-consistent/IQ-discrepant is well-grounded and it may represent a novel sensitive indicator of subtle cognitive dysfunction. However, the cognitive profiles of participants were not consistent over time. Specifically, only a proportion of those that were identified as IQ-discrepant in the first assessment were IQ-discrepant, according to the same criteria, on the second assessment after two years. Therefore, a longitudinal study must be conducted over a period of longer than two years. The study highlights strengths and weaknesses of the tests used which might have led to the apparent change in cognitive profiles. The NART is a widely used method for determining premorbid IQ, and although some studies have reported a decline in NART scores in dementia patients, it has been shown to be more resistant to the effects of ageing in a healthy adult population (Lowe and Rogers, 2011). Thus, the NART was only performed at the initial assessment and was omitted at the second assessment. On

the other hand, the WMS was performed at both assessments in order to examine potential cognitive decline. While the WMS is an appropriate test for examining subtle cognitive deficits in otherwise clinically healthy adults (Davies et al., 2005), repeated administration of the WMS could possibly lead to practice effects. This has previously been reported by Gavett et al (2016), who revealed that control and MCI, but not AD patients, performed better on the delayed recall subset over 5 annual assessments. This questions the reliability of the WMS as an appropriate neuropsychological test for repeated use and monitoring of progression at least over the short term. This issue could be overcome by using alternative stories in additional assessments, however this is not provided as part of the WMS-IV. An alternate form of the WMS IV Logical Memory subsets, the Morris Revision-IV, is currently being validated, which has demonstrated a high correlation and interrater reliability to the WMS IV Logical Memory subsets (Morris et al., 2014). New paragraphs such as these would increase the clinical utility of the WMS for monitoring cognitive decline longitudinally. Another factor which might possibly impact on the change in cognitive profiles is the fact the WMS is comprised of two separate batteries depending on the age of the participant i.e. the Adult or Older Adult Battery. As the Older Adult battery is more simplified, this could lead to unexpected changes in cognitive scores if the participant qualifies for the Older Adult battery after the intervening time. Finally, it is possible that performance on the Logical Memory subset could be affected by how the participant is feeling physically and emotionally on the testing day. Further investigation is required to determine if the cognitive profiles of individuals fluctuate over time, perhaps until a threshold is reached after which persistent deficits in cognitive decline might be evident, or if the WMS is robust enough to be used as an accurate indicator of cognitive function.

The MMSE and MoCA scores of IQ-consistent and IQ-discrepant individuals were consistently found to be within the normal range, indicating that global cognitive function was intact. Both assessments are used routinely as screening tests for MCI and AD, but the MoCA in particular is useful as it has a high sensitivity and specificity for detecting MCI compared with the MMSE (Nasreddine et al., 2005). The MoCA comprises of separate tasks which examines 6 cognitive domains: short-term

memory; visuospatial abilities; executive functioning; attention, concentration and working memory; language; and orientation to place and time (Coen et al., 2016). However it has been reported that individual subtests do not provide sufficient information for accurately assessing cognitive impairment in specific cognitive domains (Moafmashhadi and Koski, 2013), suggesting that the MoCA should solely be used for assessing global cognitive function and not for an in-depth neuropsychological assessment (Coen et al., 2016). In this study, the MoCA scores of MCI patients were lower than IQ-consistent and IQ-discrepant participants, and the MoCA scores of AD patients were decreased further, indicating a progressive worsening of cognitive function. The fact that an increase in IL-8 mRNA was observed in cells incubated with plasma from all 3 cohorts suggests that it could be used as an early indicator of cognitive decline and also to monitor progression along the AD continuum.

In relation to the majority of studies investigating potential blood-based biomarkers for cognitive dysfunction, this study adopted a different approach to directly analysing the concentrations of circulating proteins and other factors. Variability and the low rate of reproducibility between studies remains a significant hurdle in blood-based biomarker research. For example, one of the first prominent publications in the field by Ray and colleagues identified a promising protein signature that distinguished AD and predicted progression from MCI with a high level of accuracy (Ray et al., 2007). However, subsequent studies which analysed this particular panel could not replicate these findings in independent cohorts (Soares et al., 2009, Marksteiner et al., 2011, Bjorkqvist et al., 2012). As the increase in IL-8 mRNA in THP-1 cells has been replicated in two cohorts of IQ-consistent/IQ-discrepant participants and in other groups of MCI/AD patients outside of the current study, it seems reasonable to conclude that the assay might overcome the issue of reproducibility, although the reliability and specificity of the findings needs to be rigorously tested.

One of the most important issues arising from the use of plasma-treated monocytes as a method of identifying a biomarker assay is the identification of the factor(s) responsible for generating a specific increase in IL-8 mRNA. IL-8 expression in monocytes and macrophages is induced by a wide range of substances including

bacterial and viral products, lectins, phorbol esters and pro-inflammatory cytokines, most notably TNFα and IL-1β (Baggiolini and Clark-Lewis, 1992). No single protein in the plasma is likely to be responsible for eliciting an increase in IL-8 mRNA; it is more likely to be increased by a cocktail of factors. Aβ is also a potent stimulator of IL-8 in mononuclear cells (Yates et al., 2000), and in a study which reported the upregulation and downregulation of 104 genes in Aβ-treated microglia isolated from the brain tissue of non-demented individuals *post-mortem*, IL-8 showed the largest upregulation (Walker et al., 2001). However, Aβ is present at a low concentration in the plasma therefore any effects might be minimal due to the large dilution of plasma in the assay. In addition, inhibition of TLR2, a receptor for Aβ (Liu et al., 2012), in THP-1 cells did not attenuate the plasma-induced increase in IL-8 expression, suggesting that the upregulation of IL-8 mRNA is not as a result of Aβ in the plasma.

The production of IL-8 is rapidly induced following pro-inflammatory stimulation (Hoffmann et al., 2002), therefore it is possible that this particular upregulation in IL-8, (in the absence of changes in other pro-inflammatory markers) is an early inflammatory response. Transcription of IL-8 is regulated by NF-κB and AP-1, which is activated through MAPK pathways (Hoffmann et al., 2002). Under basal conditions, IL-8 expression is supressed by the NF-κB-repressing factor (NRF) (Nourbakhsh et al., 2001), therefore it is possible that the factors in the plasma inactivate NRF, resulting in the subsequent increased expression of IL-8 mRNA. Identification of the factors in the plasma inducing IL-8 is important in order to determine if this signal in THP-1 cells is indicative of any underlying mechanism related to the pathogenesis of cognitive decline. Future studies should therefore include multiplex analysis of the plasma despite the variability observed in such assays, to determine if there are any significant differences in participant and patient cohorts. Neutralising experiments could subsequently be carried out to conclusively determine if the identified factors are responsible for the upregulation in IL-8 expression. The increase in glycolysis in THP-1 cells in response to plasma from AD patients is an additional interesting element and suggests that the substance in the plasma of AD patients could possibly alter both IL-8 expression and glycolysis in THP-1 cells.

The second approach for investigation of potential blood-based biomarkers in this study adopted the *ex vivo* stimulation of MDMs and PBMCs from IQ-consistent and IQ-discrepant participants. This method has advantages and disadvantages compared with using plasma-treated monocytes. The examination of primary cells from subject groups allows for direct analysis of inflammatory and metabolic responses which is more physiologically relevant, as opposed to assessing the effect that plasma exerts on a cell culture system which is an indirect examination of biological function. However, isolation and culture of MDMs and PBMCs is time consuming and more costly compared with maintaining cell lines. THP-1 cells can be stored and used whenever they are required, and large numbers can be produced very quickly. Plasma can also be stored long-term, which means that the assay can be carried out at any time. Even though it is possible to cryogenically store PBMCs and MDMs, the response of preserved cells is impaired and is therefore not a practical option for assessment of cell physiology (Keane et al., 2015, Silveira et al., 2013). In addition, cell numbers are affected through the freeze-thaw process and although large numbers of PBMCs can be stored, it must be kept in mind that this is a mixed cell population and it is preferable to examine single cell type populations wherever possible to hopefully yield clearer results. There is also a large degree of variation in the response of *ex vivo* stimulated cells, whereas the margin of variation is lower in plasma-treated THP-1 cells which might allow for the detection of subtle differences. In conclusion both approaches have benefits and drawbacks, THP-1 cells can be used in a straight-forward and efficient manner however MDMs and PBMCs provide information on cellular function that might be related to underlying disease mechanisms.

MDMs and PBMCs derived from IQ-discrepant participants exhibited differential expression of markers compared with cells from IQ-consistent participants. Interestingly, many of the changes were only observed following stimulation with pro-inflammatory agents, which are summarised in Table 8.2. This indicates that examination of cells under basal conditions is not always sufficient to reveal potential cellular dysfunction and that the exaggerated response of cells to

inflammatory challenge is a possible marker that should be kept in mind when performing future experiments.

IQ-discrepant vs IQ-consistent	Summary of findings
Aβ-stimulated MDMs	TNFα mRNA ↑ % of $TLR2^+$ and $CD206^+$ cells ↑
Aβ+LPS-stimulated MDMs	TNFα mRNA and secretion ↑ OCR:ECAR ↓ IL-6, IL-8, IL-1β, TLR2 mRNA ↓ IL-6 secretion ↓ Pro-IL-1β protein ↓
Aβ+LPS-stimulated PBMCs	TNFα, IL-6, IL-8 secretion ↑ Glycolysis ↑ PFKFB3 mRNA ↑ PFKFB3:PFKFB1 ↑

Table 8.2 Changes in inflammatory and metabolic markers in pro-inflammatory stimulated MDMs and PBMCs from IQ-discrepant, compared with IQ-consistent, participants.

The consistent finding across MDMs and PBMCs with Aβ and/or LPS stimulation from IQ-discrepant subjects is an increase in the mRNA expression or production of TNFα. An increase in TNFα release in MDMs incubated with LPS was also reported by Downer and colleagues in 2013 who examined a separate cohort of individuals classified in the same way. TNFα is one of the major products of pro-inflammatory macrophages and plays a central role in regulating the immune response and can stimulate the production of other inflammatory cytokines (Perry et al., 2001). Higher concentrations of TNFα from PBMCs is associated with an increased risk of developing AD (Tan et al., 2007) and TNFα release from PBMCs, following either Aβ or LPS stimulation, is exacerbated in AD patients compared with controls (Reale et al., 2004, Lombardi et al., 1999). Furthermore, an increase in TNFα is consistently observed in the plasma of AD patients and it has been proposed as part of protein signatures of AD (Ray et al., 2007, O'Bryant et al., 2010, Swardfager et al., 2010). Interestingly, high baseline circulating concentrations of TNFα are associated with a faster rate of cognitive decline in the presence of a systemic infection in AD patients,

whereas those with low concentrations of TNFα do not display the same degree of cognitive decline over a 6 month period (Holmes et al., 2009). Taken together, TNFα plasma concentrations and production from PBMCs could potentially be a useful marker of AD, and the current study extends these findings to an earlier stage of cognitive dysfunction.

The data also suggest that an increase in glycolytic metabolism in MDMs and PBMCs derived from IQ-discrepant participants may be a promising marker. The metabolic function of peripheral immune cells in AD has so far only been examined by one group who reported a decrease in mitochondrial respiration in PBMCs from AD patients (Maynard et al., 2015). The recent development of Seahorse technology for assessment of glycolysis and oxidative phosphorylation offers a simple method for determining the bioenergetics of cells which could be very useful in biomarker research.

Interesting elements of the study which should be considered in future work is to stratify the participants by certain factors and to assess the relationship of the factors between participant groups. For example, it would have been beneficial to assess the APOE status of participants, particularly as the presence of an APOE ε4 allele is reported to be associated with a greater rate of cognitive decline in older adults (O'Hara et al., 1998). In addition, an asymmetric cognitive profile has previously been associated with APOE ε4 status (Jacobson et al., 2005). Significant differences in gender were observed in IL-8 mRNA in THP-1 cells following incubation with plasma; therefore it would also be interesting to examine the wider effect of gender on other biomarkers identified here in larger cohorts. In addition, future work should aim to examine possible alternatives to the WMS as a neuropsychological test to calculate a cognitive asymmetry index. The participants in the MRU underwent a battery of assessments in the initial assessment, one of which was the Free and Cued Selective Reminding Test (FCSRT; (Grober and Buschke, 1987). The FCSRT is a widely used memory test that consists of a learning phase followed by a free recall cued recall phase that assesses attention, cognitive processing, acquisition and retrieval (Lemos et al., 2014). It has been used successfully to identify prevalent dementia and MCI patients likely to develop AD, to

distinguish AD from other dementias and also to predict future dementia (Grober et al., 2010). Thus, it would be beneficial to examine its utility in determining an asymmetry cognitive index in comparison to the WMS. Finally, it would be interesting to examine other clinical parameters gathered during the testing session, such as BMI calculated by height and weight, to assess the relationship between metabolic factors and cognitive decline.

To my knowledge, this is the first study that has examined the inflammatory and metabolic response of PBMCs or MDMs in cognitively normal adults in an effort to develop a blood-based biomarker for early cognitive decline. Future experiments should aim to assess the reliability of these novel markers in larger cohorts and also in MCI and AD patients in an effort to determine if the markers could be indicative of progression along the AD continuum. Ultimately, longitudinal studies of cognitively normal older adults are essential in identifying markers of early cognitive dysfunction indicative of further decline, in order to establish a reliable blood-derived biomarker to tackle the exponential rise in AD.

Chapter 9: Bibliography

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Chapter 10: Appendix

Appendix I: List of publications and abstracts

Submitted to the Journal of Neuroimmunology, August 2017

A shift to glycolysis accompanies the inflammatory changes in PBMCs from individuals with an IQ-discrepant episodic memory

Hannah Wolfe, Caoimhe Hannigan, Michael O'Sulllivan, Liam Barry Carroll, Sabina Brennan, Brian Lawlor, Ian H Robertson, Marina Lynch

Identification of a blood-based biomarker that can detect early cognitive decline and replace expensive and invasive imaging and CSF biomarkers is a significant healthcare challenge. Here, we prepared peripheral blood mononuclear cells (PBMCs) from a cohort of healthy individuals who were identified as having a poorer than predicted performance on their delayed recall performance on the Logical Memory II Subset of the Wechsler Memory Scale (WMS) relative to their IQ estimated by the National Adult Reading Test (NART); we described these individuals as IQ-memory discrepant (IQ-discrepant), compared with IQ-memory consistent (IQ-consistent) individuals. The objective of the study was to determine whether PBMCs from IQ-consistent and IQ-discrepant individuals differentially respond to amyloid-β (Aβ) + lipopolysaccharide (LPS), specifically in terms of upregulation of inflammatory cytokines and metabolic signature. The data show that Aβ + LPS increased production of TNFα, IL-6 and IL-8, but not IL-1β, to a greater extent in PBMCs from IQ-discrepant, compared with IQ-consistent, individuals. Importantly, this was associated with a shift in metabolism towards glycolysis and the evidence indicates that 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB)3 plays a role in driving glycolysis. A similar shift to glycolysis was observed in MDMs prepared from IQ-discrepant, compared with IQ-consistent, individuals. The important finding here is that we have established an increased sensitivity to Aβ + LPS stimulation in PBMCs from individuals that underperform on a memory task relative to their estimated premorbid IQ which may be an indicator of early cognitive decline. This may be a useful took in determining the presence of early cognitive dysfunction.

Accepted for poster presentation at the Alzheimer's Association International Conference (AAIC) 2017

Evidence that a metabolic shift towards glycolysis in PBMCs may provide the basis of a biomarker of early cognitive dysfunction

Hannah Wolfe, Michael O'Sullivan, Caoimhe Hannigan, Sabina Brennan, Brian Lawlor, Ian Robertson, Marina Lynch

A major challenge in the context of the increasing aging population is to identify a method of identifying cognitive dysfunction at the earliest possible opportunity, preferably a marker that is blood-based and therefore amenable to assessment on a longitudinal basis. In this study, we identified 2 cohorts of healthy older adults that were classified as IQ-memory consistent (n=47) or IQ-memory discrepant (n=28; IQ-con, IQ-dis) on the basis of their performance in the Wechsler Memory Scale story recall test relative to their performance in the National Adult Reading Test.

We cultured THP-1 cells and incubated them for 4h with plasma (1:40) from IQ-con and IQ-dis individuals. Cells were harvested and assessed for mRNA expression of Interleukin (IL)-8 and tumour necrosis factor-α (TNFα). The data indicate that both IL-8 mRNA and TNFα mRNA were increased in THP-1 cells that were incubated with plasma from IQ-dis, compared with IQ-con, individuals. These data are indicative of an inflammatory phenotype that is linked with poorer cognitive function and to explore this further, we selected the 10 individuals from the IQ-dis cohort with the 10 highest IL-8 mRNA values and the 10 IQ-con individuals with the 10 lowest IL-8 mRNA values for further analysis.

It has been shown that inflammatory changes in several cell types is associated with a shift in metabolic profile towards glycolysis and, to determine whether the inflammation that was suggested by our data in IQ-dis individuals was linked with altered metabolism in PBMCs, we stimulated cells with amyloid-β (Aβ) and lipopolysaccharide (LPS) for 24h and assessed changes using a SeahorseXF^{e}24 Analyser. The data show that glycolysis in Aβ+LPS stimulated cells was significantly greater in PBMCs from IQ-dis individuals compared with IQ-con individuals. It is known that phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) is a significant driver of glycolysis because it increases PFKFB1 activity, which converts fructose-6-phosphate to fructose-1, 6-bisphosphate. The data show that PFKFB3 mRNA was also increased in PBMCs from IQ-dis individuals and a significant increase in the ratio of PFKFB3/PFKFB1 was also observed.

We conclude that the metabolic shift towards glycolysis on PBMCs could represent a potential biomarker assay for the detection of early cognitive decline.

Appendix II: List of Materials

Absolute ethanol	Hazmat, TCD
Aβ	Invitrogen
Biocidal ZF™	WAK-Chemie Medical
Blood collection tubes	Cruinn Diagnostics/Greiner
$CD14^+$ Microbeads	Miltenyi Biotec
cDNA Archive Kits	Applied Biosystems
Cell scrapers	Sarstedt
Cell strainers	Fisherbrand
Compensation Beads	BD Bioscience
Cryovials	Cruinn Diagnostics/Greiner
DMEM	Gibco
ELISA kits	R&D Systems
ELISA plates	Sigma-Aldrich/NUNC
FACS antibodies	Biolegend, R&D Systems, eBioscience
FACS tubes	BD Biosciences
FBS	Gibco
General laboratory chemicals	Sigma-Aldrich (unless otherwise stated)
General laboratory plasticware	Sarstedt and Fisher Scientific
Haemocytometer	Marienfled-Superior
IgG	R&D Systems
Live/Dead Stain	Life Technologies
LPS	Enzo Life Sciences
LY294002	Sigma-Aldrich
Lympoprep™	Axis-Shield
Mesoscale assay	Mesoscale Discovery
Microcentrifuge tubes	Eppendorf
Microplate sealers	Cruinn Diagnostics/Greiner
Needles (23 gauge)	BD Microlance

Nucleospin RNA Isolation Kits	Machery-Nagel
Oligomycin	Abcam
Pasteur pipettes	Fisher Scientific
Penicillin/Streptomycin	Gibco
Percoll	Sigma-Aldrich
Petri dish	Gibco
Pierce BCA assay	Fisher Scientific
Poly-D-Lysine	Merck Millipore
Protein assay plates	Sarstedt
RA1 buffer	Machery-Nagel
Recombinant human GM-CSF	R&D Systems
Recombinant human IFNγ	R&D Systems
Recombinant human IL-4 and IL-13	Biolegend
Recombinant mouse IFNγ and IL-4	R&D Systems
RPMI	Hyclone
RT-PCR plates	Fisher Scientific
RT-PCR tubes	Sarstedt
Scalpels	Swann-Morton
Sterile filter tips	Biopointe Scientific
Sterile plasticware	Sarstedt
Syringe filters	Millipore
Syringes	BD Plastipak
Taqman Gene expression Assays	Applied Biosystems
Tissue culture flasks and plates	Fisher Scientific
TLR2 antibody	R&D Systems
Trypan Blue	Sigma-Aldrich
Virkon	DuPont
Western Immunoblotting antibodies	Cell Signalling Technology and Abcam
XF24 Base Medium	Seahorse Bioscience
XF24 Calibrant and Cartridges	Seahorse Bioscience
XF24 cell culture plates	Seahorse Bioscience

Appendix III: List of Solutions

Medium A

500 ml 1 X Hank's Balanced Salt Solution (HBSS; GIBCO®, Life Technologies)

6.5 ml Glucose (45%; Sigma-Aldrich)

7.5 ml HEPES (1 M; GIBCO®, Life Technologies)

Percoll Gradients

Percoll® (Sigma-Aldrich)

Phosphate Buffered Saline (PBS, Sigma-Aldrich)

Per animal:

100% Percoll: 10.35 ml Percoll + 1.15 ml 10 X HBSS

75% Percoll: 7.5 ml 100% Percoll + 2.5 ml 1 X PBS

25% Percoll: 2.5 ml 100% Percoll + 7.5 ml 1 X PBS

MACS buffer

1 X PBS (Sigma-Aldrich)

Foetal bovine serum (FBS, 2%, GIBCO®, Life Technologies)

2mM EDTA (Sigma-Aldrich)

Lysis buffer (for protein analysis)

0.394 g Tris HCl (Sigma-Aldrich)

0.438 g NaCl (Sigma-Aldrich)

500 µl NP-40 (IPEGAL) (Sigma-Aldrich)

50 ml dH_2O

4 X Tris-glycine sample buffer

1.25 ml Tris HCl pH6.8 (1 M; Sigma-Aldrich)

5 ml Glycerol (Sigma-Aldrich)

1.6 ml SDS (25%; Sigma-Aldrich)

1.15 ml dH_2O

1 ml β-mercaptoethanol (Sigma-Aldrich)

Bromophenol blue granules (Sigma-Aldrich)

2 X sample buffer (for Aβ)

Tris HCl pH8.45 (125 mM; Sigma-Aldrich)

SDS (2%; Sigma-Aldrich)

Glycerol (20%; Sigma-Aldrich)

Bromophenol blue granules (Sigma-Aldrich)

β-mercaptoethanol (5%; Sigma-Aldrich)

10% separating gel

3.3 ml acrylamide (30%; Sigma-Aldrich)

4.1 ml dH_2O

2.5 ml SPA buffer (90.8 g Tris base, 500 ml dH_2O, 1 g SDS; pH8.8; Sigma-Aldrich)

100 µl APS (10%; Sigma-Alrich)

5 µl TEMED (Sigma-Aldrich)

12% separating gel

4 ml acrylamide (30%; Sigma-Aldrich)

3.4 ml dH_2O

2.5 ml SPA buffer (90.8 g Tris base, 500 ml dH_2O, 1 g SDS; pH8.8)

100 µl APS (10%; Sigma-Aldrich)

5 µl TEMED (Sigma-Aldrich)

4% stacking gel

1.3 ml acrylamide (30%; Sigma-Aldrich)

6.1 ml dH_2O

2.5 ml stacking buffer (15.13 g Tris base, 250ml dH_2O; 1 g SDS; pH6.8; Sigma-Aldrich)

50 µl APS (10%; Sigma-Aldrich)

10 µl TEMED (Sigma-Aldrich)

10 X Electrode running buffer

30 g Tris base (Sigma-Aldrich)

144 g Glycine (Sigma-Aldrich)

10 g SDS (Sigma-Aldrich)

1 L dH_2O

16.5 % separating gel (for Aβ)

2.5 ml peptide gel buffer (36 g Tris HCl, 100 ml dH_2O, SDS 0.3%, pH8.45, Sigma-Aldrich)

4.4 ml acrylamide (30%; Sigma-Aldrich)

0.65 ml dH_2O

1.25 ml Glycerol (80%; Sigma-Aldrich)

50 µl APS (Sigma-Aldrich)

5 µl TEMED (Sigma-Aldrich)

10% spacing gel (for Aβ)

0.66 ml peptide gel buffer (36 g Tris HCl, 100 ml dH_2O, SDS 0.3%, pH8.45, Sigma-Aldrich)

0.7 ml acrylamide (30%; Sigma-Aldrich)

0.83 ml dH_2O

10 µl APS (Sigma-Aldrich)

3 µl TEMED (Sigma-Aldrich)

4% stacking gel (for Aβ)

1.55 ml peptide gel buffer (36 g Tris HCl, 100 ml dH_2O, SDS 0.3%, pH8.45, Sigma-Aldrich)

0.9 ml acrylamide (30%; Sigma-Aldrich)

4 ml dH_2O

50 µl APS (Sigma-Aldrich)

5 µl TEMED (Sigma-Aldrich)

10 X Anode buffer (for Aβ)

121.14 g Tris HCl (Sigma-Aldrich)

500 ml dH_2O

10 X Cathode buffer (for Aβ)

60.57 g Tris HCl (Sigma-Aldrich)

89.6 g Tricine (Sigma-Aldrich)

SDS (1%; Sigma-Aldrich)

500 ml dH_2O

10 X Transfer buffer

30 g Tris base (Sigma-Aldrich)

144 g Glycine(Sigma-Aldrich)

Methanol (10%)

1 L dH_2O

10 X Tris-buffer saline (TBS)

31.52 g Tris HCl (pH7.5; Sigma-Aldrich)

87.66 g NaCl (Sigma-Aldrich)

1 L dH_2O

0.05% Tween-20 (added to 1 X TBS for TBS-T; Sigma-Aldrich)

Wash buffer

1 X PBS (Sigma-Aldrich)

0.05% Tween-20 (Sigma-Aldrich)

FACS buffer

1 X PBS (Sigma-Aldrich)

2% FBS (GIBCO®, Life Technologies)

Appendix IV: Supplementary data

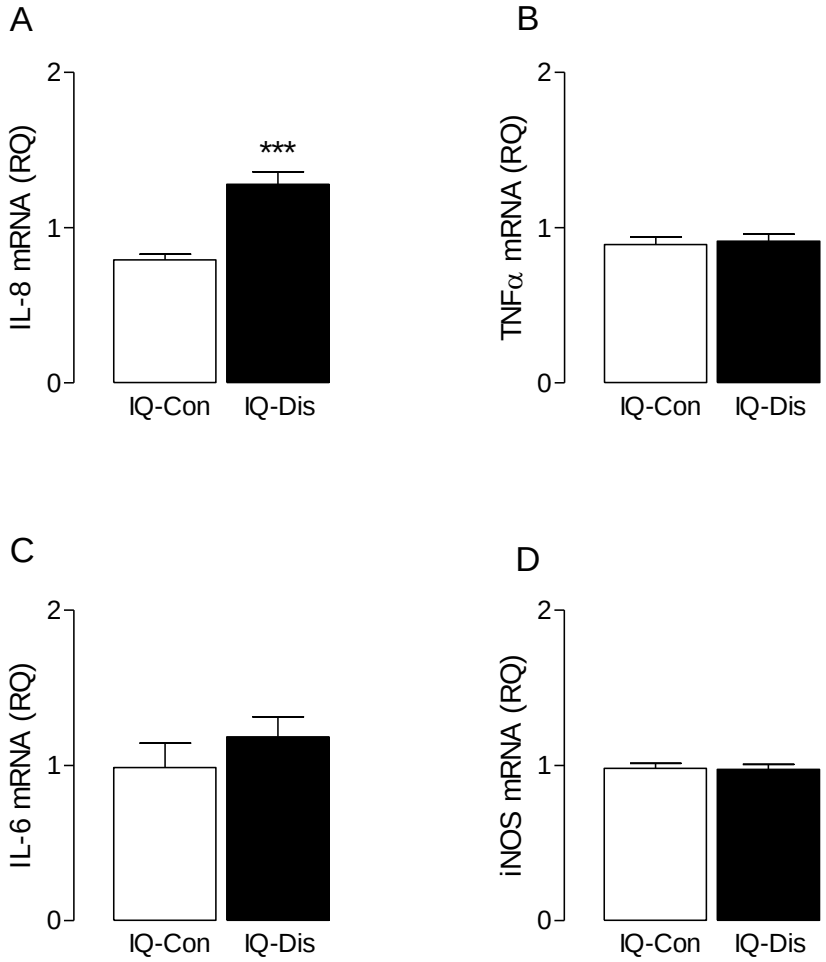

Figure S1. IL-8 mRNA is increased in THP-1 cells in response to plasma from IQ-discrepant individuals.

THP-1 cells were treated with or without plasma from IQ-consistent and IQ-discrepant participants (IQ-Con, IQ-Dis) at a 1:40 dilution for 24 h and analysis of pro-inflammatory marker expression performed using RT-PCR. Plasma from IQ-Dis participants significantly increased IL-8 mRNA in THP-1 cells in comparison to IQ-Con participants (A). No difference in TNFα (B), IL-6 (C) or iNOS (D) mRNA were observed. Data are expressed as the mean ± SEM, n = 13 for groups; ***p<0.001 vs IQ-Con; Student's t-test for independent means.

	IQ-consistent		IQ-discrepant		Group effect		LPS effect		Group x LPS	
Variable	Control	LPS	Control	LPS	F	*P* value	F	*P* value	F	*P* value
% $CD11b^+$	85.656±3.337	86.283±2.893	90.287±2.674	89.994±2.759	2.031	0.164	0.003	0.955	0.025	0.877
% $CD11b^+$ $TLR2^+$	8.225±2.472	15.002±4.535	49.312±9.881	54.435±8.515	29.710	*0.001****	0.650	0.427	0.012	0.911
% $CD11b^+$ $TLR4^+$	1.000±0.632	0.871±0.532	2.671±1.169	4.718±1.893	5.407	*0.027**	0.654	0.425	0.841	0.366
% $CD11b^+$ $CD206^+$	2.103±1.989	1.598±1.494	40.177±12.063	49.121±13.271	9.607	*0.005***	0.094	0.763	0.117	0.736
CD11b mRNA	1.134±0.067	0.654±0.042	1.015±0.065	0.707±0.065	0.234	0.599	41.09	*0.001****	1.975	0.170
TLR2 mRNA	0.965±0.106	5.422±1.040	1.853±0.587	5.766±1.488	0.418	0.523	19.280	*0.001****	0.082	0.777
TLR4 mRNA	0.903±0.039	0.883±0.053	0.862±0.095	0.752±0.039	1.895	0.179	1.071	0.309	0.523	0.472
MRC-1 mRNA	0.950±0.098	0.282±0.034	0.813±0.046	0.373±0.066	0.124	0.728	68.19	*0.001****	2.883	0.099
IL-8 mRNA	0.738±0.178	34.636±4.952	4.230±1.844	41.424±6.732	1.467	0.235	70.130	*0.001****	1.508	0.701
IL-8 pg/ml	2494.319± 252.805	36051.220± 2516.950	6550.345± 1979.341	40750.580± 2800.197	4.299	0.465	257.5	*0.001****	0.023	0.880
IL-6 mRNA	25.410±0.614	34.864±0.300	27.038±0.791	34.222±0.585	0.672	0.419	191.4	*0.001****	3.563	0.068
IL-6 pg/ml	530.203± 274.662	12484.490± 2263.872	437.125± 90.911	18678.040± 5841.845	0.860	0.361	21.060	*0.001****	0.913	0.347
TNFα mRNA	1.326±0.457	9.387±0.825	1.583±0.307	7.898±1.274	0.582	0.451	79.250	*0.001****	1.170	0.288
TNFα pg/ml	333.113± 91.50	33369.220± 5120.126	1809.316± 568.740	43805.290± 9211.200	1.263	0.269	50.100	*0.001****	0.714	0.404

Table S1. MDMs stimulated with LPS (100 ng/ml) from IQ-consistent and IQ-discrepant participants.

Data are expressed as mean ± SEM, IQ-consistent n=10, IQ-discrepant n=10. **p*<0.05, ***p*<0.01, *** *p*<0.001 vs. IQ-consistent; Two-way ANOVA with Newman Keuls *post-hoc* analysis.