Neuroinflammation, microglia and the cell biology of Alzheimer's Disease

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Abbreviations

A

AA	Amino acid
ABI3	ABI family member 3
Aβ	Amyloid beta
AD	Alzheimer's Disease
ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
ADAMTS4	A disintegrin and metalloproteinase with thrombospondin motifs 4
ASC	Apoptosis associated speck-like protein containing a CARD
ATP	Adenosine 5′-triphosphate
ANOVA	Analysis of variance
APH1B	Aph-1 Homolog B
APP	Amyloid precursor protein
ApoE	Apolipoprotein E
ApoER	Apolipoprotein E receptor
Arf	ADP-ribosylation factor

B

BACE1	β-secretase 1
BBB	Blood brain barrier
BIN1	Bridging integrator 1
BMDM	Bone marrow derived macrophage
BSA	Bovine serum albumin

C

CARD	Caspase recruitment domain
CASS4	Cas scaffolding protein family member 4
CD2AP	CD2 associated protein
CLNK	Cytokine dependent hematopoietic cell linker

CLU	Clusterin
CNS	Central nervous system
CR1	Complement receptor 1
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CTF_{β}	C terminal fragment beta

D

DAMP	Danger associated molecular pattern
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DIV	Day *in vitro*
DMEM	Dulbeccos modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

E

ECHDC3	Enoyl-CoA Hydratase Domain Containing 3
EDTA	Ethylenediamine tetra-acetic acid
eIF4B	Eukaryotic translation initiation factor 4B
ELISA	Enzyme-linked immunosorbent assay
EMP	Erythromyeloid progenitors
EOAD	Early onset Alzheimer's Disease
EPHA1	Ephrin type-A receptor 1 precursor
ER	Endoplasmic reticulum

F

FAD	Familial Alzheimer's Disease
FBS	Foetal bovine serum

G

GFP	Green fluorescent protein
GGA1	Golgi-localised gamma adaptin ear-containing binding protein 1

GTPase	Small G protein
GWAS	Genome wide association study

H

H3334	22'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate
HBSS	Hanks' Balanced Salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFIP	Hexafluoro-2-propanol
HRP	Horseradish peroxidase

I

ICPL	Isotype-coded protein labelling
IFNγ	Interferon gamma
IL	Interleukin
IL1RA	Interleukin 1 receptor antagonist
IL-1β	Interleukin 1 beta
iNOS	Inducible nitric oxide synthase
INPPD5	Inositol Polyphosphate-5-Phosphatase D

L

LDH	Lactase dehydrogenase
LOAD	Late onset Alzheimer's Disease
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat

M

Map2	Microtubule associated protein 2
MAPK	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
MHC	Major histocompatibility complex
MMP13	Matrix metallopeptidase 13

MS	Mass Spectrometry
MS4A	Membrane spanning 4A

N

NED	N-1-naphthylethylenediamine dihydrochloride
NFκB	Nuclear factor kappa B
NFT	Neurofibrillary tangles
NLR	Nucleotide-like receptor
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NMDA	N-Methyl-D-aspartate
NMDAR	N-Methyl-D-aspartate receptor
NOD	Nucleotide oligomerisation domain
NSAIDs	Non steroidal anti-inflammatory drugs

P

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PICALM	Phosphatidylinositol binding clathrin assembly protein
PI3K	Phosphatidylinositide kinase-3
PPARγ	Peroxisome proliferator-activated receptor gamma
PS	Phosphatidyl-serine
PSEN1	Presinilin-1
PSEN2	Presinilin-2
PVDF	Polyvinylidene difluorid
PX	Phox domain
PYD	Pyrin domain

R

RA	Rheumatoid arthritis
Rab	Ras-associated binding protein
ROS	Reactive oxygen species

RPMI	Roswell Park Memorial Institute

S

SCIMP	SLP Adaptor And CSK Interacting Membrane Protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Siglecs	Sialic acid-binding immunoglobulin-like lectins
SNP	Single nucleotide polymorphism
SNX	Sortin nexin
SORL1	Sortillin-related receptor

T

TFE	2,2,2-Trifluorethanol
Tcep	Tris(-carboxyethyl)phosphine hydrochloride
TNFα	Tumour necrosis factor alpha
TREM2	Triggering receptor expressed in myeloid cells 2

W

Wt	Wild type

X

XTT	(2,3-Bis-(2-Methoxy-4-Nitro-5Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide)

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Abstract

The pathology of Alzheimer's disease (AD) is characterised by progressive accumulation of misfolded proteins, which form senile plaques and neurofibrillary tangles, and chronic inflammation in the brain associated with inflammatory mediators by the activation of innate immune responses. There has been considerable interest in the role of neuroinflammation in directly contributing to the progression of AD. Studies in mice and humans have identified a role for microglial cells, the resident innate immune cells of the CNS, in AD. Activated microglia are a key hallmark of the disease and the secretion of pro-inflammatory cytokines by microglia may result in a positive feedback loop between neurons and microglia, resulting in ongoing low-grade inflammation and associated neurotoxicity. The underlying mechanisms however are poorly understood. Here the role of microglia was investigated, especially their link to ApoE – the strongest risk factor for late onset Alzheimer's disease – and the relationship between microglia, neurons and neuroinflammation.

Target replacement mice were used, where the human ApoE2, ApoE3 or ApoE4 allele replaces the mouse ApoE allele. Microglia were activated in a two-step setup. Initially cells were primed with LPS, followed by a secondary stimulus, such as ATP or Aβ. The system was used to characterise the cytokines secreted by activated microglia and to assess the impact of conditioned medium from stimulated and Aβ treated microglia on neuronal morphology.

The first results Chapter (Chapter 3) established the system – mouse microglia were isolated from brains of neonatal mice and characterised by CD11b staining. Microglia from all three ApoE genotypes were directly compared and the data from ELISA and mass spectrometry revealed an enhanced pro-inflammatory response by ApoE4 microglia and the least efficient at internalizing amyloid β.

Chapter 4 analysed the impact of conditional medium from the microglia ApoE variants on neurons and the results showed an ApoE-dependent effect on dendrite morphology. Conditioned media from immunostimulated and Aβ microglia were incubated with cortical neurons from wt animals. Both the dendrite length and number of dendrites were significantly reduced in neurons treated with conditioned medium from ApoE4 microglia.
TNFα was identified as a major cytokine and was responsible for modifying neuron morphology in cell assays. Neutralising the cytokine, with an anti-TNFα antibody abrogated the majority of morphological changes induced by the conditioned media from activated microglia. Hence the data suggests that TNFα may have a major role in mediating neuroinflammation.

The third results Chapter (Chapter 5) compared aspects of macrophage function with microglia. Here it was shown, that microglia do not require SNX5 for macropinocytosis, and most likely utilise peripheral mediated macropinocytosis as the main form of macropinocytic internalisation. A major finding was the ability of sodium chloride to augment a pro-inflammatory response not only by immunostimulated macrophages by also microglia. Inhibition of the p38 MAPK signalling pathway partially ameliorated the NaCl-induced inflammatory responses in both macrophages and microglia, together with high levels of secreted IL-1β, indicating activation of the NLRP3 inflammasome.

Overall the studies highlight a role for APoE4 allele to promote an enhanced inflammatory response by microglia cells.

Declaration

This is to certify that

(i) the thesis comprises only my original work towards the PhD except where indicated in the preface,

(ii) due acknowledgement has been made in the text to all other material used; and

(iii) the thesis is fewer than a maximum word limit in length (100,000 words), exclusive of tables, maps, bibliographies and appendices.

Alessandra Webers

Preface

The following people have contributed to this work;
Stephanie Schwartz performed genotyping of the ApoE TR mice. Xiao Peng Lin performed genotyping of $SNX5^{-/-}$ mice.

Some of the work presented in this thesis has been prepared for or submitted for publication:

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‘Life is a journey to be experienced, not a problem to be solved’

- A. A. Milne

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List of publications

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Webers A, Heneka MT and Gleeson PA (2019) The role of innate immune responses and neuroinflammation in amyloid accumulation and progression of Alzheimer's disease, Immunology and Cell Biology, https://doi.org/10.1111/imcb.12301

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Chapter 1: Literature review

1.1 Alzheimer's disease and hallmarks of the disease

Alzheimer's disease (AD) was first identified in 1906 by Alois Alzheimer who described the characteristic memory loss and confusion, as well as other psychological symptoms, in a 51year old female patient. In the same patient's brain, Alzheimer identified plaque formation, neurofibrillary pathology, tangles, astrogliosis and neuronal loss (Alzheimer A, 1906).

AD is now recognized as the most common neurodegenerative disease and is characterized by initial short-term memory loss followed by subsequent severe deficits attributed to neuronal loss (Probst *et al.*, 1991). An estimate of 46.8 million people globally were living with dementia in 2015. This number is expected to reach 131.5 million by 2050 (Ginhoux *et al.*, 2010). The continuous increase in life expectancy results in those rising numbers of dementia patients, and goes hand in hand with the increasing economical burden of dementia (Westendorp, 2006). The annual cost of dementia care is predicted to increase from $600 billion to $1 trillion over the next 15 years (Prince *et al.*, 2014).The majority of AD have a late onset (LOAD), whilst a stronger genetic prevalence is associated with the early onset AD form (EOAD).

AD is characterized by widespread neuronal degeneration, synaptic loss affecting mainly the hippocampus and cortex, resulting in diffuse brain atrophy (Braak and Braak, 1991). Accumulation and deposition of amyloid-beta (Aβ) peptides and neurofibrillary tangles were long considered the sole major hallmarks of AD. However, more recently neuroinflammation has emerged as a third hallmark of the disease.

1.2 Amyloid precursor protein

The human amyloid precursor protein (APP) gene was first identified in 1987 (Kang *et al.*, 1987) and although one of the most studied human proteins, the function of APP remains poorly defined. A number of putative physiological

functions have been assigned to APP, such as regulation of neurite outgrowth, cell adhesion, synaptogenesis and cell survival (Vetrivel and Thinakaran, 2010). APP, a type 1 membrane protein is synthesized in the ER and transported to endosomes and/or the cell surface via the secretory pathway (Sisodia, 1992; Thinakaran and Koo, 2008; Toh *et al.*, 2018). Posttranslational modifications of newly synthesized APP such as glycosylation, phosphorylation and sulfation take place during transit through the Golgi (Wang *et al.*, 2017). Proteolytic processing of APP occurs at multiple locations in the cell and results in a variety of peptide products, including the pathogenic Aβ peptides, which are then secreted from the cell (Tan and Gleeson, 2019). APP is located on chromosome 21 and exists in many different isoforms due to alternative splicing of the nascent transcript (Rajendran and Annaert, 2012). APP695 is the major neuronal isoform. Various mutations of the APP gene are known to cause familial AD (FAD) by increasing the extracellular Aβ load or shifting the ratio between $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ to the less favourable $A\beta_{1\text{-}42}$ (Scheuner *et al.*, 1996). The majority of these mutations affect the proteolytic processing by β and α secretases (Weggen and Beher, 2012). A reduction of amyloidogenic peptides is found in the Icelandic APP mutation (A673T), which confers protection against AD, due to reduced β secretase cleavage of APP (Jonsson *et al.*, 2012).

Numerous animal models have been developed an *in vivo* models to gain insights into the development of AD. A transgenic mouse model expressing high levels of human ‘high-risk’ mutant APP was developed in 1995 (Games et al., 1995). Whilst this model expressed the Indiana mutation, various APP models have been introduced expressing different APP mutations, including the Swedish and Dutch mutation. Furthermore mouse models expressing mutant PSEN have been introduced (McGowan et al., 2006). The characterisation of these models includes the formation of Aβ. Mouse models for risk genes causing LOAD have also been introduced, including ApoE target replacement mice, CD33 and TREM2 models. Whilst *in vivo* models have helped in the understanding of AD development, they do not show the dementia comparison.

1.3 β-secretase family

The generation of Aβ occurs from the processing of APP via membrane-bound proteases called secretases. Three secretases, α, β and γ, are involved in the processing of APP; β and γ secretases have a role in mediating the amyloidogenic cleavage of APP, whereas α secretase prevents Aβ generation by cleaving APP within the Aβ domain (Haass, 2004).

BACE1 (beta-site APP cleaving enzyme 1) is the major β secretase responsible for amyloidogenic cleavage of APP in the brain (Fukumoto *et al.*, 2002). BACE1 is a 501-residue type 1 transmembrane protease and belongs to the pepsin family of aspartyl proteases with optimal activity at acidic pH (Yan *et al.*, 2001; Vassar *et al.*, 2009). The protease is synthesized as a larger precursor, proBACE1, which is modified by glycosylation and cleaved by a furin-like endoprotease in the *trans*-Golgi network to generate mature BACE1. Like other aspartic proteases, BACE1 precursor is a zymogen and is synthesized in the endoplasmic reticulum (ER). The maturation of BACE1 increases the catalytic activity of the enzyme over that of immature BACE1 (Vassar *et al.*, 2009). An increased localization of BACE1 within the ER (which will represent the zymogenic non-active form) reduces generation of Aβ, whilst intracellular trafficking of BACE1 to the more acidic endosomes enhances Aβ production (Vassar *et al.*, 2009).

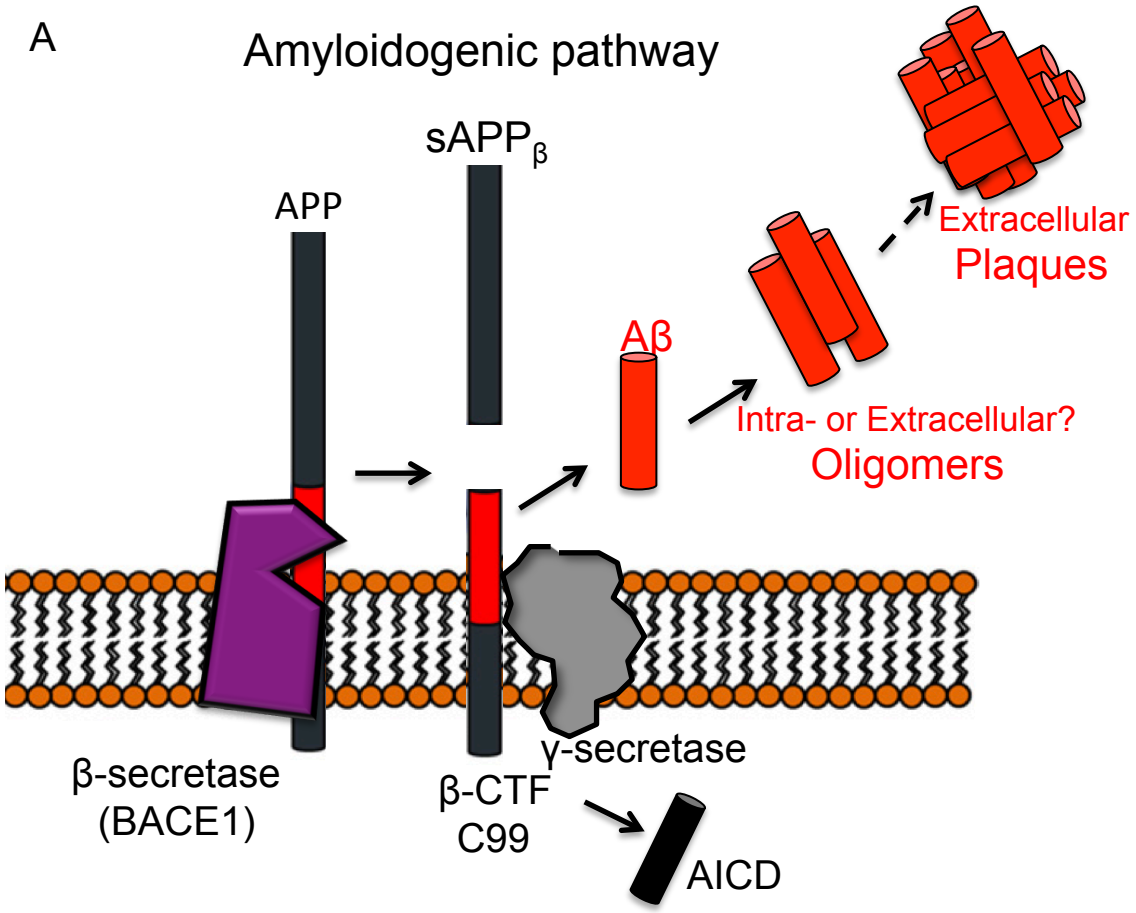
Mature BACE1 recycles between the plasma membrane and early and recycling endosomes (Toh *et al.*, 2018). The recycling is regulated by a dileucine motif within the sequence, DISLL, of the cytoplasmic tail at residues 496-500 (Toh *et al.*, 2018). BACE1 can be phosphorylated on Ser498 within this DISLL. The phosphorylation of this motif regulates BACE1 recycling between the cell surface and endosomal compartments. There are two mechanisms for transport of BACE1 from the early endosomes to the recycling endosome. A slow SNX4 (sorting nexin 4) mediated pathway that can transport both non-phosphorylated and phosphorylated BACE1, and a fast sorting signal mediated pathway dependent on the phosphorylation status of the DISLL motif of BACE1.

Golgi-localized γ-ear containing Arf binding protein 1 (GGA1) recognises the phosphorylated DISLL motif of BACE1 and promotes cargo transport to the recycling endosomes. Perturbation of the signal sorting pathway by either mutation of the Ser in the DISLL motif or by silencing GGA1 or retromer results in ~30 % reduction in the transport rate of BACE1 from the early endosomes to the recycling endosomes. The phosphorylated DISLL mediated trafficking of BACE1 is biologically relevant as elimination of the fast transport of BACE1 from early endosomes to recycling endosomes results in a three-fold increase in Aβ production (Toh *et al.*, 2018).

The analysis of BACE1$^{-/-}$ mice also provides substantial evidence for a crucial role of BACE1 in AD. Aβ generation, amyloid pathology and cognitive deficiencies are abrogated when BACE1$^{-/-}$ mice are crossed to APP transgenics. In AD, BACE1 activity is elevated. BACE activity in AD increased by 63 % in the temporal cortex and 13 % in the frontal cortex, but not in the cerebellum (Fukumoto *et al.*, 2002). As BACE1 is the only β secretase in the brain, BACE1 has been considered a therapeutic target for lowering cerebral Aβ levels in AD (Vassar *et al.*, 2009). However, an array of problems and challenges have arisen associated with the development of a therapeutic BACE1 inhibitor. The issues include the ability to cross the blood brain barrier (BBB) and the realisation that there are a large number of physiological substrates for BACE1, and inhibition of BACE1 activity will result in off target effects (Coimbra *et al.*, 2018). Notably, BACE1 knockout mice have revealed defects in synaptic function and behavioural problems (Haass, 2004).

1.4 Amyloidogenic and non-amyloidogenic processing of APP

The ~4 kDa Aβ peptide is one of the hallmarks of AD and is derived from APP. BACE1 is responsible for the initial step in Aβ production. The type-1 transmembrane aspartyl protease needs to be membrane bound and in close proximity with APP for cleavage, which highlights the importance of the spatial


A
Amyloidogenic pathway
sAPPβ
APP
Extracellular
Plaques
Aβ
Intra- or Extracellular?
Oligomers
β-secretase
(BACE1)
γ-secretase
β-CTF
C99
AICD

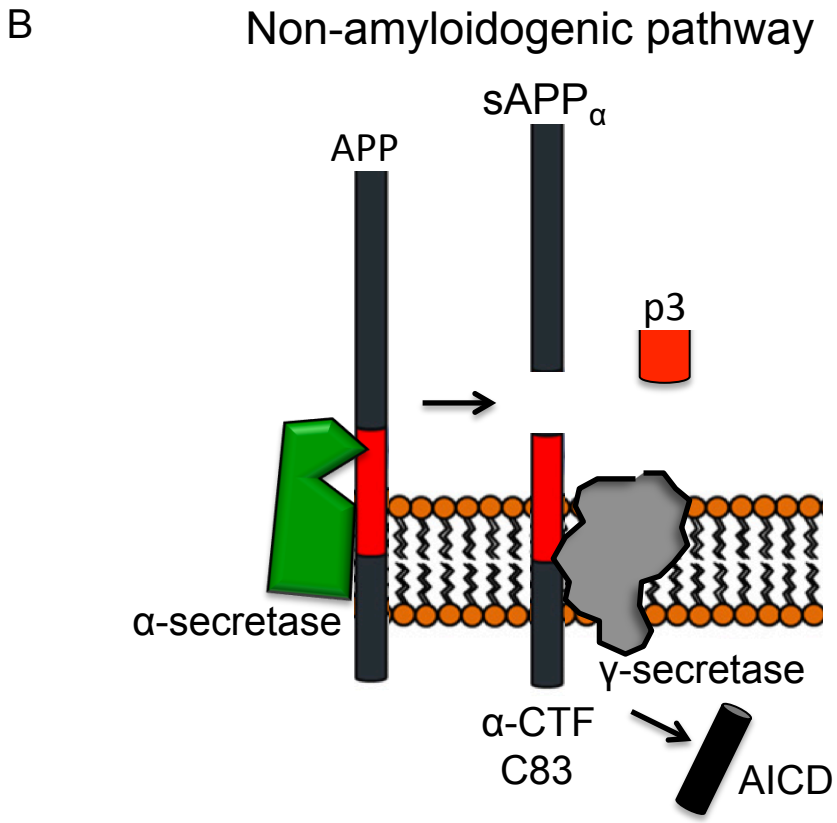

B
Non-amyloidogenic pathway
sAPPα
APP
p3
α-secretase
γ-secretase
α-CTF
C83
AICD

Figure 1.1. Processing of APP
(**A**) In the amyloidogenic pathway, APP undergoes processing by BACE1, resulting in the $sAPP_{\beta}$ and the C99 fragment. This is further processed by γ-secretase, resulting in the Aβ monomer. The Aβ monomer can assemble into oligomers and fibrils and eventually form the characteristic Aβ plaques. (**B**) In the non-amyloidogenic pathway, the amyloid beta precursor protein (APP) undergoes processing by α-secretase, resulting in the sAPPα and the C83 fragment, which is cleaved by γ-secretase resulting in the p3 peptide fragment. Adapted from Toh and Gleeson (2016).

distribution of both APP and BACE1 within the membranes and intracellular compartments for processing.

Cleavage of APP by BACE1 results in $sAPP_{\beta}$, a soluble APP luminal domain that is subsequently secreted and a membrane bound C99-residue C-terminal fragment called C99 or CTF_{β}. This C99 fragment is cleaved by γ secretase resulting in the release of hydrophobic Aβ peptides (Fig. 1.1A).

In an alternate, non-amyloidogenic, pathway, α secretase initially cleaves mature APP, resulting in the generation of a soluble luminal domain fragment, $sAPP_{\alpha,}$ and a membrane bound fragment C83 or CTF_{α} where the Aβ sequence is absent (Fig. 1.1B). Subsequent cleavage of the C83 fragment by γ secretase yields a number of short peptides excluding Aβ (Vassar *et al.*, 1999).

1.4.1 Secreted Aβ

Aβ generated from the amyloidogenic pathway is secreted from the cell and subsequently aggregates to form amyloid plaques. The majority of secreted Aβ peptides are 40 amino acids (Aβ40), and 42 amino acids (Aβ42). The later Aβ42 has the propensity to nucleate and drive production of amyloid fibrils (Jarrett *et al.*, 1993). Aβ is usually removed from the brain by export into the cerebrospinal fluid (CSF), the blood vessels and local degradation by microglia (Tarasoff-Conway *et al.*, 2015). Microglia can clear Aβ by uptake and degradation or can degrade extracellular Aβ by the release of enzymes such as neprilysin. The rapid removal of Aβ released by neurons is imperative as Aβ is a hydrophobic peptide with a tendency to aggregate. Thus, there is a fine balance between pathways to clear Aβ and the generation of Aβ aggregates, which will accumulate. An increase in secreted Aβ concentration by neurons above a certain level results in formation of Aβ oligomers and plaques (Scheuner *et al.*, 1996).

1.5 Neurofibrillary tangles

Neurodegenerative tauopathies, characterized by abnormal tau is another characteristic hallmark of AD. Tau is a microtubule-associated protein predominantly expressed in neurons and is required for microtubule stabilization as well as their assembly (Takemura *et al.*, 1992). Pathological tau protein is hyperphosphorylated, and aggregated into insoluble neurofibrillary tangles (NFT). Accumulation of toxic intracellular aggregates, together with the loss of soluble tau to stabilize microtubules, may synergistically lead to compromised neuronal survival accounting for the strong correlation between NFT burden and cognitive decline in AD (Iba *et al.*, 2013). Synaptic loss has been described with tangle accumulation. Studies of the rTg4510 tauopathy mouse model have further revealed that impaired synaptic plasticity also contributes to the neurodegenerative process in AD, and both Aβ and tau contribute to this degeneration (Crimins *et al.*, 2013). Bussian and colleagues have recently shown that tau formation is enhanced by the presence of senescent microglia and astroglial cells (Bussian *et al.*, 2018).

1.6 Microglia

There has been considerable recent attention in defining the pathways of neuroinflammation and the cell types involved. It has become clear that microglial cells are a major contributor to the inflammatory process in the brain, and are not just bystanders as originally thought. Microglia have become a major focus of neurodegenerative disease research and defining the physiological properties of microglia is crucial to understanding their potential role in neuronal loss and AD.

Microglia are the resident innate immune cells of the brain, first described as migratory phagocytic cells of the central nervous system (CNS) (del Rio-Hortega, 1932). Microglia account for approximately 10% of cells in the CNS and are the most abundant mononuclear phagocytes in this tissue (Ginhoux *et al.*, 2010; Colonna and Butovsky, 2017). These resident myeloid cells of the

CNS control the patterning and wiring of the brain in early development and contribute to homeostasis. Erythromyeloid progenitors (EMPs) develop in the yolk sac from E8.5 onwards and a subset of these cells become microglia progenitors which migrate to the brain from E9.5 onwards. This original pool of microglia is the only source of myeloid cells in the healthy brain. However, under pathological circumstances other myeloid cells such as bone marrow-derived monocytes may infiltrate the brain (Kierdorf *et al.*, 2013).

Microglia express chemokine receptors including CXCR4 and CX3CR1, as well as integrins such as CD11b, which is constitutively expressed, and CD11c, which is upregulated in activated microglia (Färber and Kettenmann, 2005; Roy *et al.*, 2006). Chemokine receptors and integrins control the migration and the position of microglia within the CNS and enhance their ability to phagocytose and eliminate target cells. Pro-and anti-inflammatory cytokines, such as IFNγ, TNFα, IL-1β, IL-10 and TGFβ tightly regulate the activity of microglia (Colonna and Butovsky, 2017). Furthermore, activated microglia are capable of releasing cytotoxicity mediators such as reactive oxygen and nitrogen species, arachidonic acid metabolites and histamine, amongst others (Nakajima and Kohsaka, 2001). Many studies have shown that the precise profile of the neurotrophic or cytotoxic factors released by microglia depends on the specific stimulus the microglia has been exposed to (Nakajima *et al.*, 1998; Zhao *et al.*, 2014). In particular, LPS stimulation is known to result in the secretion of a number of pro-inflammatory cytokines (Lee *et al.*, 2010).

Microglia, like macrophages, have previously been classified according to their M1 and M2 polarisation. The M1/M2 paradigm has helped conceptualise the pathways of microglia activation *in vitro*, but is now considered inadequate for an *in vivo* understanding as microglia rarely display bias toward either phenotype (Ransohoff, 2016). A number of transcriptome studies have revealed that microglia activation is both varied and situation-dependent (Hanisch, 2013). Different models of neurodegeneration have revealed that microglia express both neurotoxic as well as neurotrophic factors (Ransohoff, 2016). Microglia

under a non-inflammatory environment have a small soma and numerous processes extending into the microenvironment. This allows microglia to penetrate throughout the parenchyma in the normal adult brain and survey the environment, one of their main functions. The term 'resting' microglia, commonly used in the past, is therefore a misnomer (Nimmerjahn *et al.*, 2005), as microglia triggering may occur intermittently even in a healthy environment. Additional roles of microglia in healthy conditions include the maintenance of homeostasis during neurogenesis, and shaping synaptic fields through synaptic pruning (Chen and Trapp, 2016).

Recent GWAS studies have identified immune related pathways as risk factors for late onset Alzheimer's disease (LOAD), identifying microglia as central players in disease pathogenesis (Hong *et al.*, 2016).

1.6.1 Microglia and Astrocytes in AD

The precise role of microglia in contributing to chronic disease is incomplete. We need to better understand the balance between their protective role and one where healthy tissue is destroyed. This scenario is akin to the role of macrophages in the development of chronic inflammatory autoimmune disease. For microglia, it can be argued that their role is mainly protective, to remove cell debris and/or infectious agents (Chen and Trapp, 2016).

Microglia are involved in Aβ clearance (Lee and Landreth, 2010), which is both beneficial, to inhibit Aβ build up, and deleterious, when levels of Aβ are elevated, as prolonged inflammation will result. Microglia express a range of different receptors that can bind Aβ and trigger inflammation, such as different TLRs. In turn these can be stimulated by DAMPs such as ATP and induce NLRP3 activation (Heneka *et al.*, 2013; Heneka, Carson, *et al.*, 2015). Engagement of these receptors induces release of TNFα and IL-1β, which mediate neuroinflammation and neurotoxicity and cause sustained low grade

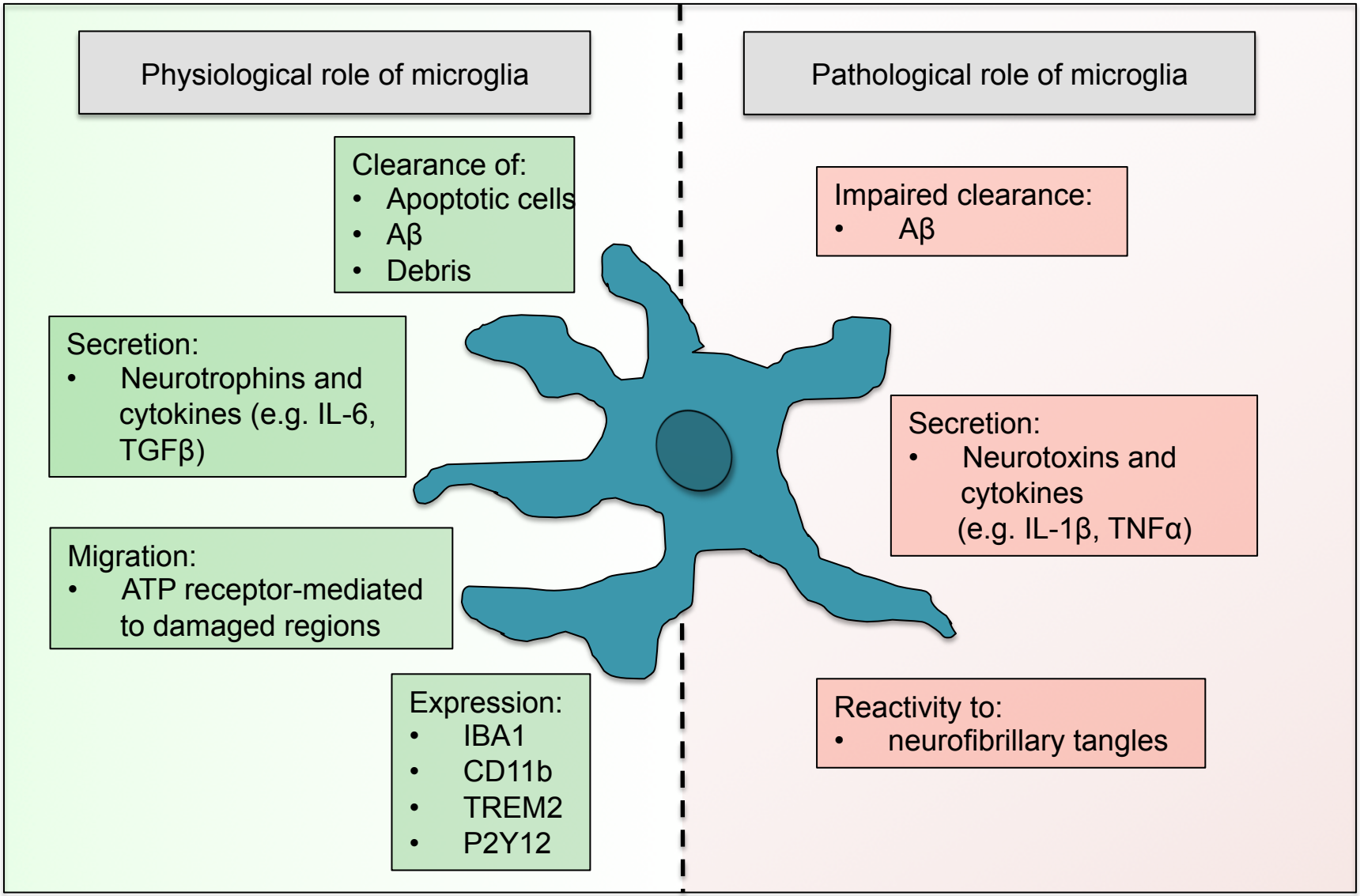

Physiological role of microglia
Pathological role of microglia
Clearance of:
• Apoptotic cells
• Aβ
• Debris
Impaired clearance:
• Aβ
Secretion:
• Neurotrophins and cytokines (e.g. IL-6, TGFβ)
Secretion:
• Neurotoxins and cytokines (e.g. IL-1β, TNFα)
Migration:
• ATP receptor-mediated to damaged regions
Expression:
• IBA1
• CD11b
• TREM2
• P2Y12
Reactivity to:
• neurofibrillary tangles

Figure 1.2 Differences of microglia function under physiological and pathological conditions
Microglia mediate immune responses by clearing apoptotic cells, debris and Aβ and secrete various neurotrophins and cytokine. Under pathological conditions, the clearance of Aβ is often impaired, leading to Aβ build up and the secretion of neurotrophins, neurotoxins together with pro-inflammatory cytokines.

inflammation (Heneka, Carson, *et al.*, 2015) (Figure 1.2). Deletion of receptors such as TLR4 reduces Aβ induced cytokine production (Fiebich *et al.*, 2018).

In addition to microglia, astrocytes may also play a role in neurodegeneration. Astrocytes contribute to synapse formation and synaptic strength regulation (Allen, 2014) and, like microglia, are involved in monitoring neuronal activity. Astrocytes both sense and modulate synaptic output and may play a role in regulating in the overall computing function of the brain (Allen, 2014). Although a role for astrocytes in neurodegenerative diseases has been largely ignored in the past, potential interactions between microglia and astrocytes are now attracting considerable attention. Astrocytes guide microglia to synapses that have been pruned via the complement pathway (Lian *et al.*, 2015). Hence astrocytes can influence the interaction of microglia with neurons, and thereby indirectly affect the delivery of neurotoxic and neurotrophic factors to neurons released by microglia.

1.6.2 Microglia and other neurodegenerative diseases

Microglia involvement has also been associated with several other neurodegenerative diseases, in addition to AD, such as amytrophic lateral sclerosis (ALS), where the release of pro-inflammatory cytokines by activated microglia leads to neuronal damage and neurotoxic activity (Boillee S Kassiotis G, 2006). They have further been implicated in Parkinson's disease as well as Huntington's disease. In these examples, neuroinflammation is the major causative neurotoxic effector, demonstrating yet again the relevance of a better understanding of the link between microglia activation and its neurotoxic effects (Colonna and Butovsky, 2017).

1.7 Neurons and their role in AD

Neurons are polarised cells with axonal and somatodendritic membrane surfaces and are considered the cellular substrate of cognitive abilities of the

CNS (Nowakowski, 2006; Schmidt and Haucke, 2007). For spatial segregation of incoming and outgoing signals, neurons retain an elaborate membrane system. Electrical signals are generated and conveyed by axons whilst synapses convert them into chemical ones. Neurotransmitter containing vesicles undergo exo- and endocytosis at the presynaptical nerve terminal. These neurotransmitter molecules bind to receptors at the postsynaptic membrane causing ion flux changes and sometimes the generation of an action potential. Kinesins are responsible for the transport of membrane vesicles containing APP or ApoER2 (apolipoprotein E receptor 2) to the axon (Schmidt and Haucke, 2007). APP and BACE1 are predominantly located in the somato/dendrites of neurons (Sannerud *et al.*, 2011).

Like microglia, the role of neurons in neuroinflammation was long believed to be that of a passive bystander, however they are able to produce inflammatory mediators themselves (Heneka *et al.*, 2010). Activation of the complement systems plays an important role in AD and neurons are capable of the production of most components of the complement cascade (Eikelenboom *et al.*, 1989). The complement system can be activated by PAMPS and/or DAMPS. C1q can directly bind to molecules like Aβ, hence there is the potential for complement to be activated as a consequence of build up of extracellular Aβ and/or release of components from dying neurons (Orsini *et al.*, 2014). Complement synthesis is low under normal conditions but increased in AD (Eikelenboom *et al.*, 1989; Orsini *et al.*, 2014) Neurons are not only involved in the production complement cascade components but have also been postulated as a source for COX-2 derived prostanoids, a subclass of eicosanoids which are vasoactive lipid mediators, and several cytokines such as IL-1β, IL-6 and TNFα (Heneka *et al.*, 2010).

Neuronal activity regulates the production and secretion of Aβ by controlling APP processing upstream of γ secretase activity. Kamenetz and colleagues identified Aβ as a modulator of synaptic strength. This negative feedback role for Aβ could suggest Aβ as a player in normal synaptic physiology, not only in

the processes leading to AD (Kamenetz *et al.*, 2003). Sustained neuronal activation is associated with plaque formation. Studies on temporal lobe epilepsy patients found plaques in patients as young as 30 years of age (Mackenzie and Miller, 1994). Taken together this suggests that neuronal activity and Aβ levels are closely interlinked and neuronal hyperexcitability is a recognised component of AD. This is most likely due to a mixture of direct and indirect effects of Aβ on neurons (De Strooper and Karran, 2016).

1.8 Neuroinflammation

Evidence that inflammation has a causal role in the pathogenesis of AD comes both genetic and immunological analysis. Genes for various immune receptors such as Trem2 (Triggering receptor myeloid 2) and CD33 are associated with AD and microglia have been shown to be capable of binding to soluble Aβ oligomers and fibrils via cell surface receptors including CD36, CD14, CD47 and Toll-like receptors (TLR2, 4, 6 and 9). Aβ binding to CD36, TLR4 or TLR6 results in activation of microglia, to produce pro-inflammatory cytokines and chemokines (El Khoury *et al.*, 2003). Pro-inflammatory cytokines secreted by activated microglia include TNFα and IL-1β; moreover, these cytokines are known to be upregulated in brains of AD patients and in transgenic mice with AD-like pathology, and in addition are secreted by primary microglia in culture (Grammas and Ovase, 2001). Although mainly pro-inflammatory an anti-inflammatory role of TNFα and IL-1β is also possible (Zheng *et al.*, 2016).

As is the case for innate immune responses in the periphery, cytokines in the CNS are key regulators of neuroinflammation. The pro-inflammatory environment in the AD brain could directly and indirectly contribute to neuronal damage. For example, IL-1β and TNFα may directly impair neuronal function (Ye *et al.*, 2013). The rate of progression from mild cognitive impairment to the dementia stages of the disease is increased in patients who have elevated TNFα levels and decreased TGFβ concentrations in the CSF. Cytokines

stimulate inducible nitric oxide synthase (iNOS) in microglia and astrocytes and iNOS is toxic to neurons at high concentrations. Notably, iNOS has been reported to be upregulated in the AD brain (Heneka, Carson, *et al.*, 2015). Consistent with this proposal, *in vitro* experiments have shown that binding of microglia to Aβ leads to the production of reactive oxygen species (ROS) (El Khoury *et al.*, 2003).

Although Aβ deposition alone may be sufficient to induce an inflammatory reaction, risk factors such as systemic inflammation, obesity and traumatic brain injury might influence the development of AD through a sustained neuroinflammatory drive (Heneka, Carson, *et al.*, 2015). Inflammation preceding the development of AD may prime microglia, causing them to be highly responsive to further activation (Colton *et al.*, 2006). Subsequent stimulation by Aβ could then result in secretion of pro-inflammatory cytokines and chemokines, which could trigger neuronal hyperexcitability and synaptic dysfunction. Recent studies have demonstrated that neurons are also able to produce inflammatory mediators (Heneka *et al.*, 2010). Activation of complement systems play an important role in AD and neurons produce most of the components of the complement cascade. Moreover, neuronal production of complement components is increased in AD (Eikelenboom *et al.*, 1989). Neurons have also been postulated to be source of COX-2-derived prostanoids, a subclass of eicosanoids which are vasoactive lipid mediators, and several cytokines such as IL-1β, IL-6 and TNFα (Heneka *et al.*, 2010).

A number of studies have shown that *in vivo* LPS treatment results in an increase level of $A\beta_{1\text{-}42}$ and a decrease in the level of $A\beta_{1\text{-}40}$ (Lee *et al.*, 2008); significantly, $A\beta_{1\text{-}42}$ is the more toxic species. This finding suggests a close connection between amyloidogenesis and neuroinflammation. However, the mechanisms responsible for LPS-induced amyloidogenesis are unknown. Lee *et al.* reported elevated β and γ secretase activity in cortical and hippocampal regions of ICR mice and Sprague-Dawley rats, as well as cell lysates upon LPS treatment, hence modified secretase activity could be a potential contributing

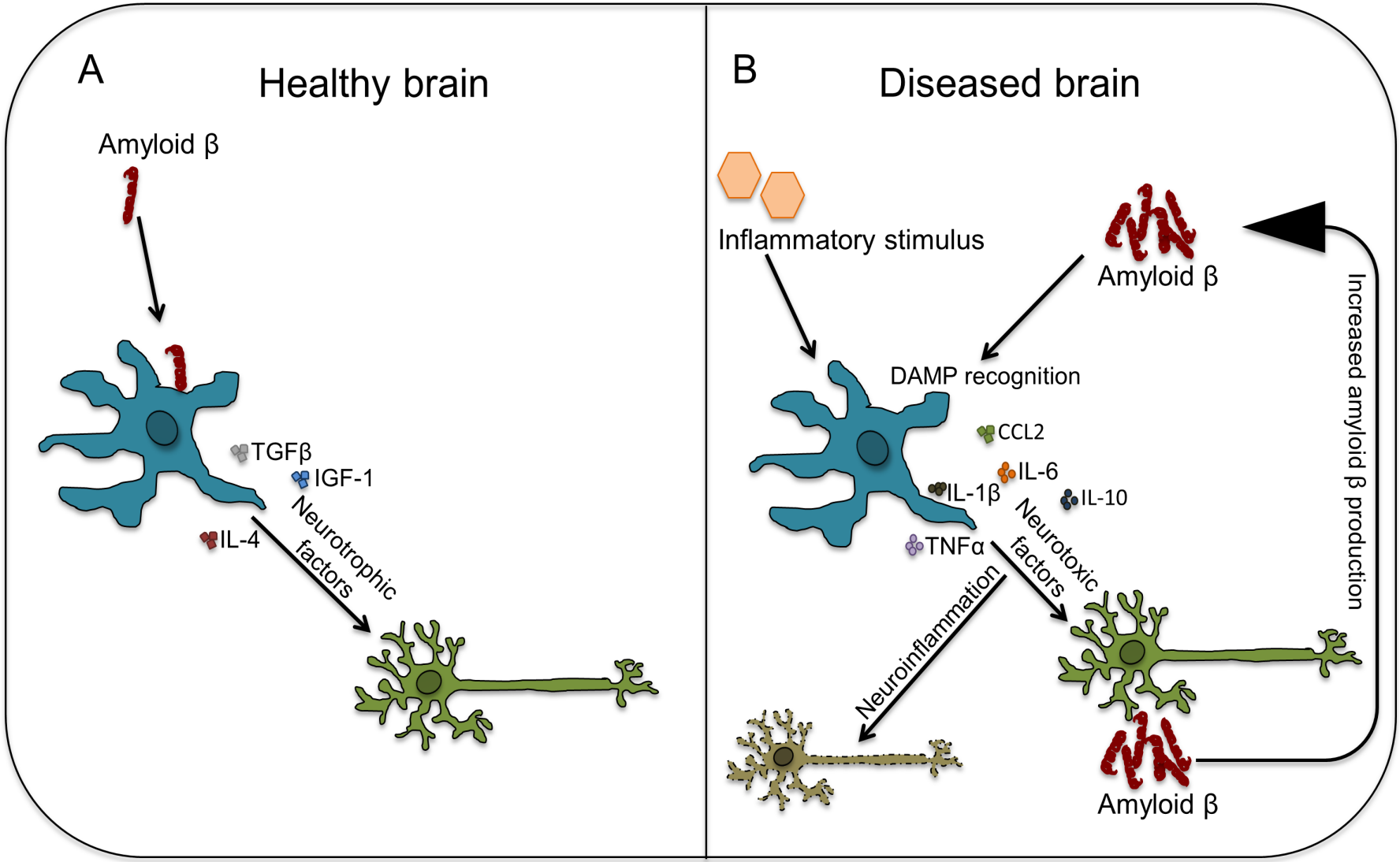

A
Healthy brain
Amyloid β
TGFβ
IGF-1
IL-4
Neurotrophic factors
B
Diseased brain
Inflammatory stimulus
Amyloid β
DAMP recognition
CCL2
IL-6
IL-1β
IL-10
TNFα
Neurotoxic factors
Neuroinflammation
Increased amyloid β production
Amyloid β

Figure 1.3 Potential positive feedback loop in the diseased brain between microglia and neurons
(**A**) In the healthy brain microglia cells clear amyloid β from the CNS microenvironment and secrete neurotrophic factors maintaining a healthy neuron population.
(**B**) A model illustrating the activation of microglia after DAMP recognition of inflammatory stimuli or amyloid β stimulation. Microglia activation induces release of pro-inflammatory cytokines and mediates neuroinflammation and neurotoxicity. The resulting increase of amyloid β leads to further aggregation and the sustained low grade inflammation found in Alzheimer's disease.

factor (Lee *et al.*, 2008). Furthermore, pro-inflammatory cytokines such as TNFα and IL-1β have been shown to increase levels of β secretase mRNA, and BACE1 protein and enzymatic activity. Lee and colleagues have proposed that LPS-induced inflammatory reactions influence APP processing through the enhancement of β and γ secretase activity and thereby affect amyloidogenesis (Lee *et al.*, 2008).

Unfolded, misfolded and aggregated proteins are recognized by the danger associated molecular pattern (DAMP) receptors found on the cell surface of innate immune cells. An important finding in the neuroinflammation field is that aggregated Aβ acts as a DAMP, resulting in the activation of the innate immune system in the brain (Heneka, 2017) with the consequence pro-inflammatory cytokine production. Hence, following an initial build up of extracellular oligomeric Aβ, there is potential to stimulate an inflammatory response via microglia. As the pro-inflammatory response is directed to self-DAMPS, and as neurons are in turn impacted by the pro-inflammatory cytokines, a positive feedback loop is likely to be established, resulting in enhancing disease progression and establishing a chronic, on-going, disease (Figure 1.3).

1.9 NLRP3 inflammasome

The innate immune system engages pattern recognition receptors (PRRs), which are expressed by cells fighting against infection and those involved in adaptive immune cells, such as macrophages and microglia (Schroder and Tschopp, 2010). Different bacteria, fungal and viral molecules that contain pathogen associated molecular patterns (PAMPs) or non-microbial danger signals (DAMPs), released by damaged cells can activate NOD-like receptors (NLRs) (Saresella *et al.*, 2016).

NLRs are characterised by a central nucleotide-binding and oligomerisation domain (NACHT), flanked by C-terminal leucine-rich repeats (LRRs) and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains (Schroder and

Tschopp, 2010; Horvath *et al.*, 2011). NLRP3 is the best-characterised inflammasome and is mainly expressed by myeloid lineage cells. It can be induced by stimulation of TLRs, cytokines and other signals (Jo *et al.*, 2016).

Activation of the NLRP3 inflammasome is a two-step process, which depends on a priming signal, required for the upregulation of NLRP3 and the production of the proinflammtory cytokines pro-IL1β and pro-IL-18. TLR4 stimulation initiates MyD88/NFκB signalling (Su *et al.*, 2018). Furthermore p38 MAPK signalling has been connected to NLRP3 activation (Li *et al.*, 2018). The secondary signal or activation step triggers assembly into the NLRP3 inflammasome complex. A secondary signal can be extracellular ATP, which activates inflammasome assembly via the purinergic receptor P2X7 (Mariathasan *et al.*, 2006). NLRP3 monomers induce oligomerisation and interact with the PYD domain of apoptosis-related speck-like protein (ASC). The adaptor ASC then recruits the cysteine protease pro-caspase-1 via a caspase recruitment domain (CARD). The resulting autocatalysis and activation of caspase-1 leads to maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18, and under certain conditions the induction of a cell death pathway known as pyroptosis (Heneka, Golenbock, *et al.*, 2015; Jo *et al.*, 2016). The caspase-1 dependent cell death is inherently inflammatory and is both morphologically and mechanistically distinctly different from other forms of cell death (Bergsbaken *et al.*, 2009). The resulting increase of IL-1β affects synaptic plasticity, as this cytokine is able to disrupt formation of dendritic spines. NLRP3 inflammasome activation is an important contributor to normal age related systemic inflammatory responses as well as brain inflammation (Heneka, Golenbock, *et al.*, 2015) (Figure 1.4).

1.9.1 NLRP3 inflammasome and its contribution to AD

The NLRP3 inflammasome plays an important role in AD pathology. APP/PS1 mice deficient in NLRP3, casp1 or ASC are largely protected from AD (Heneka *et al.*, 2013). In part, protection from AD can be attributed the role of

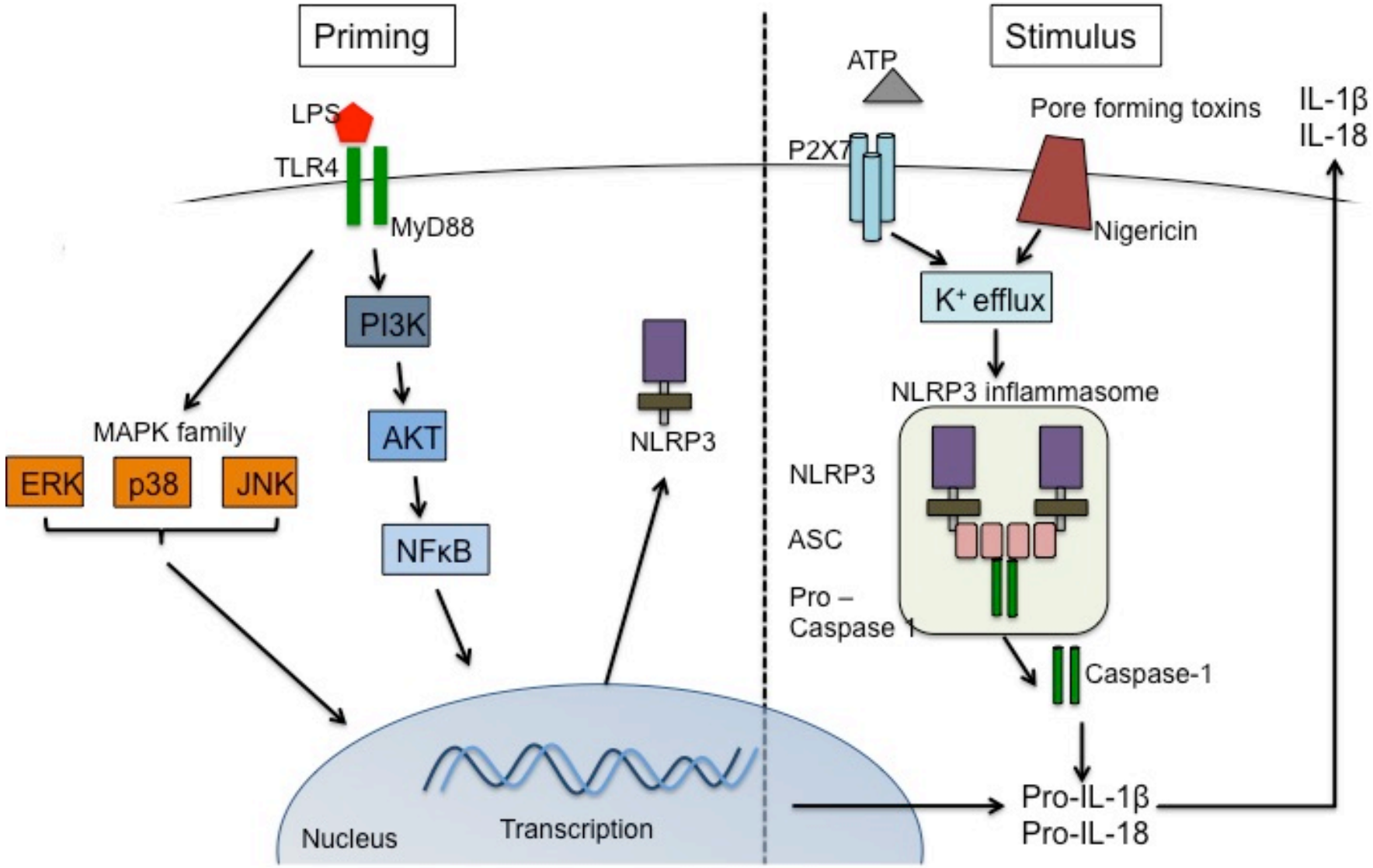

Priming
Stimulus
LPS
TLR4
MyD88
PI3K
AKT
NFκB
MAPK family
ERK
p38
JNK
NLRP3
ATP
P2X7
Pore forming toxins
Nigericin
K⁺ efflux
NLRP3 inflammasome
NLRP3
ASC
Pro –
Caspase 1
Caspase-1
IL-1β
IL-18
Pro-IL-1β
Pro-IL-18
Nucleus
Transcription

Figure 1.4 Activation and assembly of the NLRP3 inflammasome
The inflammasome is activated by a two-step mechanism. The first signal (priming) is provided by microbial or endogenous molecules such as LPS. Recognition by TLR4 in this case leads to an increase in gene transcription mediated by the adaptor protein MyD88 (myeloid differentiation factor 88) and then the activation of NFκB and p38 MAPK activities. This induces NLRP3 and pro-IL-1β, pro-IL-18 expression. The second signal (activation) is triggered by ATP, pore-forming toxins, viral RNA, or particulate matter. Different molecular and cellular events have been proposed to trigger NLRP 3 inflammasome activation including K+ efflux, ROS, Ca2+ signalling, mitochondrial dysfunction. Upon activation, the NLRP3 inflammasome assembles, and gives rise to caspase 1 which cleaves pro-IL-1β and pro-IL-18. IL-1β and IL-18 are secreted and the cell undergoes pyroptosis.

NLRP3 in IL-1β production. IL-1β has been detected in diseased brain tissue in multiple studies. IL-1β is released by activated microglia after stimulation with Aβ *in vitro* and elevated levels of IL-1β were detected in microglia surrounding Aβ plaques in patients with AD as well as animal models of AD (Griffin *et al.*, 1989) As described above, IL-1β is synthesized in an inactive form controlled by caspase-1, which in turn is controlled by the NLRP3 inflammasome. Halle and colleagues have shown that the NLRP3 inflammasome is required for Aβ induced activation of caspase-1, release of mature IL-1β and secretion of pro-inflammatory cytokines as well as potentially neurotoxic cytokines and chemokines. These authors also showed that the activation of NLRP3 inflammasome was initiated after Aβ phagocytosis. Furthermore they have demonstrated, using IL-1β ELISA of microglia treated with fibrillar Aβ (10 μM) that fibrillar Aβ can induce caspase-1 dependent release of IL-1β (Halle *et al.*, 2008). Inefficiently cleared pro-inflammatory mediators or Aβ can in turn act as DAMPs and upon receptor binding activate NLRs leading to a further increase in inflammation.

The notion that the NLRP3 inflammasome plays a key role in neuroinflammation is further supported by findings by Saresella and colleagues (Saresella *et al.*, 2016). Using patient derived monocytes these authors reported an upregulation of inflammasome genes in LPS primed and Aβ stimulated monocytes derived from AD patients but not in monocytes from mild cognitive impairment (MCI) stage individuals. Furthermore, expression of NLRP1, NLRP3 and caspase-8 was greatly increased in AD and MCI stage patients, whilst PYCARD, caspase-1 and caspase-5 expression was detected in AD but not MCI patients (Saresella *et al.*, 2016). This was also supported by Heneka *et al* (Heneka *et al.*, 2013), who found that caspase-1 expression is strongly enhanced in AD and MCI brains. Furthermore, NLRP3 and caspase-1 KO APP/PS1 mice are largely protected from loss of spatial memory and other AD associated symptoms. Reduced brain caspase-1 and IL-1β levels as well as enhanced Aβ clearance was found in these mice (Heneka *et al.*, 2013). Not only is inflammasome activation linked to AD pathology, Venegas *et al*. have shown

that intrahippocampal injections of specks of the adapter protein apoptosis-assosciated speck-like protein containing a CARD (ASC), result in the spreading of Aβ pathology in transgenic double mutant $APP_{Swe}PSEN_{de9}$ mice (Venegas *et al.*, 2017). NLRP3 inflammasome activation also results in the release of assembled ASC specks, which can be taken up by neighbouring cells and contribute to the on-going immune response (Franklin *et al.*, 2014). Studies using APP/PS1/$NLRP3^{-/-}$ mice, have revealed a protective effect of systemic inflammation and additionally revealed the the knockout improves Aβ clearance by microglia (Tejera *et al.*, 2019).

1.10 Early onset familial form of AD

EOAD affects approximately 10 % of all AD patients. As the name suggest, EOAD results in an earlier onset of the disease, affecting patients between 30 and 65 years of age (Prince *et al.*, 2016). Typical clinical presentation includes memory impairment, atypical focal cortical symptoms such as visual dysfunction, apraxia, and dyscalculia. Whilst the pathological features of late onset AD (LOAD) and EOAD are very similar, an atypical presentation is reported more frequently seen in EOAD patients (Cacace *et al.*, 2016). In comparison to LOAD, EOAD is almost entirely genetically determined with heritability ranging from 92-100 %. APP, PSEN1 and PSEN2 are the three genes mainly responsible for the early onset form of AD (Wingo *et al.*, 2012; Cruts *et al.*, 2012). PSEN mutations are inherited in an autosomal dominant manner (Cacace *et al.*, 2016). Whilst healthy neurons secrete mainly $Aβ_{1-40}$, many mutations of APP and PSENs either increase $Aβ_{1-42}$ or the ratio of $Aβ_{1-40}/Aβ_{1-42}$ (Borchelt *et al.*, 1996). The Apolipoprotein E4 (ApoE4) is known as the major risk factor for LOAD; the e4 allele of the *APOE* gene also increases the risk for EOAD in carriers of only one ApoE4 allele, in carriers with two ApoE4 alleles, the risk is further increased. The E4 allele may modify the expression of other genetic factors contributing to the disease. On the contrary the E2 allele of the *APOE* gene exerts a positive effect and delays age of onset (Corder *et al.*, 1994; Genin *et al.*, 2011)

1.10.1 Late onset sporadic form of AD

LOAD is not only more complex than EOAD but also more common. Approximately 65 % of all AD cases are considered sporadic. The sporadic form also has *APOE* as a genetic risk factor and is associated with approximately 40 % of all cases. However since this leaves 60 % of the cases unexplained for, LOAD most likely results from a combination of multiple environmental, dietary and lifestyle factors contributing to onset of the disease (Kalaria *et al.*, 2008; Piaceri *et al.*, 2013). Illiteracy or low educational achievement has also been associated with an increased risk for LOAD, as well as stroke and vascular disease (Borenstein *et al.*, 2006). Age however is the most consistent risk factor world wide, with women being slightly more likely to develop AD in particular with very old age (Kalaria *et al.*, 2008).

1.11 AD risk genes- inflammation and membrane trafficking

For many years ApoE remained the only confirmed risk factor for late onset Alzheimer's disease (LOAD). GWAS studies have now identified 29 risk genes (Lambert *et al.*, 2009; Lambert *et al.*, 2013; Jansen *et al.*, 2019). The most recent GWAS study confirmed known loci associated with AD from earlier GWAS studies and detected 9 novel loci including ADAMTS4 (α secretase) and CLNK (a regulator of immunoreceptor signalling) (Jansen *et al.*, 2019). This recent study by Jansen and colleagues had a sample size eight-fold larger than the previous GWAS (Lambert *et al.*, 2013) and included genetic data of >600,000 individuals. Analysis of the identified single nucleotide polymorphisms (SNPs) revealed that most of the variants are located in non-coding regions of the genome (Jansen *et al.*, 2019).

The identified risk genes for LOAD can be broadly grouped into four categories; innate immune response, cholesterol metabolism, endosomal trafficking and APP catabolism/processing (summarised in Table 1.1).

Examples of innate immune responses include Clusterin (CLU), TREM2 and CD33. CLU also known as apoJ, has several SNPs that have been identified and shown to confer protection against LOAD. CLU is predicted to function in synapse turnover, Aβ aggregation, clearance and toxicity (Bettens *et al.*, 2013). TREM2 (triggering receptor expressed on myeloid cells 2) is another example of a risk factor identified through GWAS studies, which belongs to the innate immune response group. TREMs are a member of the immunoglobulin superfamily of receptors encoded by a gene cluster linked to the major histocompatibility complex (MHC) (Jay *et al.*, 2015). TREM2 is a receptor expressed by myeloid cells, including microglia in the brain. TREM2 stimulates phagocytosis and plays a role in the reduction of inflammation by suppressing toll-like receptor induced inflammatory cytokines and enhancing anti-inflammatory cytokine transcription (Jay *et al.*, 2015). TREM2 is also required for migration, lipid sensing and ApoE binding (Schlepckow *et al.*, 2017; Ulland and Colonna, 2018). The risk associated with TREM2 mutations depends on the genetic background, as different populations show differences in their risk of developing AD for a given TREM2 mutation. For example, the R47H variant of TREM2 confers an increased risk of developing AD similar to that of ApoE4 in Caucasians, however there is no association between R47H status and risk of developing the disease in East Asian individuals (Ulland and Colonna, 2018).

CD33 has also been identified as a risk factor for LOAD. Two SNPS in the CD33 gene have been associated with LOAD. Like TREM2, the CD33 variant associated with increased risk has been directly linked to impaired uptake of Aβ by microglia cells. CD33 is a member of the sialic acid-binding immunoglobulin-like lectins (Siglecs) and mediates cell-cell interactions that inhibit or restrict immune responses (Griciuc *et al.*, 2013).

Table 1.1 Summary of selected genetic risk factors associated with LOAD

Functional class	Risk gene
Innate immune response	Triggering receptor expressed in myeloid cells 2 (TREM2) (El Khoury *et al.*, 2003)
	Ephrin type-A receptor 1 precursor (EPHA1) (Jansen *et al.*, 2019)
	Complement receptor 1 (CR1) (Jansen *et al.*, 2019)
	SLP Adaptor And CSK Interacting Membrane Protein (SCIMP) (Jansen *et al.*, 2019)
	CD33 (Griciuc *et al.*, 2013)
	HLA-DRB1 (Jansen *et al.*, 2019)
	Inositol Polyphosphate-5-Phosphatase D (INPPD5) (Jansen *et al.*, 2019)
	ABI family member 3 (ABI3) (Jansen *et al.*, 2019)
	Membrane spanning 4A (MS4A) (Lambert *et al.*, 2013)
	Cytokine dependent hematopoietic cell linker (CLNK) (Jansen *et al.*, 2019)
	Cas scaffolding protein family member 4 (CASS4) (Jansen *et al.*, 2019)
Cholesterol metabolism	Apolipoprotein E (ApoE) (Corder *et al.*, 1993)
	Enoyl-CoA Hydratase Domain Containing 3(ECHDC3) (Jansen *et al.*, 2019)
	Clusterin (CLU) (Lambert *et al.*, 2009)
	ATP binding cassette subfamily A member 7 (ABCA7) (Lambert *et al.*, 2013)
	Sortilin protein related receptor (SorL1) (Lambert *et al.*, 2013)
Endocytosis	Sortilin protein related receptor (SorL1) (Lambert *et al.*, 2013)
	Bridging integrator 1 (BIN1) (Lambert *et al.*, 2013)

	Sortin nexin 3 (SNX3) (Lambert *et al.*, 2013)
	Phosphatidylinositol binding clathrin assembly protein (PICALM) (Lambert *et al.*, 2013)
	CD2 associated protein (CD2AP) (Lambert *et al.*, 2013)
	A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) (Jansen *et al.*, 2019)
APP processing	A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) (Jansen *et al.*, 2019)
	Aph-1 Homolog B (APH1B) (Jansen *et al.*, 2019)

1.11.2 ApoE

The major genetic risk factor for AD is the ApoE. The gene is located on chromosome 19q13.2 (Strittmatter *et al.*, 1993). ApoE is a 299 amino acid lipoprotein and regulator of lipid metabolism and is involved in different roles in the CNS such as cholesterol transport and inflammation. Its function in the brain remains unclear, despite ApoE being highly expressed in the CNS (Holtzman *et al.*, 2012; Piaceri *et al.*, 2013). The ApoE lipoprotein contains two structural domains linked by a protease sensitive loop (Wetterau *et al.*, 1988). The N-terminal domain (aa 1-191) contains the receptor-binding region and forms a four helical antiparallel bundle. The C-terminal domain (aa 225-229) contains the major lipid binding region (Mahley *et al.*, 2009a). Due to its conformational flexibility ApoE can bind to lipoproteins of variable sizes and shapes. ApoE is mainly produced by astrocytes and to a lesser extent by microglia under control of PPARγ and LXR (Terwel *et al.*, 2011). The three most common alleles of ApoE encode the following three isoforms, ApoE2, ApoE3 and ApoE4. Weisgraber *et al* elucidated that single aa substitutions result in the structural differences between the isoforms (Weisgraber *et al.*, 1981). The differences of aa at residues 112 and 158 distinguishes the isoforms. ApoE3 has cysteine at

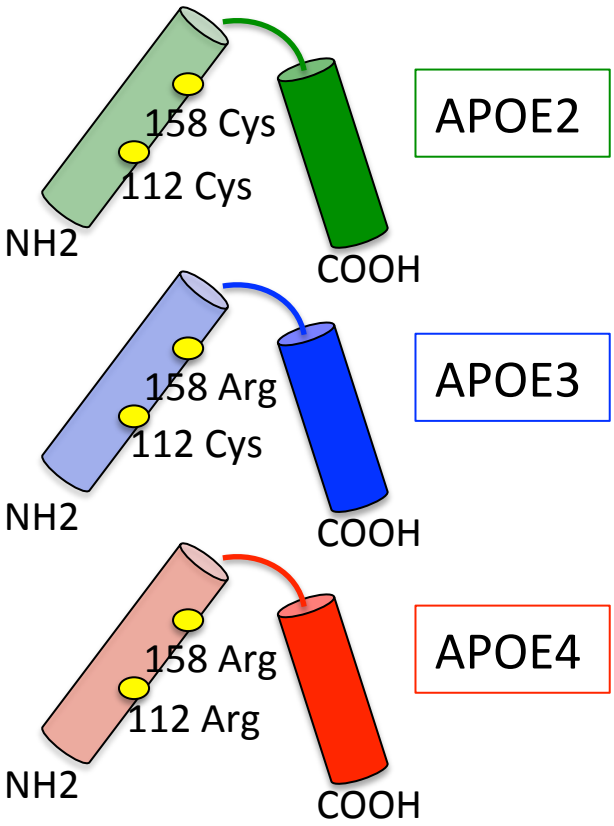


Figure 1.5 Single nucleotide polymorphism gives rise to the three major ApoE alleles
The single AA substitution at position 158 and 112 leads to substitution of Cys to Arg and induces binding to other nearby AA via salt bridges which dramatically affects the protein structure.

position 112 and arginine at 158, whilst ApoE4 has arginines at both sides, and ApoE2 cysteines (Weisgraber *et al.*, 1981), as depicted in Figure 1.5.

ApoE allele frequencies in the population differ, with the e3 allele being the most common (~65 %), followed by e4 (15-20 %) and e2 the rarest (5-10 %). The ApoE genotype determines the risk of developing AD (Mahley *et al.*, 2009b). Individuals with one ApoE2 allele and one ApoE3 allele are 20 % less likely to develop LOAD compared with individuals with one allele of ApoE4. The addition of an ApoE4 allele increases the AD risk of an individual by a factor of 2.84. Thus patients with two ApoE4 alleles are over eight times more likely than those without ApoE4 alleles (for example ApoE2/E3 or ApoE3/E3) (Corder *et al.*, 1993). ApoE4 is associated with increased deposition of Aβ, hyperphosphorylation of tau as well as impairment of neuronal plasticity and is also found in senile plaques and intracellular NFTs (Strittmatter *et al.*, 1994; Arendt *et al.*, 1997; Piaceri *et al.*, 2013). In early age, the ApoE4 variant confers an advantage, as ApoE4 positive individuals performed better in behavioural tests executive attention, verbal fluency and memory tasks (Jochemsen *et al.*, 2012). The beneficial effect of ApoE4 is reversed in old age. This so called antagonistic pleiotropy hypothesis could explain ApoE4s persistence in the human population (DiBattista *et al.*, 2016).

Not only does ApoE4 constitute the highest risk of developing the disease, it also has an effect on the clinical course of the disease, causing earlier onset of dementia, increased brain atrophy, thinner cortices as well as faster cognitive decline (Jack *et al.*, 1998). In contrast, the ApoE2 isoform has a protective effect, manifesting in lower incidences of MCI and AD, later age at onset of the disease and a slower cognitive decline (Martins *et al.*, 2005).

Brain regions typically associated with Aβ plaque deposition coincide with regions that metabolise glucose by aerobic glycolysis. This process seems to be disrupted in AD patients by ApoE4. Studies on patients carrying the ApoE4 allele revealed reduced cerebral glucose metabolism (Piaceri *et al.*, 2013).

ApoE binds to Aβ and influences the clearance of soluble Aβ and its aggregation. ApoE4 carriers exhibit accelerated and more abundant Aβ deposition (Karch and Goate, 2015). Furthermore it was shown that ApoE4 enhances the oligomerisation of Aβ, suggesting that ApoE4 predisposes AD by increasing toxic Aβ oligomers (Huang *et al.*, 2017) and that ApoE4 has an impaired ability to facilitate the proteolytic degradation of Aβ by neprilysin and insulin degrading enzyme (Du *et al.*, 2009). It has also been shown that ApoE4 is less effective at inducing cholesterol efflux than ApoE3, suggesting that the pathological effects of ApoE4 are related to lipid metabolism (Safieh *et al.*, 2019).

ApoE has also been associated with neuroinflammation. Ophir and colleagues have demonstrated, using human ApoE3 and ApoE4 transgenic mice, that following inflammatory stimulation with LPS, ApoE4 mice had an enhanced and prolonged neuroinflammatory response (Ophir *et al.*, 2005). The inflammation is most likely driven by the effect of ApoE4 on microglial activation and possibly enhanced levels of pro-inflammatory cytokines. It was also demonstrated that NFκB activation was more pronounced in ApoE4 mice, further suggesting that ApoE can regulate inflammatory processes isoform specifically (Ophir *et al.*, 2005). Interestingly, levels of ApoE in the CSF also differ in an isoform dependent manner, suggesting that ApoE concentration may contribute to some of the ApoE genotype effects. ApoE4 carriers have lower serum and ApoE levels in brain tissue (Martínez-Morillo *et al.*, 2014).

Several studies have shown that ApoE4 does not always increase the risk for developing AD. Some populations seem to be protected by other overruling factors. Sub-Saharan Africans belong to that group, as well as Caribbean Hispanics and African Caribbean people of Jamaican origin, for whom the ApoE4 allele is only weakly associated with an increased risk of developing LOAD (Kalaria *et al.*, 2008). Differences in populations associated with ApoE4 and risk of AD suggests genetic interactions with the ApoE4 allele, which modulate the risk.

1.12 Impact of neuroinflammation on trafficking of APP and BACE and Aβ production

There is evidence that an increase of Aβ production arises as a direct result of neuroinflammation (Alasmari *et al.*, 2018). An increase in β and γ secretase activities may be one reason for the increased amyloidogensis. In addition, neuroinflammatory cytokines have been reported to increase APP levels in neuronal cell models, suggesting that the expression of APP is upregulated (Alasmari *et al.*, 2018). In addition, another key issue is whether neuroinflammation has an effect on the trafficking of APP and BACE and Aβ production.

Neuronal BACE1 has been reported to be increased in the proximity of activated glia cells (Sastre *et al.*, 2008), hinting the possibility of an inflammation dependent BACE1 upregulation. This suggestion was further supported by a study using neuronal cultures where exposure to pro-inflammatory cytokines and under oxidative stress, resulted in an increase in BACE1 protein (Sastre *et al.*, 2008). BACE1 has gained a lot of attention as a target for therapeutic inhibitors. Whilst BACE inhibition has been shown to reduce Aβ levels, many clinical trials failed to rescue cognitive decline (Zhu *et al.*, 2018). Selective targeting of BACE1 expression could present a more successful approach. An increase of MMP13 has been observed in both human AD brains and AD mouse models. The overexpression of MMP13 stimulates PI3K (phosphatidylinositide kinase-3) signalling which in turn promotes the eukaryotic translation initiation factor 4B (eIF4B), which facilitates BACE1 mRNA translation. Selective targeting of MMP13 decreases eIF4B phosphorylation and led to a reduction of BACE1 synthesis. These findings highlight the potential of both MMP13 and PI3K/Akt signalling as therapeutic targets (Zhu *et al.*, 2019).

In order to understand the direct connection between neuroinflammation and Aβ production, several studies have investigated the effect of LPS on Aβ

production. *In vivo* animal experiments have shown that LPS injections induce memory loss and also the generation of $A\beta_{1-42}$ in the cortex and hippocampus. LPS treatment was found to increase both β and γ secretase activities. This may be related to the transcriptional upregulation of β secretase mRNA levels (Lee *et al.*, 2008). Taken together, these findings indicate that LPS augmented inflammatory reactions could influence APP processing through the enhancement of β and γ secretase activity thereby affecting amyloidogenesis (Lee *et al.*, 2008).

In addition to perturbations in the activity of the membrane-bound secretases, defects in membrane trafficking are known to be linked to enhanced Aβ production. A number of trafficking machinery genes have been identified as LOAD risk genes (Small *et al.*, 2017). The regulation of membrane trafficking plays an important role in APP processing and there is an intimate relationship between neuron activation and Aβ production. Increased neuronal activity results in increased Aβ production (Kamenetz *et al.*, 2003). Aβ production resulting from increased neuronal activity has been linked to endosomal processing of APP and Aβ release from the cells (Cirrito *et al.*, 2008). Enhanced co-localization of exogenously expressed APP and BACE1 was observed following glycine-induced N-methyl-D-aspartate receptor (NMDARs) activation or potassium activation (Das *et al.*, 2013), findings suggesting that the trafficking of APP and BACE1 could be altered under different physiological conditions. Of relevance, is that the Gleeson laboratory has demonstrated that the phosphorylation of the BACE1 sorting motif, DISLL, is regulated by signalling in neurons (Toh *et al.*, 2018) and that the phosphorylation of DISLL influences endosomal trafficking of endogenous BACE1 (Toh *et al.*, 2018). These studies highlight that external stimuli can induce changes to the trafficking itinerary of APP and BACE1 in primary neurons, which can also affect Aβ production. The question that arises from these findings is whether cytokines secreted from activated microglia can also drive signalling events in neurons to influence trafficking and convergence of APP and BACE1. Of relevance is that TNFα, a pro-inflammatory cytokine secreted by microglia, has

been shown to stimulate BACE1 expression and is also linked to enhanced Aβ production (Yamamoto *et al.*, 2007). Collectively, these reports suggest that the inflammatory environment contributes to Aβ production. This goes in hand with the controversy around the validity of the amyloid hypothesis due to the lack of successful targeted therapies (Kametani and Hasegawa, 2018). However, the amyloid targeted drugs have been tested on later stages of the disease rather than testing the impact of amyloid inhibition during the onset of disease or as preventative treatment. Evidence of amyloid being the initial driver of the disease is very strong. And neuroinflammation appears to be play a crucial role in sustaining the disease.

Aβ induced apoptosis is associated with cyclooxygenase-2 upregulation through activation of NFκB signalling, or other complementary pathways including ERK and p38 MAPK that can intersect with various parts of the NFκB pathway. The increase of apoptotic neuronal cell death via elevation of $A\beta_{1-42}$ could be an important mechanism in LPS induced memory impairment (Jang and Surh, 2005).

In summary, systemic inflammatory stimuli elevate amyloidogenesis through a number of likely mechanisms, including activation of β and γ secretases, inhibition of α secretase, alterations in membrane trafficking of APP and BACE1, leading to elevated $A\beta_{1-42}$ levels both *in vivo* and *in vitro*. The elevated inflammation and amyloidogenesis would then be responsible for neuronal cell death and thus memory impairment.

1.13 Relationship between chronic inflammation in the periphery and AD

Given the importance of neuroinflammation in AD, it is important to consider the potential impact of peripheral chronic inflammation on the progression of AD. What do we know about conditions associated with chronic inflammation and susceptibility to AD? There is evidence of a correlation between systemic chronic inflammation, as determined by elevated C-reactive

protein (CRP), and AD risk (Tao *et al.*, 2018). Inflammatory conditions are also increasingly linked to AD. Oral health and infection, such as periodontitis, is correlated with AD (Teixeira *et al.*, 2017). The relationship between chronic inflammatory autoimmune diseases, such as Rheumatoid Arthritis (RA), which is characterised by both elevated CRP and TNFα, and AD have been studied. Whilst one study found that AD was more prevalent among RA patients than those individuals not affected by RA (Chou *et al.*, 2016). Kao and colleagues claim an inverse correlation between the two diseases (Kao *et al.*, 2016).

Is there any evidence of protection from AD by long term anti-inflammatory medication? Several epidemiological studies have provided evidence for a reduced prevalence of AD among non-steroidal anti-inflammatory drug (NSAID) users (Rogers *et al.*, 1993; Gómez-Isla *et al.*, 2008). The effects appear to be strongly dependent on both the duration of treatment and the ApoE genotype. Individuals with at least one ApoE4 allele, benefited significantly more from NSAID use (Imbimbo *et al.*, 2010). Why ApoE4 carriers benefit more from NSAIDs treatment is unclear, it can be hypothesised that this is due to the higher risk of Aβ deposition and accumulation. However, whilst some studies suggest beneficial effects of NSAIDs such as Ibuprofen, others studies did not find a correlation. A more recent report, involving very large cohort of 8.5 million participants (Chou *et al.*, 2016), not only confirmed an increased AD risk among RA patients, but also indicates there could be an important connection between anti-TNFα therapy for RA and reduced risk of AD amongst RA patients. RA patients on anti-TNF therapy with Etanercept have a lowered risk of AD (Chou *et al.*, 2016). This represents an exciting observation that needs further investigation.

1.14 Macrophages

Macrophages are a resident population in non-neuronal tissues that bear similarities to microglia. Given the depth of information on macrophages,

knowledge of the cell biology of this myeloid cell may provide insights into the behaviour and biological properties of microglia.

Macrophages are large phagocytic cells that form a resident population in many tissues of the body (Gordon, 1986). Mononuclear cells, like macrophages, originate from haematopoietic stem cells and develop along distinct differentiation pathways (Geissmann *et al.*, 2010). They have variable turnover rates and are replaced by cells in the circulation. Postnatally monocytes are derived from the bone marrow and can differentiate into macrophages (Roca *et al.*, 2009).

Resident macrophages play an important role in tissue homeostasis via clearance of apoptotic cells and production of growth factors (Geissmann *et al.*, 2010). They survey their environment by ingesting dead cells, debris and pathogens (Davalos *et al.*, 2005).

Macrophages in non-neuronal tissue reprogram their function in response to pathogens and tissue damage. Macrophages can be polarised into at least two functional states, M1 and M2. Bacterial stimuli such as LPS polarise macrophages to the M1 phenotype. IL-4 or CCL2 drive M2 polarisation (Roca *et al.*, 2009). Macrophages activated by LPS and by IL-4 are functionally distinct. M1 polarised cells produce pro-inflammatory cytokines and chemokines such as IL-12, IL-23, TNFα, CXCL9 and CXCL10 and show enhanced antigen presentation capacity (Biswas and Mantovani, 2010). M2 polarised cells produce anti-inflammatory cytokines, such as IL-10, and display increased phagocytic activity and express scavenger receptors such as CD163 (Biswas and Mantovani, 2010). Whilst the concept of polarised M1 and M2 functional states is very useful to conceptualise the principles of macrophage activation, it is now debated whether it is, like for microglia, an oversimplification of cell activation (Yin *et al.*, 2017).

A variety of myeloid populations reside in the brain, including microglia, perivascular cells, meningeal macrophages and choroid plexus macrophages, sharing a multitude of markers such as IBA1, F4/80 and CX3CR1 (Prinz *et al.*, 2011). Despite the variety of myeloid cells in the brain, microglia are the only myeloid cells that reside behind the BBB (Prinz *et al.*, 2011). The BBB is composed of specialised endothelial cells, linked by complex tight junctions comprised of adhesion molecules including cadherins (Engelhardt and Ransohoff, 2005). The BBB structure limits entry of solutes, and occludes 98 % of antibodies and small molecules but at the same time ensuring the efflux of others (Bechmann *et al.*, 2007). A second interface, the blood CSF barrier (BCSFB), is at the choroid plexus where tight junctions are formed between the apical surface of the epithelial cells. A third barrier is formed by the arachnoid membrane under the dura mater (Abbott *et al.*, 2010). Following injury to the CNS the activation of endothelial cells and associated cells such as astrocytes can lead to reduced tight junction integrity facilitating the migration of leukocytes across and through the BBB into the brain (Wilson *et al.*, 2010). Under pathological conditions, excessively produced Aβ acts as a chemotractant to macrophages which then transmigrate across the BBB (Fiala *et al.*, 1998). Infiltration of macrophages in the AD brain suggests a systemic immune response rather than the originally recognised local immune reaction (Fiala *et al.*, 2002; Bechmann *et al.*, 2007). Macrophages are considered to play an important role in the reduction of vascular amyloid. A study eliminating perivascular macrophages showed an accumulation of vascular amyloid (Hawkes and McLaurin, 2009). Interestingly macrophages from healthy individuals have been reported to be more efficient at Aβ phagocytosis and less susceptible to apoptosis upon Aβ exposure than macrophages from AD individuals (Lai and McLaurin, 2012).

1.15 Uptake mechanisms

Phagocytes such as macrophages and microglia are responsible for the uptake of cellular debris and elimination of pathogens. In order to do so they utilise

different endocytic uptake mechanisms, which also allow them to survey their environment. Phospholipids play an important role in internalisation processes, by directing traffic of individual cellular compartments and the recruitment of proteins that control tubulation, fusion and fission events. Unsurprisingly dysregulation of phospholipid synthesis and catabolism during endocytosis is associated with a multitude of disorders, including infections, and neurological pathologies (Bohdanowicz and Grinstein, 2013). Hence, the understanding of endocytic pathways is critical to normal brain function.

1.15.1 Endocytosis

Endocytosis is the uptake of particles and cellular debris and involves the exchange between different membrane compartments of the cells, including plasma membrane, endosomes, lysosomes and the Golgi apparatus (Ryter, 1985). All endocytic pathways share the requirement for a budding structure on the plasma membrane, or membrane ruffles as is the case in macropinocytosis, to generate endocytic structures. Endocytic pathways can be classified into two categories – clathrin-dependent endocytosis and clathrin-independent endocytosis (Doherty and McMahon, 2009). Not all cells perform the entire spectrum of endocytic pathways (Kuhn *et al.*, 2014).

1.15.2 Clathrin mediated endocytosis

Clathrin-dependent endocytosis uses clathrin-coated vesicles to facilitate the uptake of material into the cell. The name originates from the fact that the coat around these vesicles consists of the protein clathrin (Pearse, 1976). Vesicle formation is a five-step process; beginning with a membrane pit formation and cargo selection by adaptor complexes such as AP-2, which in turn recruits clathrin. The small GTPase, dynamin, is then recruited to the vesicle and induces membrane scission. After the formation of a clathrin-coated vesicle, accessory proteins as well as clathrin are recycled back to the cytosol

and can be reused. The internalised cargo is then delivered to endosomes where it is sorted (McMahon and Boucrot, 2011).

Interestingly clathrin-dependent endocytosis can be used for the uptake of particles. One study demonstrated that 40 nm nanoparticles are taken up by clathrin mediated endocytosis in macrophages (Kuhn *et al.*, 2014).

1.15.3 Clathrin independent endocytosis

In addition to the classic clathrin-mediated endocytosis, there are several rapid internalisation pathways that are clathrin-independent. Clathrin-independent endocytosis can be further divided into dynamin-dependent and dynamin-independent pathways (Mayor and Pagano, 2007). Caveolae-mediated endocytosis and a pathway dependent on the small GTPase RhoA are both dynamin-dependent mechanisms (Lamaze *et al.*, 2001). A dominant negative mutant of dynamin was used to demonstrate a dynamin and clathrin independent pathway contributing to pinocytosis in HeLa cells (Damke *et al.*, 1995). Dynamin- and clathrin-independent endocytic pathways are regulated by small GTPases such as CDC42 or ARF6 (Mayor and Pagano, 2007). The GTPase dynamin pinches off vesicles at the plasma membrane, which is required for internalisation. The question therefore arises, how transport carriers are budded in dynamin independent endocytosis? Here, internalisation occurs via tubular structures, in the absence of a defined coat. Physical tension curves the membrane and promotes the spontaneous generation of transport vesicles (Mayor *et al.*, 2014; Johannes *et al.*, 2015).

The clathrin-independent pathway(s) provide very rapid endocytosis (~100 ms) and can be used to restore the surface area of the membrane after exocytosis, as observed in mouse hippocampal neurons and plays an important role in the regulation of cell growth and development (Watanabe *et al.*, 2013).

1.15.4 Phagocytosis

Phagocytosis is an actin-dependent pathway used for uptake of large particles (>0.5 um), such as apoptotic cells and bacteria, into intracellular structures known as phagosomes (Botelho and Grinstein, 2011). Professional phagocytic cells such as macrophages, monocytes, neutrophils, dendritic cells and microglia, are extremely efficient at surveying their environment and provide a first line of defence against infection (Stuart and Ezekowitz, 2005). Phagocytes can distinguish between pathogenic components that result in the induction of a pro-inflammatory response and apoptotic cells that trigger an anti-inflammatory response. Pathogen associated components include bacteria, which are recognised by TLRs. Apoptotic cells are recognised by the expression of phosphatidyl-serine (PS) (Stuart and Ezekowitz, 2005). PS is an anionic phospholipid, normally confined to the inner leaflet of the plasma membrane of healthy cells (Cocco and Ucker, 2001). The translocation to the outer leaflet, exposing it to the cell surface, facilitates removal of cellular debris (Elliott *et al.*, 2005).

Phagocytosis is initiated upon receptor recognition on the cell surface following ligand binding. Actin polymerisation is induced under the membrane at the site of contact and a phagocytic cup, an actin rich membrane extension, then forms on the surface membrane that will protrude and curve around the target particle. Highly dynamic actin filaments drive the growth of membrane pseudopods that encircle the target. The phagosome then closes by fusion of pseudopods from both sides of the target particle and detaches from the surface membrane. The target can then be internalised and the phagosome matures to become a phagolysosome (Aderem, 2003; Botelho and Grinstein, 2011).

1.15.5. Pinocytosis

Pinocytosis describes both fluid phase uptake and receptor mediated endocytosis (or clathrin mediated endocytosis) and includes micropinocytosis and macropinocytosis. Macromolecules of a size less than 0.2 μm in diameter

are internalised by micropinocytosis (Steinman *et al.*, 1983). Macropinocytosis on the other hand facilitates the internalisation of large quantities of solutes and membrane, due to the large size of macropinosomes, which can be between 0.2 – 5 μm in diameter. Macropinocytic vesicles have no apparent coat structure (Hewlett *et al.*, 1994; Swanson and Watts, 1995).

1.15.6 Macropinocytosis

Compared with other endocytic pathways, the size of vesicles formed by macropinocytosis are much larger (Mayor and Pagano, 2007). Macropinocytosis is a non-selective, signal-dependent process that can be activated by various stimuli including high doses of growth factors, integrin substrates, PS containing apoptotic cells, viruses and bacteria (Haigler *et al.*, 1979; Dharmawardhane *et al.*, 2000; Watanabe and Boucrot, 2017). In the immune system, antigen presenting cells such as macrophages not only use macropinocytosis to capture antigens from their surrounding as a means of surveillance, but they are capable to constitutively do so (Norbury *et al.*, 1995; Lim and Gleeson, 2011).

Macropinocytosis begins with actin mediated membrane ruffling of the plasma membrane. This can occur both on the peripheral and dorsal surface of the cell (Abella 2010, Luo 2014). Ruffle extension and retraction is regulated by the phosphorylation and dephosphorylation of phosphoinoisitides. PI5-kinase stimulates actin polymerisation for ruffle extensions, whilst PI3-kinase is required for closure of the macropinosome (Araki *et al.*, 2000). Rac, a member of the Rho family of small GTPases, is activated and leads to the formation of lamellipodial extensions (Araki *et al.*, 2000; Araki *et al.*, 2006).

The so formed lamellipodia fold back onto themselves and fuse with the basal membrane creating large vesicles capturing the target. The closed vesicle is then internalised. Once formed, the macropinosome undergoes a maturation process, by recruiting late endosomal and lysosomal markers, including Rab5 at the early stages of maturation and later Rab7. The macropinosome initially

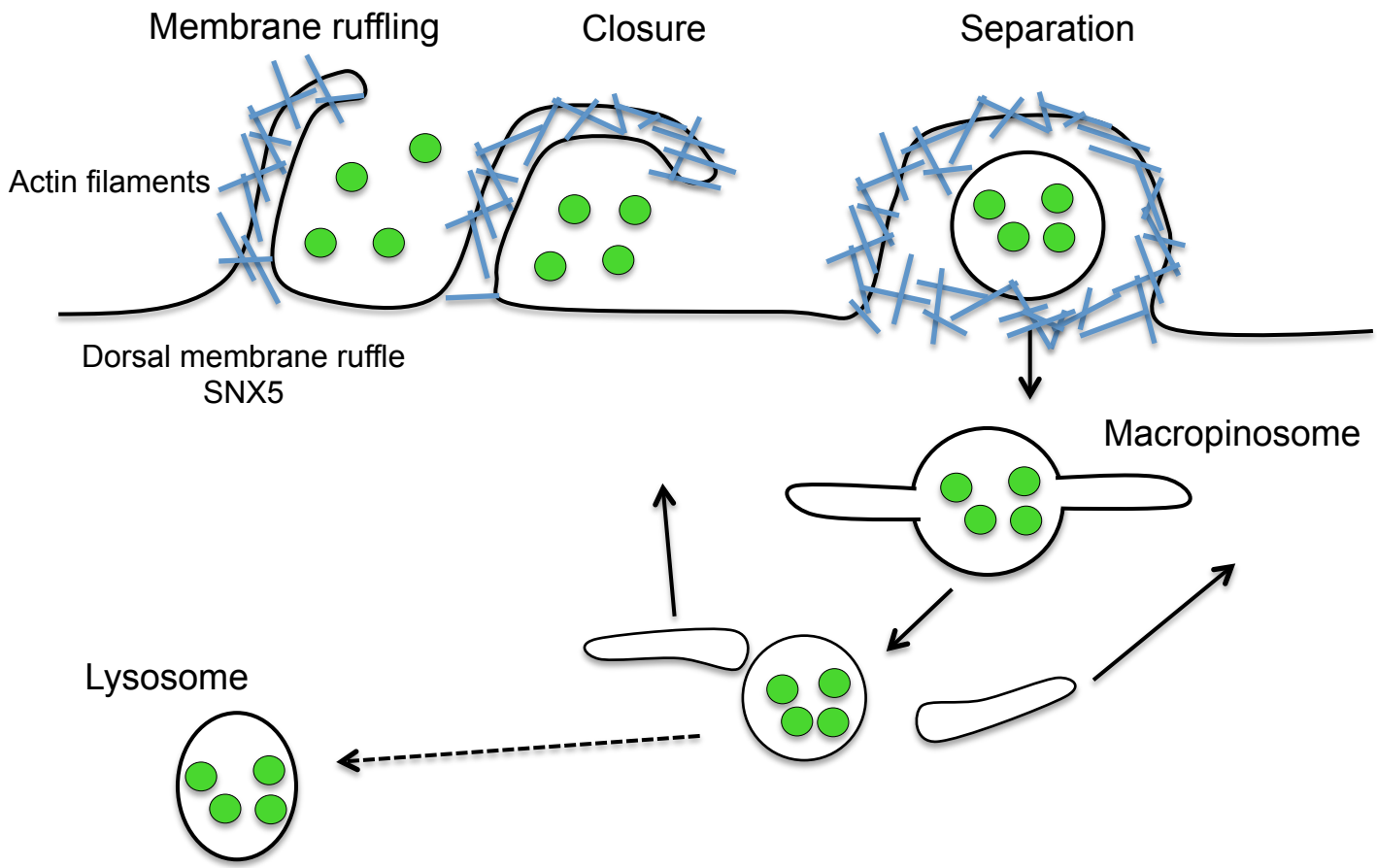


Figure 1.6 Schematic model of macropinocytosis at the dorsal membrane
Macropinocytosis is an endocytic pathway that arises from actin mediated membrane ruffling of the plasma membrane. Lamellipodia fold back over themselves and fuse with the plasma membrane, capturing the target and creating a large vesicle. The vesicle is internalised and the macropinosomes matures.

acts as a maturing vessel and then fuses with tubular lysosomes (Racoosin and Swanson, 1993; Wang *et al.*, 2010) (Figure 1.6).

1.15.6.1 Physiological relevance of macropinocytosis

As macropinocytosis is associated with actin dependent ruffling of the plasma membrane it is also implicated with cell motility. Therefore, macropinocytosis is not only relevant under physiological but also pathological conditions such as tumour progression and metastasis. Macropinocytosis is considered important in chemotactic response of highly mobile cells, such as neutrophils (Carpentier *et al.*, 1991). This is believed to increase the level of pathogen ingestion by neutrophils.

Pathogens such as bacteria, viruses and prions opportunistically exploit macropinocytosis to evade and invade the host immune system. The ebola virus is a particular interesting example of this, as it primarily enters the host cell via macropinocytosis, but has also adapted to an alternative route of entry via clathrin mediated endocytosis (Hoffmann *et al.*, 2001; Lim and Gleeson, 2011; Aleksandrowicz *et al.*, 2011).

Macropinocytosis is also crucial for the ability of antigen presenting cells to sample their immediate environment for antigens (Norbury *et al.*, 1995). Macrophages are extremely efficient doing this, as they are able to undergo extensive constitutive macropinocytosis, internalising up to 200 % of their surface area every hour (Steinman *et al.*, 1976).

1.15.6.2 Regulation of macropinocytosis

A key difference between clathrin-dependent endocytosis and macropinocytosis is that the latter requires actin cytoskeleton reorganisation. The dynamics of the actin cytoskeleton can be visualised in cells expressing fluorescently labelled actin probes (Gaidarov *et al.*, 1999). The actin associated

protein coronin was identified in playing a key role in the formation of the macropinosome (Hacker *et al.*, 1997). In macrophages F-actin was shown to associate with dextran positive macropinosomes and decreases when the structure matures (Araki *et al.*, 2000; Lee and Knecht, 2002).

1.15.6.3 Sorting nexins and macropinocytosis

Recently members of the sorting nexin (SNX) family have been localised to macropinosomes and also implicated in their formation (Merino-Trigo *et al.*, 2004). SNXs are phox homology (PX) domain containing proteins, which belong to a diverse group of cellular trafficking proteins. They have the ability to bind to specific phospholipids, through their PX domain, and can form protein-protein complexes aiding them in their role in membrane trafficking (Wishart *et al.*, 2001; Worby and Dixon, 2002).

A number of SNXs have been implicated in the biogenesis of macropinosomes including SNX1, SNX5, SNX9, SNX18 and SNX33 (Wang *et al.*, 2010). GFP-SNX5 expressing HEK cells were used to show that SNX5 colocalises with macropinosomes (Merino-Trigo *et al.*, 2004). In a model of HEK-GFP-SNX5 stable cells an increase in macropinocytosis was observed, which was associated with increased formation of macropinosomes. This suggests that SNX5 has a role in macropinocytosis regulation. In macrophages SNX5 is abundantly present and correlates to high intrinsic macropinocytosic activity (Lim *et al.*, 2008). Using SNX5-knockout mice, Lim and colleagues have generated both macrophages and dendritic cells and shown that SNX5 plays an important role in the biogenesis of macropinsomes from dorsal ruffles. Interestingly, the lack of SNX5 did not have an effect on the phagocytic activity of these cells. Macrophages deficient in SNX5 have a dramatically reduced macropinocytic activity compared to wt macrophages. Whilst SNX5 was found both on macropinosomes derived from dorsal and peripheral ruffles, it was shown that SNX5 is essential to dorsal-ruffle derived macropinosomes and not peripheral mediated macropinsomes (Lim *et al.*, 2012; Lim *et al.*, 2015).

1.16 Aims of this project

ApoE is well established as one of the strongest risk factors for AD. Microglia are known to be key players associated with ApoE risk, but how the ApoE alleles affect microglia function remains largely unknown. Many studies have performed experiments comparing only two of the three APOE genotypes. The aim of this study is to directly compare all three ApoE alleles and additionally compare them to wt C57/BL6 microglia, harbouring the single isoform wt mouse ApoE allele. Neuroinflammation is a key hallmark of AD. Therefore, the first goal to understand the molecular basis for chronic inflammation in AD is to assess how microglia from the different genotypes respond to immunostimulation, and to determine how they respond to Aβ stimulation.

Neurons are another key cell player in AD pathology, but the effect ApoE has on primary neurons is not well characterised. In particular, the interplay between microglia and neurons and the role of ApoE in such interactions remains an open question. Thus, the second aim of this project is to investigate the effect immunostimulated, or Aβ treated, microglia from different genetic backgrounds on neurons, in particular the impact on neuron morphology.

Thirdly, the characterisation of microglia and comparison between microglia and macrophages is relevant to a better understanding of the cell biological properties of microglia. There is a wealth of knowledge on macrophages that can potentially be utilised to further understand microglia biology and thus helping further understand AD pathology. Macrophages, like microglia, are an integral part of the innate immune system, in part due to their ability to survey their environment by utilising different endocytic mechanisms. Macropinocytosis is one of these endocytic pathways. The Gleeson lab had previously shown that macropinocytosis in macrophages is SNX5-dependent. There is little known

about macropinocytosis and the role of SNX5 in microglia, and therefore another aim is to assess the role of SNX5 in macropinocytosis in microglia.

Another potentially relevant property of macrophages is the ability of sodium chloride to trigger an inflammatory response, a characteristic, which exacerbates inflammation in the periphery. Another component of Aim 3 was to examine the impact of sodium chloride on inflammatory responses of microglia.

Chapter 2: Materials & Methods

2.1 Antibodies for immunocytochemistry and immunoblotting

The following primary antibodies were used at the indicated dilutions: MAP2 (1:1500 Rabbit polyclonal antibody, cat. no.: ab32454, Abcam, Cambridge, UK), Gm130 (1:300 mouse monoclonal antibody, cat. no.: 610823, BD, Franklin Lakes, US), Rabbit anti-mouse SNX5 antibody is as described previously (Lim *et al.*, 2012), Phalloidin (1:1000, Sigma, Darmstadt, Germany), Integrin αM (1:150, mouse monoclonal antibody, cat. no.: sc-20050, Santa Cruz, Santa Cruz, US). Secondary antibodies for immunofluorescence were Goat anti-mouse IgG-Alexa Fluor 488 nm, Goat anti-rabbit IgG-Alexa Fluor 488 nm and Goat anti-rabbit IgG-Alexa Fluor 647 nm. All secondary antibodies were diluted in Blocking Solution (5% FCS, 0.02% Sodium Azide in PBS) and obtained from Life Technologies. Horse-radish peroxidase (HRP) conjugated sheep anti-rabbit Ig and anti-mouse Ig were purchased from DAKO Corporation (Carpinteria, US).

2.2 General reagents

2.2.1 Amyloid beta preparation

Amyloid beta protein (Aβ 1-42) (HFIP-treated) was obtained from Bachem (Bubendorf, Switzerland) and prepared following protocol by Nitsch et al 2001 (Nitsch, 2001). Aβ was diluted with dPBS and incubated for 30 minutes whilst shaking, followed by a further dilution to 250 µM and incubated at 37 °C for 84 hours at 1000 rpm.

2.3 Animals

All animals were kept under a temperature (20-24 °C) and humidity controlled vivarium in group housing. Both male and female mice were included in the study.

2.3.1 ApoE target replacement mice

ApoE 2, ApoE 3 and ApoE 4 target replacement mice express the human apolipoprotein E (2, 3 or 4 respectively) isoform under the control of the murine ApoE regulatory sequence. All animals are on a C57BL/6J genetic background. These animals were obtained from Taconic Biosciences (Albany, US) and maintained through mating of homozygotes. The original ApoE 2 target replacement chimera mouse was backcrossed to C57BL/6 for seven generations and twice more (N9) by Taconic, where embryo transfer was derived. The colony is since maintained through mating of homozygotes (Biosciences, n.d.).

2.3.2 SNX5$^{-/-}$ mice

The SNX5 'Knock-out First' strain was created from ESC clone (a2EPD0033_3_D07) and obtained from the NCRR-NIH-supported KOMP repository and generated by the CSD consortium for the NIH-funded KOMP project. Mice were bred as homozygotes on a C57/BL6 background (Lim *et al.*, 2015). (University of Melbourne ethics ID: 1613960).

2.3.3 Wild type animals

Wild type C57BL/6J (Charles River, Wilmington, US) animals were used as control animals. All animals were maintained in a temperature and humidity controlled vivarium.

2.4 Mammalian cell methods

2.4.1 Production of L-conditioned medium

L929 cells (Sigma, Darmstadt, Germany) were cultured in DMEM GlutaMAX™ (Gibco, Carlsbad, US) supplemented with 10% FBS (Gibco,

Carlsbad, US) and 1% Penicillin/Streptomycin (Gibco, Carlsbad, US) at 37 °C, 5% CO_2, humidified air. After 14 days, supernatant was collected, centrifuged at 1500 rpm for 5 minutes and the supernatant was filtered using a 0.22 µm syringe driven filter unit (Merck Millipore, Burlington, US). Supernatants were kept at -20 °C until needed.

L-conditioned medium for macrophage cultures were prepared by culturing L929 cells in RPMI (Gibco, Carlsbad, US) supplemented with 10% FBS (Gibco) and 1% Penicillin/Streptomycin (Gibco, Carlsbad, US) at 37 °C, 5% CO_2. Confluent cells were harvested by trypsinisation (5 minutes at 37 °C, 5% CO_2 and cells grown in 2 litre roller bottles in a rotating incubator) on a roller incubator for 6 weeks. The medium was centrifuged at 1620 x g for 10 minutes, supernatant then filtered using a 0.22 µm syringe driven filter unit (Merck Millipore) and stored at -20 °C.

2.4.2 Generation of bone marrow derived macrophages

Eight to twelve-week old mice were killed by CO_2 asphyxiation in accordance with animal ethics guidelines (University of Melbourne ethics ID: 1613960). Cells were obtained from the femurs and tibias of mice. Erythrocytes were lysed and cells pelleted by centrifugation. Cells were resuspended in freezing media (90% FBS, 10% DMSO) and frozen in liquid nitrogen until needed. Once thawed, cells were maintained in complete bone marrow medium (RPMI supplemented with 15% FCS, 20% L-cell conditioned medium, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine) in a humidified incubator at 37 °C, 5% CO_2.

2.4.3 Generation of primary cortical neuron cultures

Eight to twelve-week old pregnant mice were killed by CO_2 asphyxiation in accordance with animal ethics guidelines (University of Melbourne ethics ID: 1613960). Cortical tissue was taken from the brains of E15-17 mouse embryos and digested with 0.0125 % (w/v) trypsin (Sigma, Darmstadt, Germany) for 5

minutes at 37 °C. Tissue was suspended in HBSS (Life Technologies, Carlsbad, US) containing 1 mM sodium pyruvate (Life Technologies, Carlsbad, US), 10 mM HEPES (Life Technologies, Carlsbad, US), 1.16 mM $MgSO_4$ (Sigma, Darmstadt, Germany), 7.6 mM D-Glucose (Sigma, Darmstadt, Germany), followed by mechanical tituration. Cell viability and density was determined using a trypan blue exclusion assay. Cells were plated on poly-D-lysine (Sigma, Darmstadt, Germany) coated plates or coverslips in complete-Neurobasal medium (Life Technologies, Carlsbad, US), typically 0.7x10^5 cells/ml and incubated at 37 °C, 10% CO_2 overnight. The following day 50% of the medium was replaced with fresh medium and then every three days 50% of the medium was changed.

2.4.4 Generation of primary microglia cultures

Microglia were isolated from mouse brains of day 1-3 (P1-3) neonatal mice according to Giulian and Baker (Giulian and Baker, 1986). In brief, brains were isolated and stripped of their meninges. The tissue was then trypsinised (0.25%, Gibco, Carlsbad, US) for 10 minutes and cells were plated on Poly-L-lysine (Sigma, Darmstadt, Germany) coated flasks and incubated in DMEM GlutaMAX™ (Gibco, Carlsbad, US) supplemented with 10% FBS (Gibco, Carlsbad, US) and 1% Penicillin/Streptomycin (Gibco, Carlsbad, US) overnight at 37 °C, 5% CO_2, humidified air. The medium was changed on the following day and supplemented with 1.5% (v/v) L929 conditioned medium. Cells were harvested after 7-10 days by gently tapping the culture flasks to dissociate the microglial cells. The purity of microglia was microscopically assessed by CD11b staining (Integrin αM, Santa Cruz, Santa Cruz, US). Typically 98% of cells were CD11b positive. Prior to the use of microglia in any assay, microglia were serum-starved overnight and the medium supplemented with N2 (Thermo Fisher Scientific, Waltham, US).

2.5 Indirect immunofluorescence microscopy

Cells were cultured as monolayers on coverslips and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature, followed by quenching in 50 mM NH_4Cl/PBS for 10 min at room temperature. Monolayers were then permeabilised with 0.1% Triton X-100/PBS for 4 minutes and incubated in Blocking Solution (5% FCS, 0.02% Sodium Azide in PBS) for 30 minuets to reduce non-specific binding. Cell monolayers were incubated with primary antibodies diluted in blocking solution for 4 hours or overnight at 4 °C and then washed 6 times in PBS. Fluorochrome-conjugated secondary antibodies were added at the appropriate dilution and incubated for 1 h at room temperature. Following another washing step in PBS, cells were stained with DAPI (Life Technologies, Carlsbad, US) for 4 minutes. Coverslips were then washed 6 times with PBS, followed by a washing step in milli-Q water, before mounting in Mowiol (Calbiochem, SAN Diego, US). Confocal microscopy was performed using a Leica SP8 system. Images were collected sequentially for multi-colour imaging using a x63/1.4 NA HCX PL APO CS oil immersion objective. FITC and Alexa Fluor 488 were excited with the 488 nm line of an Argon laser, Alexa Fluor 647 with a 633 nm HeNe laser and DAPI with a 405 nm UV laser. Images were collected with pixel dimensions of at least 512 x 512.

2.5.1 Image analysis

FIJI (ImageJ, version 2.0.0-rc-59/1.51k, Wayne Rusband, National Institute of Health, USA) was used for image analysis. The simple neurite tracer plugin was used to measure dendrite length in neurons (Longair M et al, 2011). Cells for analysis were carefully chosen and analysis done using a projection line of beginning and end of dendrites through a 3D reconstruction of the image. Each experiment was repeated at least 3 times.

2.6 Treatment of cells

2.6.1 Microglia conditioned media preparation

Primary microglia were treated with LPS, LPS and ATP, Aβ or LPS and Aβ as outlined above and collected after 4 hours. Conditioned media (1.5 ml) were collected, spun down at 1200 rpm and snap frozen in liquid nitrogen. Conditioned media were stored at -80 °C until used. Primary cortical neurons were subjected to microglia supernatants for 72 hours before they were fixed and stained as outlined above. At least 3 independent experiments were carried out for each treatment condition.

2.6.2 Microglia immunostimulation

Primary microglia were cultured as monolayers, serum starved overnight and then stimulated with 150 ng/ml lipopolysaccharide (LPS) from E.coli K12 (InvivoGen, San Diego, US) for 3 hours, LPS (3 hours) and 1 μM or 10 μM Adenosine 5′-triphosphate (ATP) (Sigma, Darmstadt, Germany) for 1 hour, or LPS and 10 μM nigericin (Sigma, Darmstadt, Germany) for 1 hour. Supernatants were either collected immediately after treatment or medium replaced with complete DMEM for 24 hours before supernatant collection.

2.6.3 Microglia amyloid beta stimulation

Primary microglia were cultured in monolayers, serum starved overnight and stimulated with 5 μM or 10 μM Ab (Bachem, Bubendorf, Switzerland) for 1 or 24 hours or pre-stimulated with 150 ng/ml lipopolysaccharide (LPS) from E.coli K12 (InvivoGen, San Diego, US) for 3 hours, followed by Aβ stimulation.

2.6.4 Neuronal TNFα treatment

Primary cortical neurons were cultured as monolayers and incubated with 2 ng/ml, 1 ng/ml or 500 pg/ml purified mouse TNFα (Sigma, Darmstadt, Germany) for 72 hours. The neurons were then fixed and stained as outlined above.

2.6.5 Sodium chloride treatment.

Microglia were cultured overnight in serum-free complete DMEM, in the presence of 1% N2 supplement, to render cells quiescent. After a 3 hour incubation with LPS (150 ng/ml), cells were treated with an additional 40 mM of NaCl, 10 μM Aβ, or 40 mM of NaCl and 10 μM Aβ, for 24 hours. To examine the impact of osmolarity and tonicity, experiments were also performed in the same manner, with either 80 mM urea or 80 mM mannitol added.

2.6.6 Treatment with p38 inhibitor

p38 MAP Kinase inhibitor (SB203580, InvivoGen, San Diego, US) was incubated with cells 1 hour prior to cell treatment. p38 MAP Kinase inhibitor was used at a final concentration of 5 μM.

2.6.7 TNFα neutralizing antibody

TNFα neutralizing antibody (Cell Signalling Technology, Danvers, US) was incubated with the supernatants or purified mouse TNFα 1 hour prior to the experiment. Mouse TNFα neutralizing rabbit antibody was used at a final concentration of 10 ng/ml.

2.7 Uptake experiments

2.7.1 Fluorescently labeled Amyloid uptake in ApoE microglia

Primary microglia were plated in optical-bottom 96-well plates (Nunc, Rochester, US). Cell monolayers were serum starved overnight, washed and incubated with Beta Amyloid (1-42) Hi-Lyte Fluor 488 (AnaSpec, Fremont, US) for 1-2 hours. The suspension was then removed, and subsequently, 100 μl per well of trypan blue suspension (250 μg/ml, pH 4.4) was added for 1 minute to

quench the extracellular probe. After aspiration of trypan blue, the fluorescence was measured at 486 nm excitation and 534 nm emission using a microplate reader (Tecan Infinite M200, Magellan™ V6.55).
Cell number was determined by a 15 minute incubation with H33342 solution (25 µg/ml in PBS/ 0.1% TritonX-100) (Sigma, Darmstadt, Germany) followed by fluorescence measurement at 360 nm excitation and 418 nm emission using a microplate reader (Tecan Infinite M200, Magellan™ V6.55).

2.7.2 Fluid-phase uptake assays

Cells were initially rendered quiescent by culturing primary microglia (150,000 cells per well in a 12 well plate) overnight in serum-free DMEM, in the presence of 1% N_2 supplement and bone marrow derived macrophages (180,000 cells per well in a 12 well plate) in RPMI. Cells were stimulated with 150 ng/ml LPS for 3 hours, followed by incubation with either 500 µg/ml 70kDa dextran, Aβ (1-42) Hi-Lyte Fluor 488 (AnaSpec, Fremont, US) for 15 or 45 minutes. Following the incubation, cells were placed on ice for 5 minutes and were washed with ice-cold medium and PBS. Uptake was quantified following the method described by Wang et al., using Fiji's particle analyser (Wang *et al.*, 2014). For macropinocytosis assays, cells were also pre-treated with the inhibitor 5-(N-Ethyl-N-isopropyl) amiloride (Sigma, Darmstadt, Germany) at 100 µM for 30 mins prior to the addition of 70 kDa dextran.

2.8.1 Cytotoxicity assay.

Cytotoxicity of supernatants were analysed using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, Waltham, US) according to the manufacturers protocol.

2.8.2 Nitric oxide determination

Nitric oxide released into the cell culture mediums was measured using the Griess Reagent System (Promega, Madison, US) according to the manufacturer's protocol. Briefly; sulfanilamide solution was added for 5 minutes, followed by a 5 minute incubation with NED solution. Absorbance was then measured at 550 nm using a microplate reader (Tecan Infinite M200, Magellan™ V6.55/ FLUOstar® Omega, BMG Labtech). The nitrite concentration was determined by interpolation using a nitrite standard curve.

2.8.3 Quantification of secreted IL-1β and TNFα cytokines

Conditioned medium collected from treated microglia or macrophages was analysed for IL-1β (mouse IL-1β DuoSet ELISA) and TNFα (mouse TNFα DuoSet ELISA) using commercially available mouse colorimetric sandwich ELISA plates (R&D Systems, Minneapolis, US). Absorbance was measured at 450 nm using a microplate reader (Tecan Infinite M200, Magellan™ V6.55/ FLUOstar® Omega, BMG Labtech). The cytokine concentration was determined by interpolation using a standard curve

2.8.4 Viability assay

Cell viability was assessed by an XTT assay kit (XTT Cell Viability Kit, Cell Signaling, Danvers, US). Following the treatment of cells, the medium was replaced with FluoroBrite DMEM (Gibco, Carlsbad, US) and the tetrazolium salt XTT added. Absorbance was measured at 450 nm using a microplate reader (Tecan Infinite M200, Magellan™ V6.55). The XTT concentration was determined by interpolation using a standard curve.

The viability of BMDMs and primary microglia treated with NaCl and or Aβ, with and without LPS priming was assessed by FACS. Cells were serum starved overnight, treated as outlined above and detached using TrypLE™ Express (Gibco, Carlsbad, US). Cell supernatants, as well as the trypsinised cells were centrifuged at 300 x g for 10 minutes at 4 °C. The cell pellets were resuspended in Fixable Viability Dye eFluor™ 780 (Invitrogen, Carlsbad, US) for 30 minutes

on ice. Following the staining step, cells were centrifuged at 300 x g for 10 minutes at 4 °C and resuspended in FACS buffer (0.5 % FBS, 0.5 mM EDTA in PBS). Cells were then analysed on FACSCanto™ II Cell Analyser (BD Bioscience, Franklin Lakes, US). Data was analysed using FlowJo V9.3.2.

2.9 Mass Spectrometry

2.9.1 Sample preparation

$1.5x10^6$ microglia cells were seeded on 6 well plates and treated as outlined above. Supernatants were collected (after 24 hours) and snap frozen in liquid nitrogen. The supernatants were stored at -20 °C. Supernatants were thawed and acetone precipitated by incubation in ice cold acetone (ratio of supernatant to acetone 1:4) for 60 minutes. This was followed by a 10 minute centrifugation step at 15,000 x g at 4 °C. Supernatants were decanted and the remaining acetone allowed to evaporate for 30 minutes at room temperature. The pellet was then resuspended in buffer comprising 2,2,2-Trifluorethanol (TFE, Sigma, Darmstadt, Germany), 20 mM Tris(-carboxyethyl)phosphine hydrochloride (Tcep, Sigma, Darmstadt, Germany) and 20 mM Tetraethylammonium (TEAB, Sigma, Darmstadt, Germany), followed by an overnight trypsin digest at 37 °C by the addition of trypsin (1 µg) dissolved in 20 mM TEAB. The following day samples were centrifuged at 15,000 x g for 5 mins and stored at 4 °C until processing. For each genotype 4 samples from independent preparations were processed.

2.9.2 Technical details

Samples were analysed by LC-MS/MS using Q-Exactive plus mass spectrometer (Thermo Scientific, Waltham, US) fitted with nanoflow reversed-phase-HPLC (Ultimate 3000 RSLC, Dionex). The nano-LC system was

equipped with an Acclaim Pepmap nano-trap column (Dionex – C18, 100 Å, 75 µm × 2 cm) and an Acclaim Pepmap RSLC analytical column (Dionex – C18, 100 Å, 75 µm × 50 cm). Typically for each LC-MS/MS experiment, 1 µl of the peptide mix was loaded onto the enrichment (trap) column at an isocratic flow of 5 µl/minute of 3% CH_3CN containing 0.1% formic acid for 6 minutes before the enrichment column was switched in-line with the analytical column. The eluents used for the LC were 0.1% v/v formic acid (solvent A) and 100% CH_3CN/0.1% formic acid v/v. The gradient used was 3% B to 20% B for 95 min, 20% B to 40% B in 10 min, 40% B to 80% B in 5 min and maintained at 80% B for the final 5 min before equilibration for 10 min at 3% B prior to the next analysis. All spectra were acquired in positive mode with full scan MS spectra scanning from m/z 375-1400 at 70000 resolution with AGC target of 3e6 with maximum accumulation time of 50 ms. Lockmass of 445.120024 was used. The 15 most intense peptide ions with charge states ≥2-5 were isolated with isolation window of 1.2 m/z and fragmented with normalized collision energy of 30 at 17500 resolution with AGC target of 1e5 with maximum accumulation time of 100ms. Underfill threshold was set to 2% for triggering of precursor for MS2. Dynamic exclusion was activated for 30s.

2.9.3 Mass Spectrometry analysis

A target list was created including 13 pro- and anti-inflammatory cytokines. Many additional proteins overlapped with the predicted masses and were included in the analysis. Mascot protein identification search engine (Matrix Science, v2.4) was used for Mass Spectometry analysis, using the SWISSPROT database. Search parameters used were 10-ppm peptide mass tolerance and 0.2-Da fragment ion mass tolerance. Oxidation (M) was set as variable modification and 2 missed cleavages were permitted. The search taxonomy was restricted to Mus musculus. Peptides with mascot ion scores greater than the homology score were considered significant and only those that appeared in at least 3 out of 4 independent experiments were included in the analysis.

2.10 Statistical analysis

Data were analysed using, unpaired t-test (for comparison between two groups) or one-way ANOVA (for comparison between three groups or more), after performing Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefor P value normality testing using GraphPad Prism 7.0 software (GraphPad Software). Data were expressed as mean ± SEM, and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

2.11 Protein methods

2.11.1 Cell lysates

Equal numbers of cells were lysed in RIPA buffer (50 mM Tris-HCl, ph 7.3, 150 mM NaCl, 0.1 mM EDTA, 1% [w/v] sodium deoxycholate, 1% (v/v) Triton X-100, 0.2% (w/v) NaD and 100 uM Na3VO4) supplemented with Complete™ protease inhibitor (Roche). Cell lysates were incubated on ice for 10 minutes and centrifuged for 10 minutes at 16,000 x g.

2.11.2 Bradford assay

Bradford assay was performed to determine protein concentrations prior to SDS PAGE, following the manufacturer's instructions, using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, US). BSA was used to generate a standard curve.

2.11.3 Immunoblotting

Cell lysates from primary cortical neurons were mixed with 4x buffer containing β-mercaptoethanol and boiled for 10 min at 100 °C. The samples were then resolved on a 4-12% Bis-Tris SDS-PAGE gel (Life Technologies,

Carlsbad, US) and transferred onto Immnobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Burlington, US) at 400 mA for 1 hour. Following this the membrane was blocked for 1 hour in 5% skimmed milk/ PBS. The membrane was incubated with primary antibodies diluted in 1 % BSA/ PBS (Sigma, Darmstadt, Germany) overnight at 4 °C and then washed three times for 10 minutes each in 0.1 % (v/v) PBS-Tween 20. HRP conjugated secondary antibodies were then added to the membrane for 1 hour and washed as above. Blots were scanned using ChemiDoc™ Gel Imaging Systems (BioRad, Hercules, US). Where reprobing of the membrane was necessary, the membrane was stripped using Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific, Waltham, US). Western blot quantification was performed using FIJI (ImageJ, version 2.0.0-rc-59/1.51k, Wayne Rusband, National Institute of Health, USA).

Chapter 3: Characterisation of microglia of different ApoE genotypes

3.1 Introduction

Alzheimer's disease is the most common cause of dementia. Despite considerable efforts in understanding the disease and developing treatments, there is no cure. Over the past 15 years (between 2000 and 2015) deaths resulting from AD have increased by 123 % (Anon, 2018). The three pathological hallmarks of AD include Aβ plaques, neurofibrillary tangles and neuroinflammation (Ardura-Fabregat *et al.*, 2017). Inflammation is found in the AD brain, particularly near Aβ deposits, which are also rich in activated microglia. The activated microglial cells release a variety of pro-inflammatory mediators including complement components, cytokines, free radicals and nitric oxide. These inflammatory mediators then contribute to neuronal dysfunction and establish a vicious cycle. Minor signs of neuroinflammation can also be found in the normal aging brain. The AD brain however faces a much stronger activation of inflammatory systems indicating that an increasing level and/or qualitatively different immunostimulants are present (Heneka and O'Banion, 2007).

Microglia, the resident macrophage-like population in the CNS, play a critical role in the initiation as well as the progression of neuroinflammation. Under pathological conditions microglia become activated and migrate to locations where damaged or dead cells are present, and clear debris from the area. Activated microglia up-regulate a variety of surface receptors and upon immunostimulation release a variety of pro-inflammatory mediators. Several cytokines are implicated in AD progression and include different interleukins, TNFα and TGFβ. TNFα in particular is strongly associated with AD pathology (Combs *et al.*, 2001).

The three biggest risk factors for LOAD are ageing (Hebert *et al.*, 2010), a family history of AD (Fratiglioni *et al.*, 1993) and the ApoE4 variant of the *APOE*

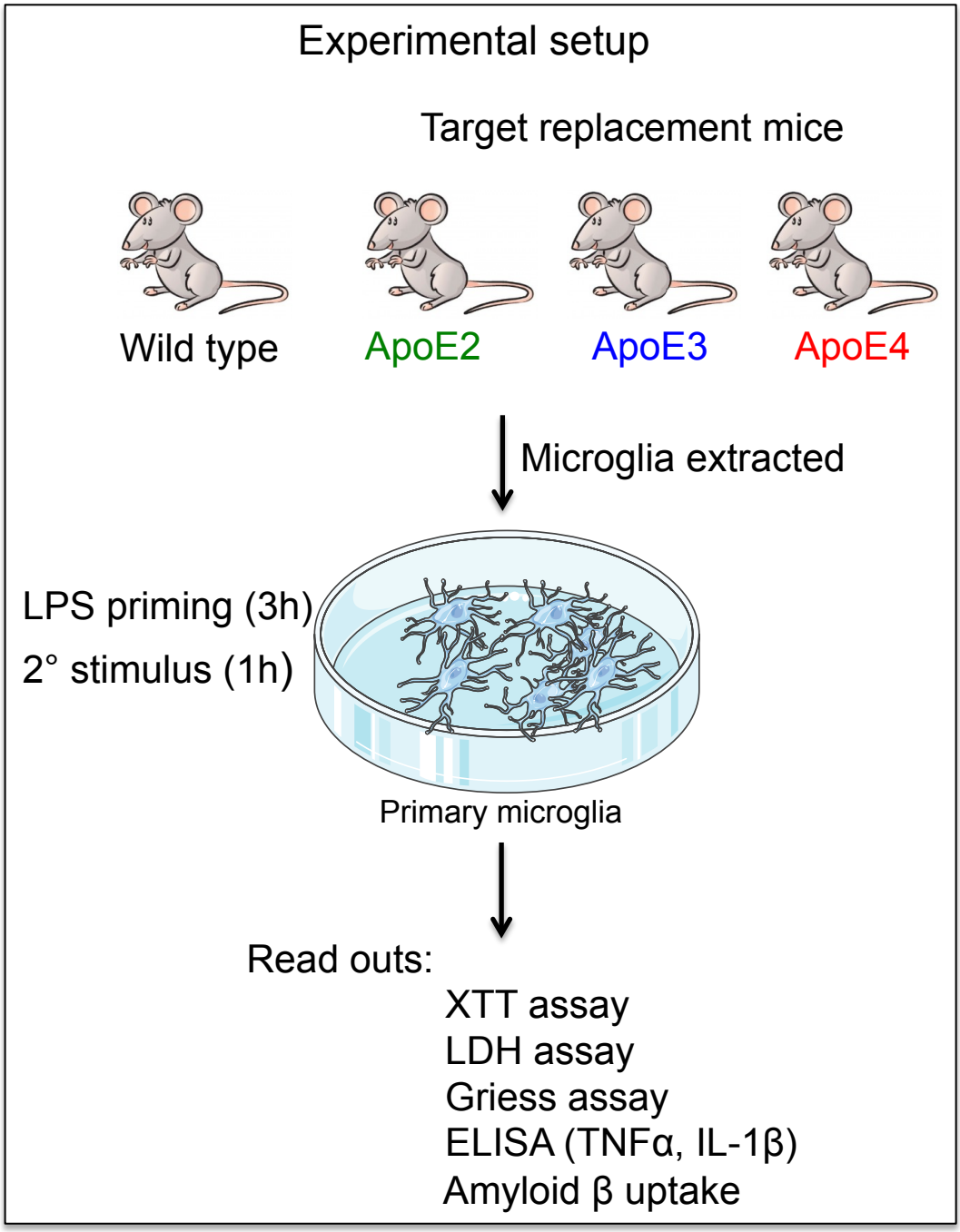
gene (Saunders *et al.*, 1993). At least one copy of the ApoE4 allele was found in 65 % of AD patients in a study which analysed 1770 pathologically confirmed AD cases, highlighting the potential risk factor of ApoE4 allele (Mayeux *et al.*, 1998).

The *APOE* gene is considered the strongest genetic risk factor for LOAD (Holtzman *et al.*, 2012). The three ApoE alleles contribute fundamental differences to the risk of developing the disease. ApoE3 is the most common allele, which doesn't alter the relative risk of developing the disease. ApoE2, whilst rare, confers protection against LOAD. ApoE4 increases the risk of developing AD (Corder *et al.*, 1993). ApoE functions as a ligand in receptor mediated endocytosis of lipoprotein particles and is used to support synaptogenesis (Mauch *et al.*, 2001). Aside from ApoE there are other apolipoproteins in the CNS including ApoJ (CLU), which is another risk factor for LOAD (Lambert *et al.*, 2009), suggesting an important role for apolipoproteins in AD pathology.

Furthermore, there is a positive correlation with ApoE4 and Aβ deposition in the brain and AD onset (Reiman *et al.*, 2009; Morris *et al.*, 2010). ApoE2 carriers on the other hand rarely develop fibrillar Aβ (Morris *et al.*, 2010). Despite efforts to determine the underlying mechanism how ApoE influences Aβ aggregation and accumulation, it remains unclear. Some studies suggest that ApoE isoforms do not influence Aβ production, rather they affect Aβ clearance (Castellano *et al.*, 2011). This has been suggested to occur by pathways involving ApoE mediated enhanced degradation and enzyme facilitated clearance via neprilysin (Jiang *et al.*, 2008; Kim *et al.*, 2009).

The effects of ApoE on microglia function are still largely unknown and, in particular, studies comparing all three ApoE alleles directly are very limited. The aim of this chapter was to compare the three ApoE alleles on microglia function, amyloid uptake and cytokine secretion. The results presented in this chapter show that the ApoE isotype affects different microglia functions. The strongest

A


Experimental setup
Target replacement mice
Wild type
ApoE2
ApoE3
ApoE4
Microglia extracted
LPS priming (3h)
2° stimulus (1h)
Primary microglia
Read outs:
XTT assay
LDH assay
Griess assay
ELISA (TNFα, IL-1β)
Amyloid β uptake

B

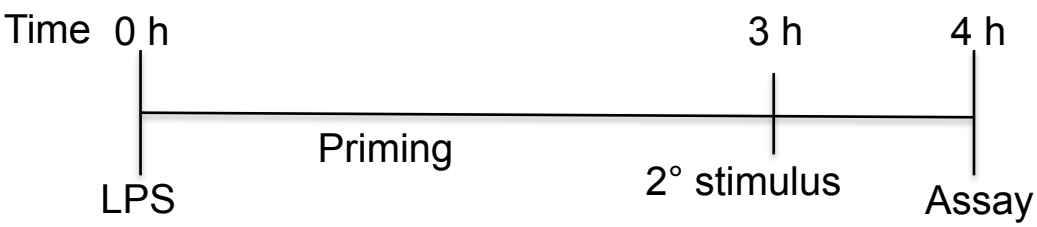

Time
0 h
3 h
4 h
Priming
LPS
2° stimulus
Assay

Figure 3.1 Experimental setup of microglia treatments
Primary microglia were extracted from ApoE target replacement mice and wt animals. Microglia were then primed with LPS (150 ng/ml) for 3 hours, followed by a secondary stimulus for 1 hour (ATP, nigericin or Aβ). (**A**) Assays were then performed on the cells, or with cell culture medium. (**B**) Timeline for stimulation of primary microglia.

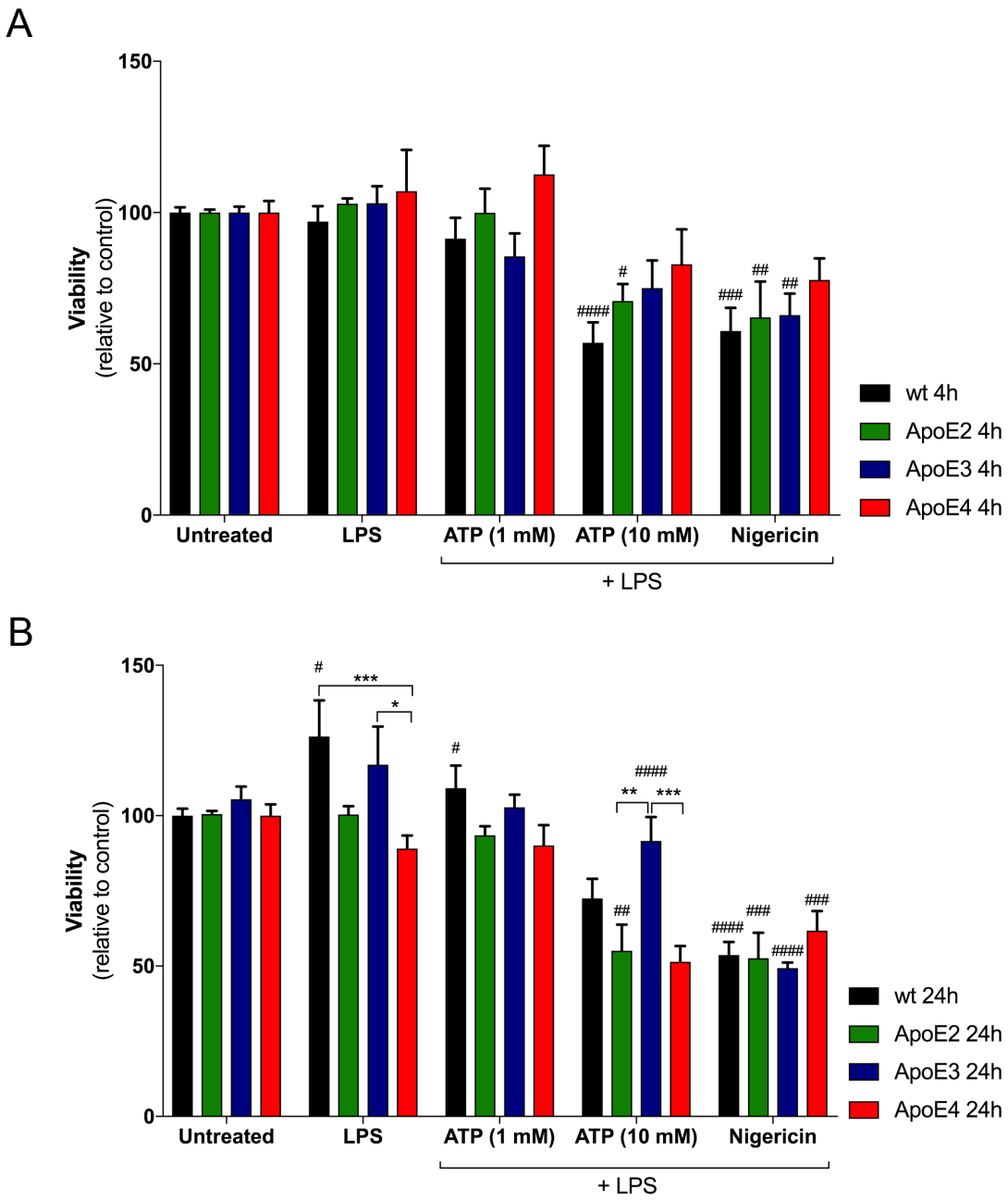


Figure 3.2 Viability of immunostimulated primary microglia
Primary microglia cells were primed with LPS (150 ng/ml) for 3 hours, with or without stimulation by ATP (1 mM, 10 mM) or nigericin (10 µM) for 1 hour. Viability was determined by XTT assay. The viability test was performed either (**A**) immediately after treatment or (**B**) after 24 hours following the treatment. Cell viability is presented as percentage (Untreated control set to 100%). Statistical significance was determined using a 2way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001. # is compared to untreated cells from each genotype. n=7 and is from at least 3 independent experiments.

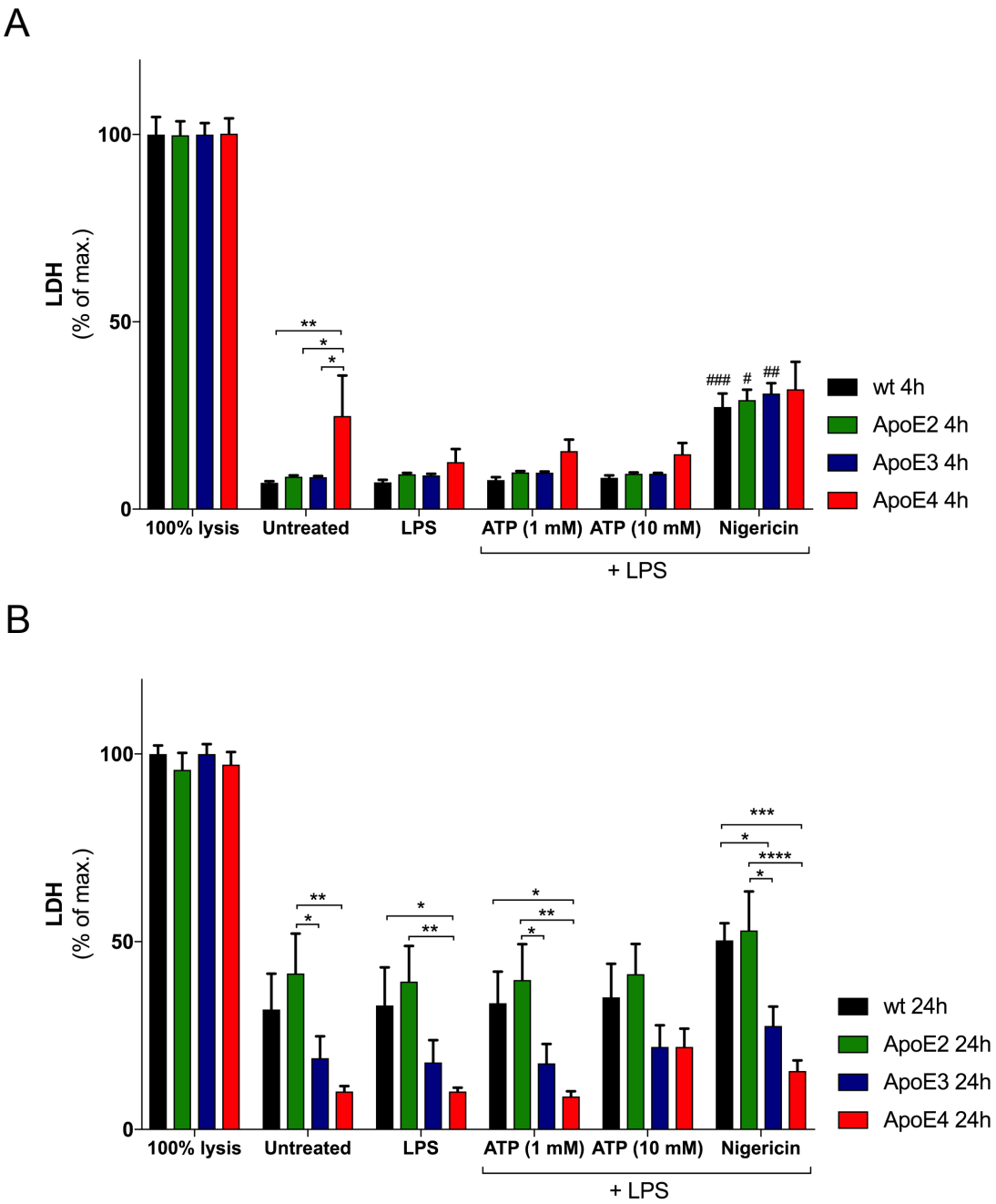


Figure 3.3 LDH release in microglia culture medium after immunostimulation Primary microglia cells were primed with LPS (150 ng/ml) for 3 hours, with or without stimulation by ATP (1 mM, 10 mM) or nigericin (10 µM) for 1 hour. Cell free supernatants were collected (**A**) after treatment or (**B**) 24 hours after treatment. LDH secretion was determined and is presented as percentage of 100 % lysed cells. Statistical significance was determined using a 2way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. # is compared to untreated cells from each genotype. n=6 from at least 3 independent experiments.

genotype dependent effect was observed in Aβ uptake. Aβ uptake is most efficient in ApoE2 followed by ApoE3 with ApoE4 the least efficient. Differences in cytokine secretion were also determined; in particular TNFα secretion is significantly increased in ApoE4 microglia. Using mass spectrometric analyses, a protocol was successfully developed to identify and compare cytokines secreted by immunostimulated and Aβ treated microglia of all three genotypes. The study demonstrates that mass spectrometry can be utilised to define cytokine profiles of different microglia populations.

3.2 Results

Microglial activation *in vitro* is commonly achieved by priming with LPS, followed by a secondary stimulus, such as ATP or nigericin for NLRP3 inflammasome activation (Perregaux *et al.*, 1992). In addition to these stimuli we have also examined Aβ as a potential secondary stimulus. Here wt microglia from C57/BL6 mice and microglia of different ApoE genotypes were analysed after LPS priming treatment alone and also by the classical LPS priming and secondary stimulus with ATP or nigericin. ApoE2, 3 and 4 microglia were generated from target replacement (TR) mice, where the human ApoE allele replaces the mouse allele. This humanised mouse model is commonly used to study the effects of the different ApoE alleles (Bour *et al.*, 2008; Hu *et al.*, 2015). Mouse colonies were established in the facility in Bonn and genotyping was performed by PCR from tail DNA to confirm the ApoE genotypes. C57BL/6 mice carry the murine ApoE form of the gene (Desurmont Caroline *et al.*, 2000). The murine ApoE allele most closely resembles the human ApoE 3 allele (Fernandez *et al.*, 2019).

The same experimental setup was followed for all experiments in this chapter: primary microglia were primed for 3 hours with LPS (150 ng/ml) and treated with ATP (1 mM or 10 mM), nigericin (10 µM) or Aβ (5 µM or 10 µM). Assays were then performed on cell monolayers or conditioned medium (Fig. 3.1).

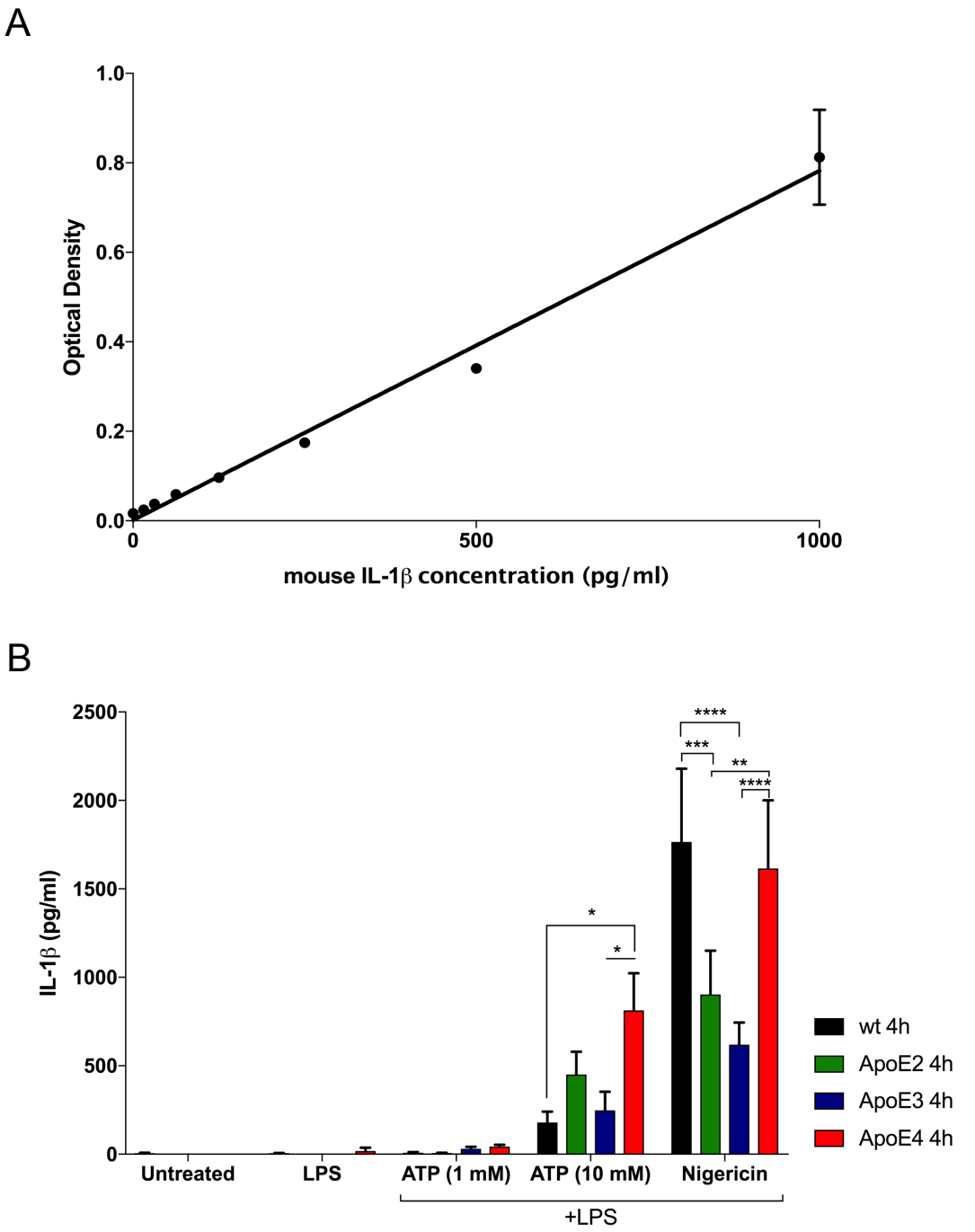


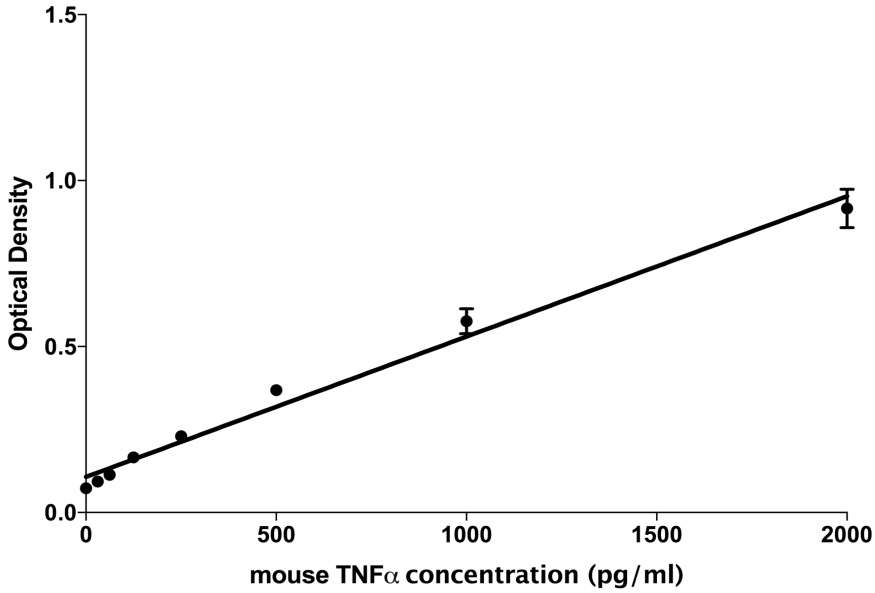
Figure 3.4 IL-1β secretion varies between ApoE genotypes
A representative example of a standard curve is displayed. (**A**) Standards were measured in duplicates in concentrations ranging from 0 to 1000 pg/ml. (**B**) Primary microglia cells were primed with LPS (150 ng/ml) for 3 hours, with or without stimulation by ATP (1 mM, 10 mM) or nigericin (10 µM) for 1 hour. IL-1β secretion was measured in cell free supernatants by ELISA. Statistical significance was determined using a one way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. n=7 from at least 3 independent experiments.

3.2.1 Viability of immunostimulated microglia is genotype independent.

Cell viability was determined by assessing metabolic activity using XTT (Altman, 1976) and by measuring lactate dehydrogenase (LDH) secretion into the culture medium. Both assays were performed either immediately after the treatments (4 hours) or 24 hours following the final treatment. Primary microglia from wt C57/BL6 mice and ApoE2, 3 and 4 mice were primed with LPS (150 ng/ml) for 3 hours, followed by treatment with a secondary stimulus (1, 10 mM ATP or 10 μM nigericin). Following the 4 hours of total treatment, cell viability as assessed by the XTT assay showed no decrease in viability of cells treated with LPS alone or LPS and 1 mM ATP. However, there was a 20 - 40 % decrease in viability after treatment with LPS and either 10 mM ATP or nigericin. Reduced viability was particularly apparent in wt, ApoE 2 and ApoE 3 microglia compared to untreated cells. (Fig. 3.2A). At 24 hours after treatment the viability remained high for LPS primed and LPS and ATP (1 mM) treated samples (Fig. 3.2B). All genotypes showed >90 % viability, which is comparable with untreated control samples. However, following LPS priming combined with either a higher ATP concentration (10 mM) or nigericin treatment there was a reduction in viability of ~30 – 45 % compared with untreated control group with the exception of ApoE3, which maintained a high viability based on this assay (Fig. 3.2B).

LDH secretion following the treatments was also assessed. The cytoplasmic enzyme LDH is released following damage to the cell membrane and thus elevated LDH levels in the cell supernatant indicate reduced viability (Decker and Lohmann-Matthes, 1988). The maximum level of cellular LDH was determined from a supernatant of lysed cells. Very little LDH was detected in medium of microglia cell cultures at 4 hours, with the exception of LPS and nigericin treatment, where there was ~20 % LDH release from wt C57/BL6 and all three genotypes (Fig. 3.3A). These findings are consistent with the observations for the XTT assay (Fig. 3.2A). However, 24 hours following treatment LDH release is increased to ~40 % in wt and ApoE2 microglia,

A



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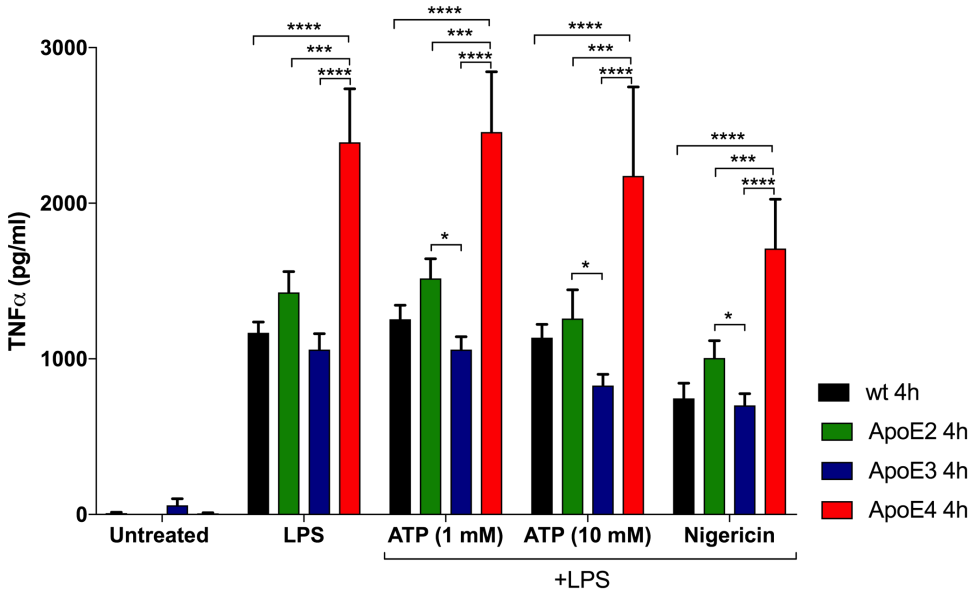


Figure 3.5 Immunostimulated ApoE4 microglia secrete higher levels of TNFα than other ApoE genotypes

(**A**) A representative example of a standard curve is displayed. Standards were measured in duplicates in concentrations ranging from 0 to 2000 pg/ml. (**B**) Primary microglia cells were primed with LPS (150 ng/ml) for 3 hours, with or without stimulation by ATP (1 mM, 10 mM) or nigericin (10 µM) for 1 hour. TNFα secretion was measured in cell free supernatants by ELISA. Statistical significance was determined using a one way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. n=5 from at least 3 independent experiments.

regardless of the treatment and consistently low (<25 %) in ApoE3 and ApoE4 microglia (Fig. 3.3B). Hence, for ApoE3 and ApoE4 differences were observed between the LDH assay and the XTT assay (Fig. 3.2B). LDH release following ATP or nigericin treatment is expected, as the microbial toxin is a known NLRP3 inflammasome inducer (Perregaux *et al.*, 1992). Several independent experiments with microglia from independent preparations were assessed by the LDH release assay and variations were observed between genotypes and the XTT assay. Due to the reduced viability of microglia cell populations at 24 hours, the subsequent experiments focused on the 4 hour time point. In summary, the viability data from both the XTT assay and LDH release shows that the treatments had modest effect on viability of microglia preparation over the short term of 4 hours, but there was substantial loss of viability over 24 hours.

3.2.2 Cytokine secretion differs between immunostimulated microglia from different ApoE genotypes

Immunostimulation with a priming step to activate the NFκB p38 MAPK signalling pathway followed by a secondary stimulus is known to activate the NLRP3 inflammasome, which ultimately leads to IL-1β secretion (Sander *et al.*, 2011). IL-1β secretion is known to be increased in AD. To investigate the effect of immunostimulation on microglia and whether the different ApoE alleles influence the microglia response, cells were primed with LPS (150 ng/ml) for three hours, followed by treatment with ATP or nigericin for 1 hour, as described in Figure 3.1. (Perregaux *et al.*, 1992). Priming alone with LPS did not results in IL-1β secretion (Fig. 3.4), as expected (Perregaux *et al.*, 1992; Sander *et al.*, 2011). Whilst microglia from all genetic backgrounds secrete IL-1β in response to immunostimulation with 10 mM or nigericin, there are genotype specific differences. ApoE4 microglia secrete more than three times more than ApoE3 microglia, in response to ATP (10 mM) stimulation. After nigericin treatment, IL-1β secretion is higher in all microglia populations than after 10 mM ATP stimulation. In wt C57/BL6 microglia, IL-1β secretion is 1500 pg/ml higher after

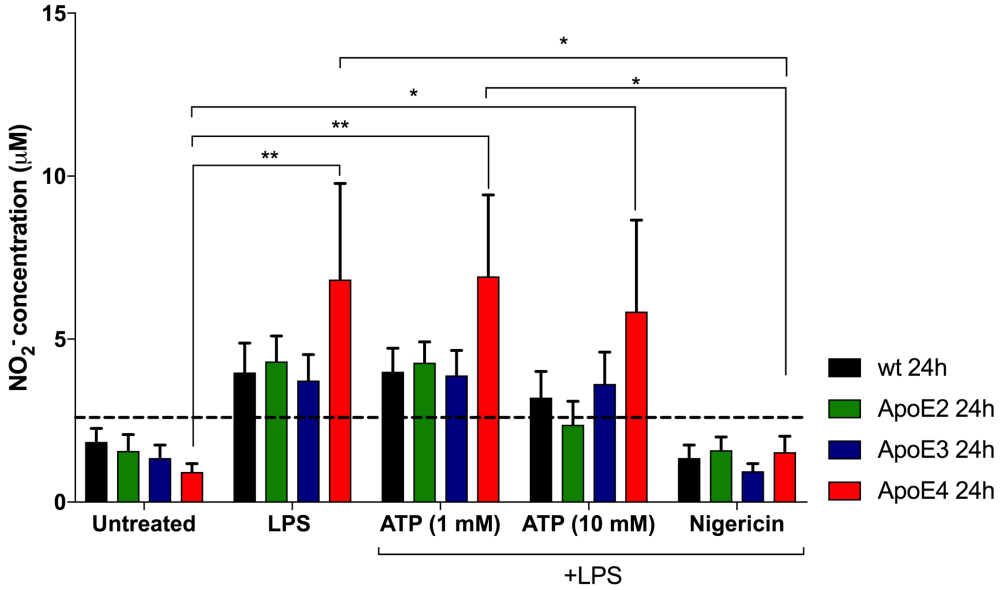


Figure 3.6 Nitrite secretion in primary microglia
Primary microglia cells were primed with LPS (150 ng/ml) for 3 hours, followed by immunostimulation with ATP (1 mM or 10 mM) or nigericin (10 μM). Nitrite concentration was determined by Griess assay in cell free supernatants, which were collected 24 hours after treatment. Dashed line represents the detection limit of the assay. Statistical significance was determined using a 2way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. n=8 from at least 3 independent experiments.

nigericin treatment than after 10 mM ATP treatment (Fig. 3.4B). The high levels of IL-1β secretion following nigericin treatment are consistent with the reduced viability of nigericin-treated populations at the 4 hour time point, as NLRP3 inflammasome activation leads to IL-1β production and pyroptotic cell death.

Like IL-1β, the pro-inflammatory cytokine TNFα is implicated in many inflammatory diseases, such as AD (Franchi *et al.*, 2009). To further assess the different ApoE alleles on microglial response to immunostimulation, TNFα secretion was determined in conditioned media of microglia cell cultures by ELISA, following the same setup as outlined above and illustrated in Figure 3.1. High levels of TNFα were detected in the conditioned medium at 4 hours following LPS treatment in all samples. Of note is that ApoE4 microglia secrete almost twice as much TNFα as the other genotypes. TNFα secretion was also observed in response to LPS and the secondary stimulus; however, the profiles of each ApoE microglia population were very similar to LPS alone. ApoE 2 microglia secrete more TNFα in response to ATP and nigericin, under primed conditions, than ApoE 3 microglia. TNFα follows a genotype dependent pattern, with ApoE 4 microglia secreting consistently more TNFα than the other genotypes, regardless of the stimulus (Fig. 3.5B).

In summary, the data described here suggests a genotype dependent effect on immunostimulated microglia, whereby ApoE 4 microglia have the strongest pro-inflammatory response and secrete significantly more IL-1β and TNFα than the other ApoE genotypes.

3.2.3 Nitrite secretion is genotype independent after immunostimulation

Inflammation in brain tissue is accompanied by increased NO synthesis in microglial cells via induction of iNOS. Nitric oxide has been implicated as a chemoattractant for activated microglia. Microglia will migrate toward the site of injury upon sensing secreted NO (Gutièrrez-Mecinas *et al.*, 2007). Furthermore NO is known to be involved in NMDAR mediated neurotoxicity (Yamada *et al.*,

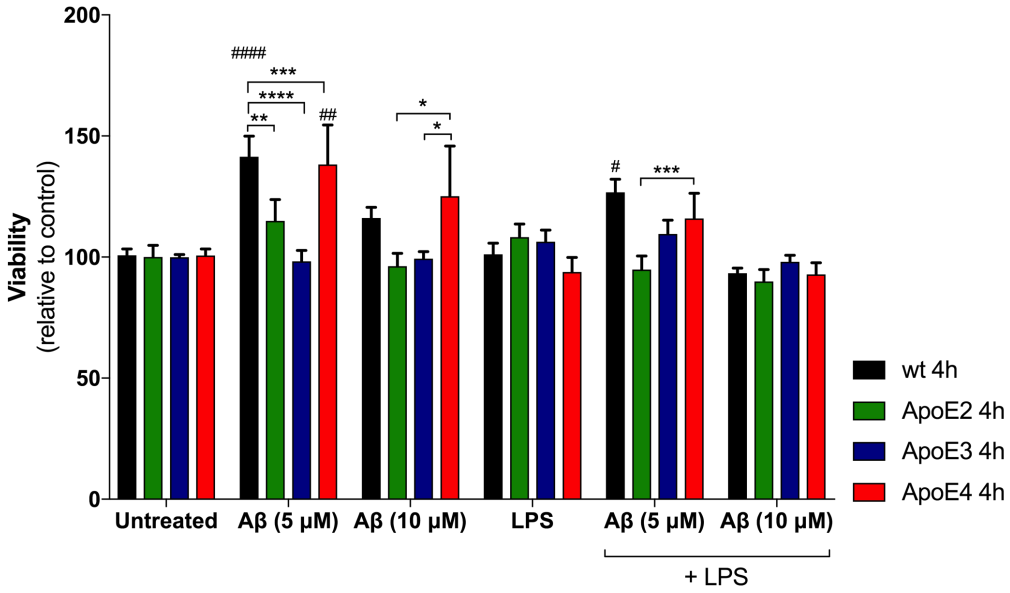


Figure 3.7 Viability of primary microglia after Aβ treatment
Primary microglia cells were treated with different concentrations of Aβ (5 μM, 10 μM) for 1 hour either directly or after 3 hours LPS pre-stimulation. Viability was determined by XTT assay. The viability test was performed immediately after treatment. Cell viability is presented as percentage (Untreated control set to 100%). Statistical significance was determined using a 2 way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001. # is compared to untreated cells from each genotype. n=6 from at least 3 independent experiments.

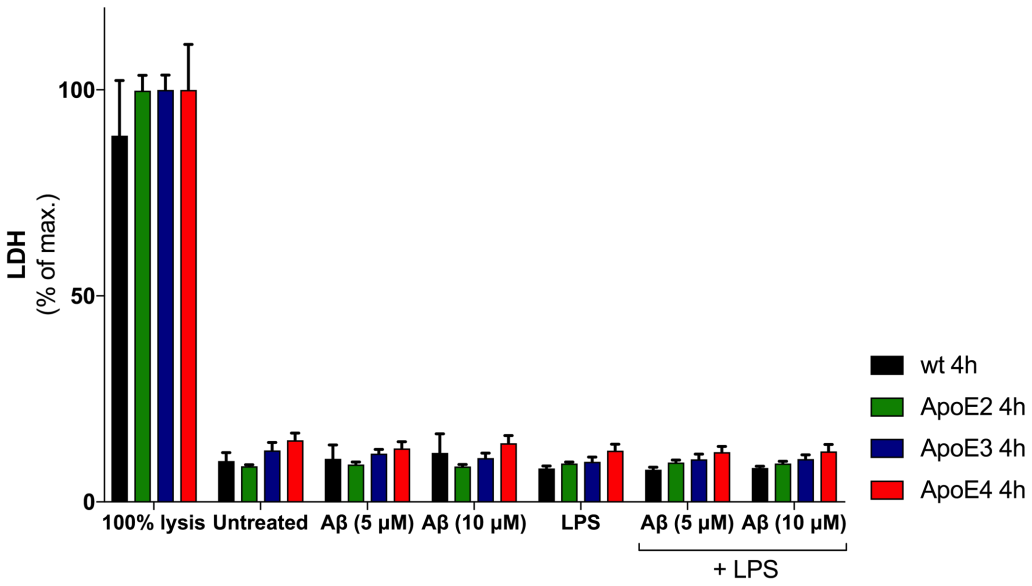


Figure 3.8 LDH release in Aβ stimulated primary microglia
Primary microglia cells were treated with different concentrations of Aβ (5 μM, 10 μM) for 1 hour, either directly or after 3 hours LPS pre-stimulation. Cell free supernatants were collected after treatment and LDH secretion determined by LDH cytotoxicity assay. LDH secretion is presented as percentage (Technical control = 100 %). Statistical significance was determined using a 2way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. n=5 from at least 3 independent experiments.

1997). Here I assessed whether the ApoE genotype has an effect on microglial NO secretion. Using the Griess assay for nitrite production, endogenous NO formation was measured in the conditioned media of immunostimulated microglia. Nitrite secretion is not as rapid as other cellular processes and therefore it was necessary to measure nitrite secretion 24 hours after treatment. Unsurprisingly, the levels of nitrite detected at the 4 hour time point were below the detection limit of the assay (Figure A1 see appendix). Nitrite concentrations detected at 24 hours were very low, ranging from 4 to 7 μM (Fig. 3.6). LPS primed ApoE 4 microglia secrete more NO than untreated cells. The addition of a secondary stimulus however did not result in a further increase in NO. Indeed, it has been reported that treatment with the NLRP3 activator nigericin (Perregaux *et al.*, 1992) as a secondary stimulus leads to a decrease in nitrite secretion, likely due to the increased cytotoxicity.

3.2.4 Aβ treatment has no effect on microglia viability

To examine the effect of Aβ on microglia responses, I initially assessed the viability of microglia following treatment with Aβ (5 μM or 10 μM) for 1 hour with or without prior LPS priming for 3 hours (Fig. 3.7). Microglia viability was assessed by XTT, measuring the cell's metabolic activity and by determining LDH secretion into the culture medium after 4 hours. The XTT assay revealed >90 % viability across all genotypes and treatments (Fig. 3.7). This was confirmed by LDH; microglia from all genotypes showed ~10 % LDH in the conditioned medium, regardless of the treatment, which is comparable to LDH levels of untreated controls (Fig. 3.8). Therefore, it can be concluded that Aβ, under these experimental conditions, does not have an effect on viability of microglia from the three ApoE isoforms and C57/BL6 wt microglia.

3.2.5 Aβ uptake is lower in ApoE4 microglia compared with ApoE3 and ApoE4

It has been proposed that the increased risk of ApoE4 is partially due to reduced Aβ clearance associated with the ApoE4 genotype (Deane *et al.*,

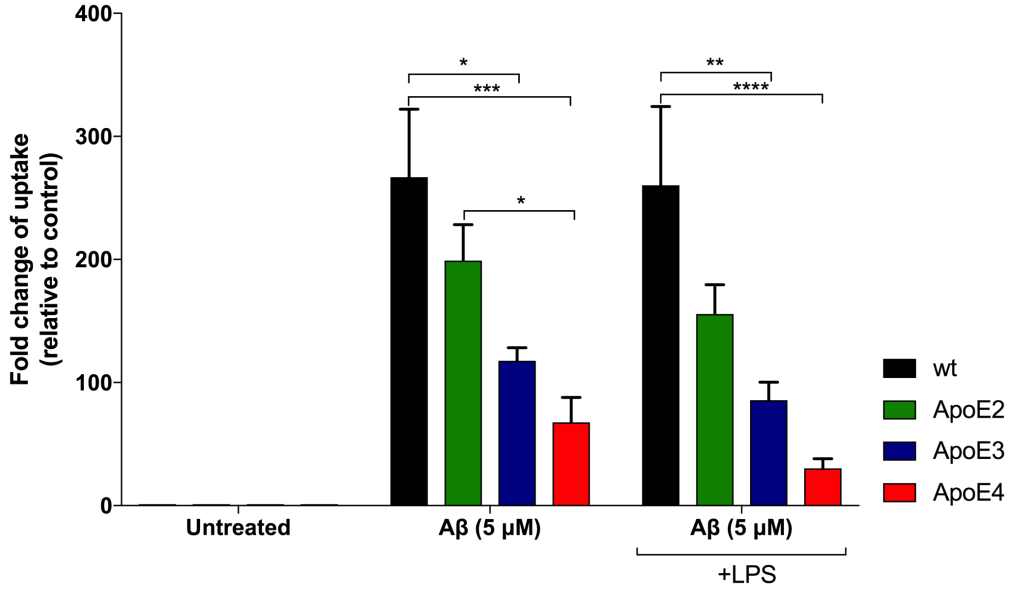


Figure 3.9 Amyloid beta uptake is genotype dependent
Primary microglia monolayers were treated with Aβ (5 μM) for 1 hour, either directly or after 3 hours LPS priming. Fluorescence emission of internalised Aβ was detected using a microplate reader. Statistical significance was determined using a 2way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. n=5 from at least 3 independent experiments.

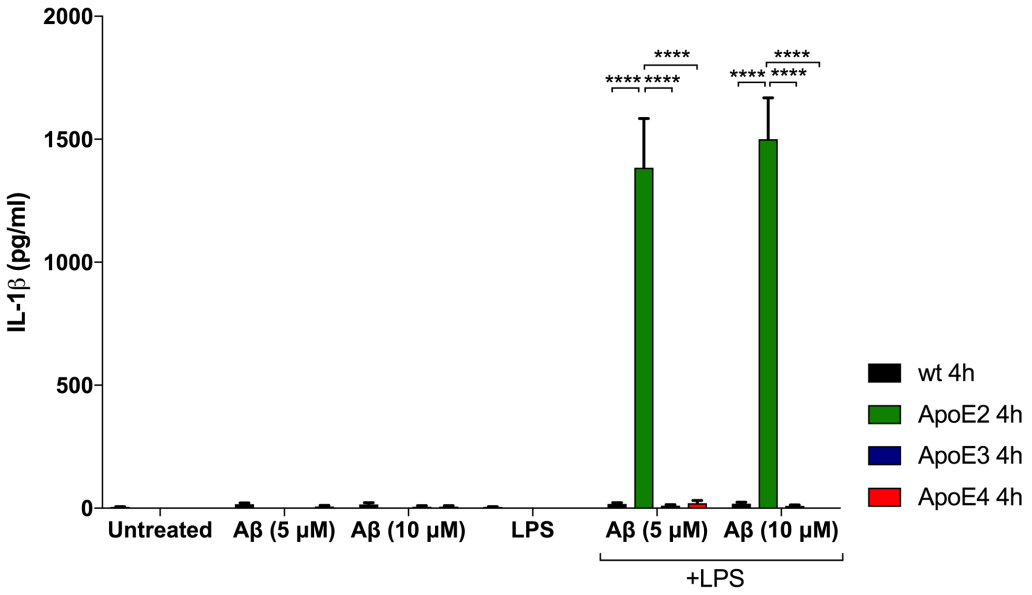


Figure 3.10 IL-1β secretion by microglia treated with amyloid beta
Primary microglia cells were treated with different concentrations of Aβ (5 μM, 10 μM) for 1 hour, either directly or after 3 hours LPS pre-stimulation. IL-1β secretion was measured in cell free supernatants by ELISA. Supernatants were collected immediately after treatment. Statistical significance was determined using a 2way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. n=3 from 3 independent experiments.

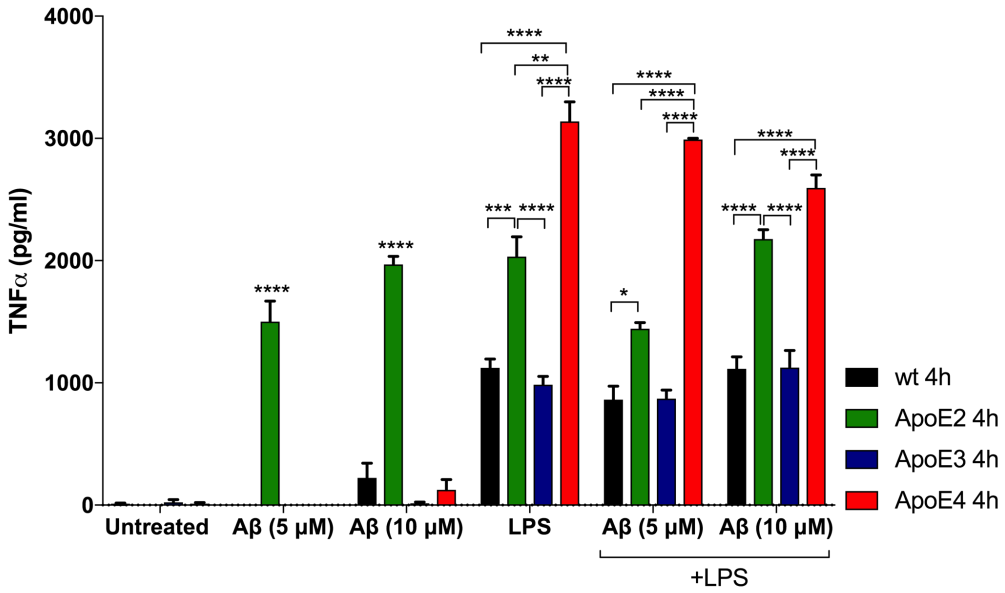


Figure 3.11 Microglial TNFα secretion is ApoE genotype dependent
Primary microglia cells were treated with different concentrations of Aβ (5 μM, 10 μM) for 1 hour, either directly or after 3 hours LPS pre-stimulation. TNFα secretion was measured in cell free supernatants by ELISA. Supernatants were collected immediately after treatment (A). Statistical significance was determined using a 2way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. n=3 from 3 independent experiments. Outliers were removed based on Grubb's test for outliers.

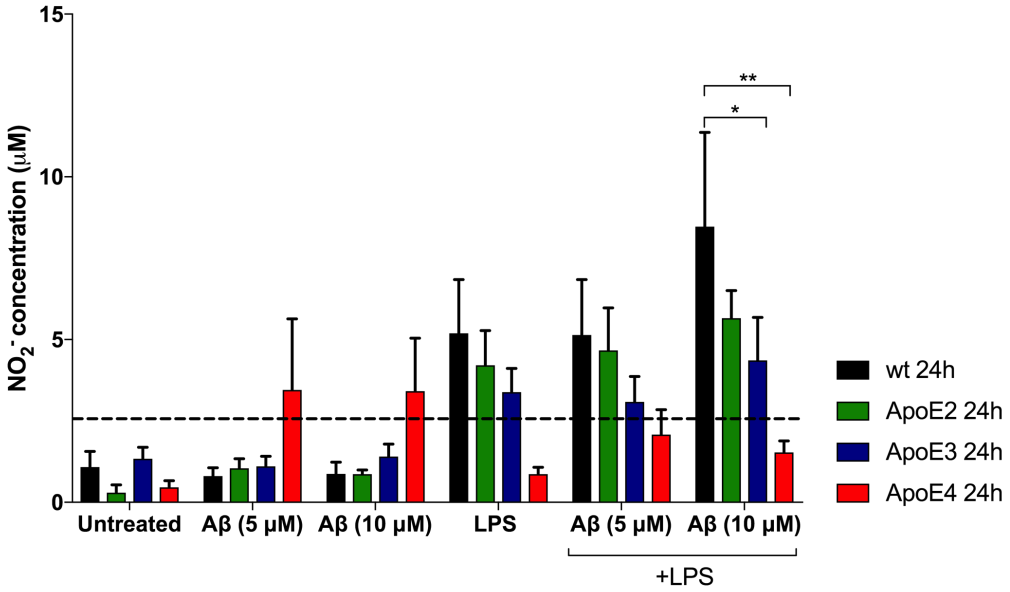


Figure 3.12 Nitrite secretion in primary microglia
Primary microglia cells were treated with amyloid β, with and without LPS pre-stimulation. Nitrite concentration was determined by Griess assay in cell free supernatants which were collected 24 hours after treatment. Dashed line represents the detection limit of the assay. Statistical significance was determined using a 2way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. n=4 from at least 3 independent experiments.

Target list	Identified
TNFα	✓
IL-1β	✓
IFNγ	
CCL2	✓
CD14	✓
CD36	
CD68	
IL-10	
IL-11	
TGFβ	✓
IL-6	✓

Table 3.1 Original target list for MS
A target list was created for MS as shown. Targets, from that original list that were identified by MS are indicated.

2008). Here, Aβ internalisation by microglia of the three ApoE genotypes, as well as microglia wild type animals, was determined by incubating primary microglia in the presence of fluorescently labelled $A\beta_{1-42}$. The uptake of amyloid beta was assessed with and without LPS stimulation prior to the assay. The data, presented in Fig. 3.9 indicates uptake by microglia of all genotypes. Non-treated microglia (i.e. no priming) showed abundant Aβ uptake by wt microglia and ApoE2, E3, E4 microglia. Interestingly there was no difference in uptake between primed and un-primed microglia. ApoE4 microglia were the least efficient at Aβ uptake, and ApoE2 microglia together with wt C57/BL6 microglia, the most efficient. ApoE 2 microglia showed a 3-4 fold higher uptake of Aβ compared with ApoE 4 microglia, and almost twice as much as ApoE 3 microglia (Fig. 3.9). Overall, this data shows that Aβ uptake by microglia is genotype dependent with the corresponding efficiency of uptake as follows, ApoE 2 > ApoE 3 > ApoE 4.

3.2.7 Pro-inflammatory cytokine secretion is ApoE isoform dependent

IL-1β secretion was also determined in conditioned medium of Aβ-treated microglia. Here, microglia were treated with Aβ (5 or 10 μM) for one hour, with or without prior LPS priming. IL-1β secretion was observed in primed and Aβ treated cells ApoE2 microglia. ApoE2 microglia respond to the treatment with large quantities of IL-1β secretion (~1500 pg/ml), whilst very little IL-1β was detected in the microglia culture media from the other genotypes (<20 pg/ml). No IL-1β secretion was detected from microglia treated with either Aβ or LPS alone (Fig. 3.10). Taken together, this suggests that Aβ treatment under primed conditions leads to IL-1β secretion in a genotype dependent manner, and that ApoE 2 microglia respond the strongest to this treatment.

TNFα secretion by Aβ treated microglia was also determined. As for IL-1β secretion, ApoE2 microglia responded strongly to Aβ. Even without priming, Aβ induced TNFα secretion of ~1600 pg/ml. Upon LPS priming, all ApoE genotypes secreted TNFα, with ApoE4 microglia secreting the highest levels, with ~1.5

Table 3.2 Summary of identified cytokines by Mass Spectometry
Ctrl – untreated control samples, Imm. – immunostimulated samples (150 ng/ml LPS + 10 mM ATP), L+Aβ – (150 ng/ml LPS + 10 μM Aβ)

Cytokine	Wt Ctrl	Wt Imm.	Wt L+Aβ	A2 Ctrl	A2 Imm.	A2 L+Aβ	A3 Ctrl	A3 Imm.	A3 L+Aβ	A4 Ctrl	A4 Imm.	A4 L+Aβ
TNFα		✓	✓		✓	✓		✓	✓		✓	✓
IL-1β						✓			✓			✓
IL-12			✓		✓				✓			✓
CCL-2	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓
CD14	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
IL-12β									✓			✓
IL-1α						✓			✓			
ITGB2	✓						✓	✓				✓
IL-6		✓	✓		✓	✓			✓			✓
TGFβ									✓			
C3	✓	✓	✓		✓	✓		✓	✓		✓	✓
IL1RA	✓	✓	✓			✓			✓			✓

Table 3.3 TNFα peptides identified by MS
Primary microglia from wt, ApoE 2, 3 and 4 mice were primed with LPS (150 ng/ml) and treated with Aβ (10 μM). Supernatants were collected and MS performed after acetone precipitation. Shown is the number of significant peptides and the protein score for TNFα.

TNFα	Significant peptides (total)				Protein score			
	Experiment							
	1	2	3	4	1	2	3	4
wt	4	7	6	6	132.72	295.45	241.67	204.04
ApoE2	5	5	1	4	225.81	218.73	46.25	161.75
ApoE 3	8	8	9	3	296.27	292.44	329.84	152.20
ApoE 4	5	5	7	6	195.04	178.52	196.27	265.64

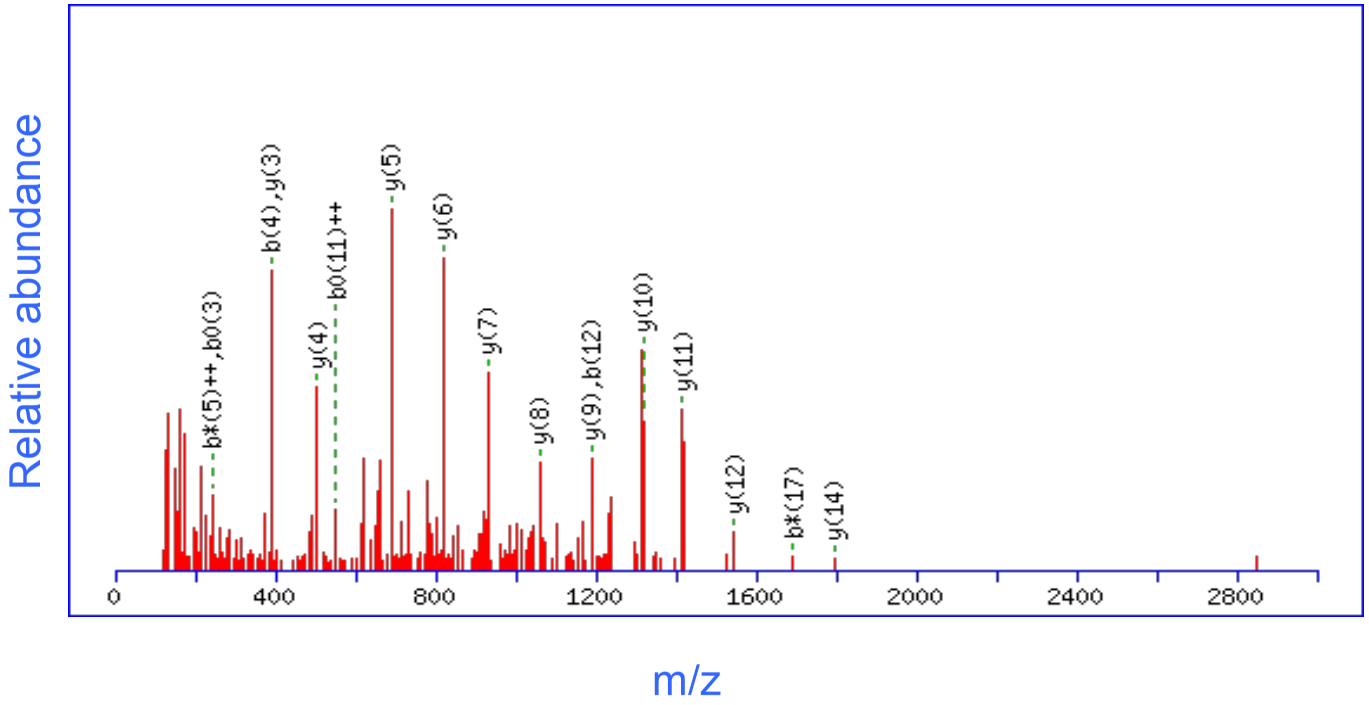


Figure 3.13 Mass Spectrum of TNFα
A representative MS spectrum from LPS and Aβ stimulated ApoE4 microglia derived cell culture medium is shown.

times higher TNFα compared with ApoE2 microglia and ~3 times higher TNFα compared with wt and ApoE3 microglia. A similar trend was observed when microglia were primed and treated with Aβ (Fig. 3.11, see also Fig. A2). In summary, TNFα secretion is genotype dependent with the corresponding levels of TFNα secretion as follows; ApoE4 > ApoE2 > ApoE3.

3.2.8 Nitric oxide production is slightly increased upon Aβ treatment in primed microglia

NO production was also assessed in Aβ stimulated microglia. Cells were treated with Aβ (5 or 10 μM) for 1 hour, either directly or following LPS priming for 3 hours. Cell culture media were replaced with fresh medium after the treatment and conditioned medium collected 24 hours following the treatment. After LPS priming, small amounts (~ 3 to 5 μM) of nitrite were detected in conditional medium from wt and E2 and 3 microglia, but not ApoE4 microglia. Nitrite secretion of microglia treated with LPS and Aβ (5 μM) was higher than cells only primed with LPS. Upon the addition of 10 μM Aβ, nitrite secretion is slightly increased (~ 5-10 μM) in wt, ApoE2 and 3 microglia, but remains below the detection limit of the assay for culture medium derived from ApoE4 microglia (Fig. 3.12). These results suggest, that nitrite secretion is below the limits of detection for ApoE4 microglia, and that Aβ treatment does not induce a strong nitrite secretion in any of the genotypes.

3.2.9 Mass spectrometry analysis of microglia supernatants

The production of pro-inflammatory cytokines by activated microglial cells is known to lead to neuroinflammation and to contribute to AD. IL-1β and TNFα have been implicated in the disease, together with various other pro-inflammatory cytokines (Akiyama *et al.*, 2000; Smith *et al.*, 2012). The balance of pro- and anti-inflammatory cytokines secreted by microglia may influence the development and progression of AD. In order to ascertain pro- and anti-inflammatory cytokines secreted by microglia after immunostimulation and Aβ

treatment, the conditioned medium from microglia cultures was analysed by mass spectrometry (MS). Microglia from wt C57/BL6 and the three ApoE isotypes were either left untreated, immunostimulated with LPS and ATP, or primed with LPS and treated with Aβ. Culture media was collected after treatment and proteins were acetone precipitated for MS analysis. A list of potential protein targets was generated based on a literature search of cytokines known to be secreted by microglia and with links to AD pathology (Table 3.1). The target list included the pro-inflammatory cytokines and chemokines; TNFα, IL-1β, IFNγ, CCL2, CD14, CD36, CD68 and the anti-inflammatory cytokines IL-10, IL-11, TGFβ and IL-6. These targets were selected based on their documented involvement in the regulation of inflammation by microglia. The MS analysis targeted the mass of the peptides predicted from a trypsin digestion of these targets.

A number proteins were identified using this targeted MS approach including TNFα, IL-1b, CCL2 and IL-6. The other members of the target list, IFNγ, CD36 and IL-10 were not detected (Table 3.1). However, a variety of proteins not included in the original target list were also identified, due to overlapping masses of the tryptic peptide fragments. These additional proteins are summarised in Table 3.2. The majority of cytokines were identified across all genotypes. The myeloid differentiation antigen CD14 (Haziot *et al.*, 1988) was found in all samples, and also the protein kinase CCL2 (Roca *et al.*, 2009). The pro-inflammatory cytokine TNFα was found across all genotypes in both immunostimulated and Aβ treated samples but not the untreated control. IL-12 was consistently identified in conditioned media from LPS primed and Aβ treated microglia, with the exception of ApoE2 derived samples. IL-12β was only found in samples from ApoE3 and ApoE4 microglia that had been primed and treated with Aβ. IL-6 was identified in samples from all genotypes. In wt C57/BL6 and ApoE2 samples IL-6 was detected after immunostimulation and Aβ treatment. In ApoE3 and ApoE4 derived samples, IL-6 was only detected in samples from Aβ treated microglia (Table 3.2). The majority of cytokines identified were treatment dependent. In addition, ApoE3 and ApoE4 microglia

display the largest variety of identified cytokines (Table 3.2). Overall, these analyses demonstrate that MS can be utilised for identifying cytokine profiles from conditioned media of small microglia cultures. Furthermore, the analysis has revealed a number of cytokines that can be further investigated.

3.3 Discussion

ApoE is the strongest risk factor for LOAD (Strittmatter *et al.*, 1993) but how the individual isoforms modify susceptibility to development of AD is still unclear. The role of ApoE in acute and chronic neurological pathologies can be linked to a glial cell response to brain injury (Laskowitz *et al.*, 2001). However, the mechanisms behind this are also unclear. To understand the effect of ApoE genotype on microglia function, I performed a number of studies assessing microglial responses after immunostimulation and after Aβ treatment. A particular focus was on the inflammatory response of the stimulated cells, and how the ApoE genotype affects this response. From the studies presented in this chapter, it can be summarised that ApoE4 induces the strongest pro-inflammatory phenotype in response to immunostimulation, in comparison to wt C57/BL6, ApoE2 and ApoE3 microglia. Aβ treatment also induces a strong pro-inflammatory phenotype, which is strongest in ApoE4 but also in ApoE2 microglia. Furthermore, it was confirmed that Aβ uptake is ApoE genotype dependent.

Pro-inflammatory cytokine secretion was determined by ELISA. IL-1β is of relevance to AD as it has been proposed to regulate Aβ production and promote expression and phosphorylation of tau proteins (Sheng *et al.*, 2000; Griffin *et al.*, 2006). In this study, IL-1β secretion was found to be ApoE isoform specific, with ApoE4 microglia secreting significantly more (>50 %) IL- 1β, in the presence of ATP (10 mM) and nigericin under primed conditions, than the other ApoE genotypes (Fig. 3.4B). The increased IL-1β secretion suggests NLRP3 inflammasome activation, which is characterised by IL-1β secretion and

pyroptotic cell death (He *et al.*, 2016). The viability data (Fig. 3.2A), however does not indicate increased cell death in immunostimulated ApoE4 microglia, neither was an increase in LDH secretion detected (Fig. 3.3A). Conos and colleagues however propose that caspase-1 mediated IL-1β secretion can be cell death independent (Conos *et al.*, 2016). Detecting levels of NLRP3 and caspase-1, for example by western blot could confirm NLRP3 inflammasome activation.

In addition to IL-1β, secretion of TNFα was also determined. It was shown that dimerization of the pro-inflammatory cytokine TNFα can cause IL-1β cleavage and its secretion (Conos *et al.*, 2016). TNFα has also been linked to neuroinflammation and AD progression in multiple studies and microglia are known to release this pro-inflammatory cytokine upon activation. It has been reported that biologically relevant concentrations of ApoE2 suppress the secretion of TNFα in enriched primary microglia cultures and BV-2 cells, a microglial cell line (Laskowitz *et al.*, 2001). Microglia were generated from neonatal mice, as this is a standard protocol for high purity microglial cultures (Lian *et al.*, 2016). Here primary microglia cells from newborn mice expressing the human AopE alleles were used. The data shown here further underlines an ApoE genotype dependent effect on TNFα secretion, as ApoE4 microglia secreted significantly higher levels (~50 %) of TNFα upon activation (LPS, LPS + ATP, LPS + nigericin) (Fig. 3.5B). This is consistent across the different immunostimulatory protocols.

However ApoE2 microglia were found to secrete higher levels of TNFα than ApoE3 microglia. Published data on the effect of ApoE on macrophages are in line with the findings described here. Using J774A.1 cells (a mouse macrophage cell line), Tsoi *et al* have demonstrated that both ApoE4 and ApoE2 cells produce higher amounts of TNFα and attribute these effects to modulation of the ERK 1/2 MAPK signalling pathway. They further suggest that ApoE isoforms differently modulate the activation of parallel signalling cascades triggered by LPS (Tsoi *et al.*, 2007). Importantly, this study extends these

previous findings from cell lines of macrophages, to primary microglial cells. The findings presented here directly demonstrate that different ApoE isoforms influence levels of TNFα secreted by stimulate primary microglia.

The study in this Chapter confirms that ApoE4 microglia are the least efficient at Aβ clearance (Fig. 3.9). A significant decrease in Aβ uptake is observed between ApoE2 and ApoE4 microglia that were left unprimed. Together with the elevated TNFα secretion this is in line with the findings by Hickman and colleagues suggesting an inverse correlation between cytokine production and Aβ clearance in PS1-APP mouse microglia, an established mouse AD model expressing both the APP bearing Swedish mutation and PSEN1 mutation (Radde *et al.*, 2006; Hickman *et al.*, 2008). Further experiments should aim to analyse the degradation of internalised Aβ and whether the ApoE isotype influences the level of degradation. One possible limitation to the interpretation of the study is that a fluorescent conjugate of Aβ was used for the assay, and it is possible that the conjugation has an effect on the properties of Aβ and uptake mechanism.

Not only was the uptake of Aβ investigated here, but also the microglial response to Aβ treatment. Whilst TNFα secretion was not further increased by the addition of Aβ to primed cells, ApoE2 microglia produced elevated TNFα secretion in response to Aβ treatment alone. This was not observed in the other genotypes. IL-1β secretion was also found to be significantly higher in ApoE2 microglia, compared with the other genotypes, after LPS priming and Aβ treatment (Fig. 3.10). After immunostimulatory treatment, the pro-inflammatory response was much lower in ApoE2 microglia compared to the other genotypes, suggesting that ApoE has an effect on the response to different stimuli. This was further supported by the MS findings, which revealed a more diverse range of secreted cytokines were detected by ApoE4 cells compared with ApoE2 microglia. Increased pro-inflammatory response to Aβ by ApoE2 microglia could lead to the recruitment of more microglia to the site of Aβ

accumulation to clear the Aβ load. This would be consistent with the increased Aβ clearance described above.

The MS analysis detected both pro- and anti-inflammatory cytokines. TNFα and IL-1β, which had previously been identified by ELISA, were also detected by MS. Additionally a variety of pro- and anti-inflammatory were detected, such as TGFβ and IL-6, demonstrating that sufficient material can be collected from spent cultures for detailed proteomics. Furthermore, the balance between pro- and anti-inflammatory cytokines should be further investigated, both in response to immunostimulation and Aβ treatment. An imbalance between pro- and anti-inflammatory cytokines is most likely the cause of persistent neuroinflammation in ApoE4 carriers.

Expanding the MS analysis and performing a quantitative comparison between the different genotypes would be well worthwhile in the future. Quantitative MS can be performed by isotopic labelling techniques such as isotype-coded protein labelling (ICPL) (Schmidt *et al.*, 2005). For this approach the lysine side chains and N-termini are labelled with ICPL reagents. (Chahrour *et al.*, 2015). ICPL is suitable for different protein samples including body fluids, and would be compatible with the experimental setup chosen here (Rainczuk *et al.*, 2013).

Another approach would be to investigate the expression of genes encoding pro- and anti-inflammatory cytokines by RNA seq analysis. A previous study has identified four genetic profiles at risk for AD by combining the ApoE genotypes and genes encoding pro-inflammatory cytokines (Licastro *et al.*, 2007). This interesting approach allows for the identification of gene variants modulating innate immunity and ApoE polymorphism and creating a risk prediction for individuals. It further highlights that AD has multiple genetic factors in disease development.

In summary, here it was shown that ApoE4 microglia have the strongest pro-inflammatory response to immunostimulation and that ApoE2 microglia respond

the strongest to Aβ treatment, whilst also being the most efficient at clearing Aβ. These findings are compatible with the role of E4, but not E2, as a risk factor in AD. Both immunostimulation and Aβ treatment do not cause ApoE genotype specific effects on microglia viability. Furthermore this study demonstrates that MS can be utilised for cytokine detection of cell culture media and could provide a valuable means to creating large cytokine profiles. However all experiments have been performed *in vitro* and on mouse cells rather than cells of human origin.

Chapter 4: Effects of primary microglia conditioned media on primary cortical neurons

4.1 Introduction

Alzheimer's disease is believed to have its onset 2-3 decades before the first symptoms of memory loss appear (Selkoe and Hardy, 2016). Hence further understanding of the disease progression is crucial. Neuronal degradation and loss represents one of the key pathological hallmarks of AD and includes early alterations such as synaptic and dendritic injury (DeKosky *et al.*, 1996; Serrano-Pozo *et al.*, 2011). Several studies have shown that Aβ can impair synaptic structure and function. However aside from Aβ, neuroinflammation also contributes to neuronal impairment. Immunohistochemical studies support the early suspicion by Oskar Fischer, that plaque formation could be the result of an inflammatory process in the brain (Fischer, 1907). Neuroinflammation involves sustained activation of glial cells (Eikelenboom *et al.*, 2010) and is strongly mediated by microglia cells responding to stimuli in their microenvironment by the release of pro-inflammatory cytokines. These cytokines then in turn can have a detrimental effect on neurons. The secretion of pro-inflammatory cytokines such as TNFα and IL-1β mediates neuroinflammation and neurotoxicity and causes sustained low grade inflammation (Heneka, Golenbock, *et al.*, 2015). TNFα in particular accounts for most of the neurotoxic activity secreted by microglia (Combs *et al.*, 2001). However TNFα has also been reported to have neuroprotective properties (Akiyama *et al.*, 2000). These controversial findings need further investigation and are addressed in this Chapter.

Microglia play an important part in the maintenance and plasticity of neuronal circuits and thus contribute to synapse remodelling (Ji *et al.*, 2013). The interaction between microglia and neurons is considered critical for healthy brain function and often impaired during disease (Eyo and Wu, 2013; Tao *et al.*, 2018). This relationship works in both ways, microglia having an effect on neurons and *vice versa*. Neuronal ageing, for example, can drive microglia

priming (Heneka, Carson, *et al.*, 2015). This relationship under pathological conditions can result in a vicious cycle. Neurons can prime and activate microglia, which leads to increased secretion of pro-inflammatory and neurotoxic factors and thus more neuronal ageing, secretion of Aβ and further microglial activation. Understanding the molecular details of this interaction should reveal approaches, which could be used therapeutically.

Similarly, further investigation of the mechanisms, which are responsible for the most important genetic risk factor for LOAD, APOE, is important. Studies investigating the effects of ApoE alleles on neurons have revealed that human ApoE4 cultures secreted two times more $A\beta_{1-42}$ than ApoE3 cultures. The same study also demonstrated that using gene editing to convert ApoE4 to ApoE3 ameliorated this effect, suggesting an ApoE4 specific effect (Wang *et al.*, 2018). In this Chapter, the relationship of ApoE allele and generation of inflammatory factors was further expanded on.

The aim of this Chapter was to determine the effects of activated microglia from different ApoE backgrounds on primary neurons. Activated primary microglia are known to secrete a variety of neurotrophic and neurotoxic factors into their culture medium (Nakajima *et al.*, 2001; Heneka, Carson, *et al.*, 2015). Culture media were collected and added to primary cortical neuron cultures.

Here, it was shown that immunostimulation and Aβ treatment of microglia cells resulted in secreted factors in the culture medium, which have an effect on neuron morphology. Moreover, these effects are ApoE genotype dependent. In particular, the pro-inflammatory cytokine TNFα was shown to reduce dendrite number and length. This can be partially prevented by the addition of an anti-TNFα antibody. The degree of protection mediated by the anti-TNFα antibody, from the morphological changes to neurons is also ApoE genotype dependent.

4.2 Results

4.2.1 Establishing an experimental setup for neuron treatment

The aim of this Chapter was to elucidate the effect of microglia culture conditioned media on neurons. Primary microglia from the three ApoE isotypes (ApoE2, 3 and 4), as well as wt C57/BL6 microglia were immunostimulated (150 ng/ml LPS and 10 mM ATP) or treated with Aβ 10 μM, either alone or after priming with LPS (150 ng/ml). Conditioned medium was then collected and added to primary cortical neurons at a 1:2 ratio for 72 hours (Fig.4.1A). Primary cortical neurons were cultured in neurobasal medium, whilst primary microglia were cultured in DMEM with the addition of N2 supplement, which contains growth factors. In order to determine whether the mix of different cell culture media would affect neuron viability, neurons were cultured in neurobasal medium, or neurobasal medium supplemented with N2 and DMEM. After 72 hours of incubation, LDH release was measured. The analysis revealed that the addition of N2 or DMEM medium does not affect neuron viability (Fig. 4.1B).

4.2.2 Conditioned media of microglial cultures affect neuron morphology in an ApoE dependent manner .

Conditioned media from untreated and immunostimulated (150 ng/ml LPS for 3 hours and 10 mM ATP for 1 hour) microglia were added to primary cortical neurons from DIV 7 (Days *in vitro*) for 72 hours. Neurons were then processed for confocal microscopy by staining for the microtubule associated protein 2 (map2). Map2 belongs to a family of phosphoproteins expressed in the cell body and dendrites of neurons and was therefore used to assess dendrite morphology (Li *et al.*, 2008). Morphological changes were determined using Fiji software. Both the number of dendrites per cell and the length of dendrites decreased in neurons treated with conditioned medium from immunostimulated (LPS and ATP) microglia, compared with conditioned medium from untreated microglia, regardless of microglia genotype (Fig 4.2). Microscopic images were analysed and the number of dendrites per cell and

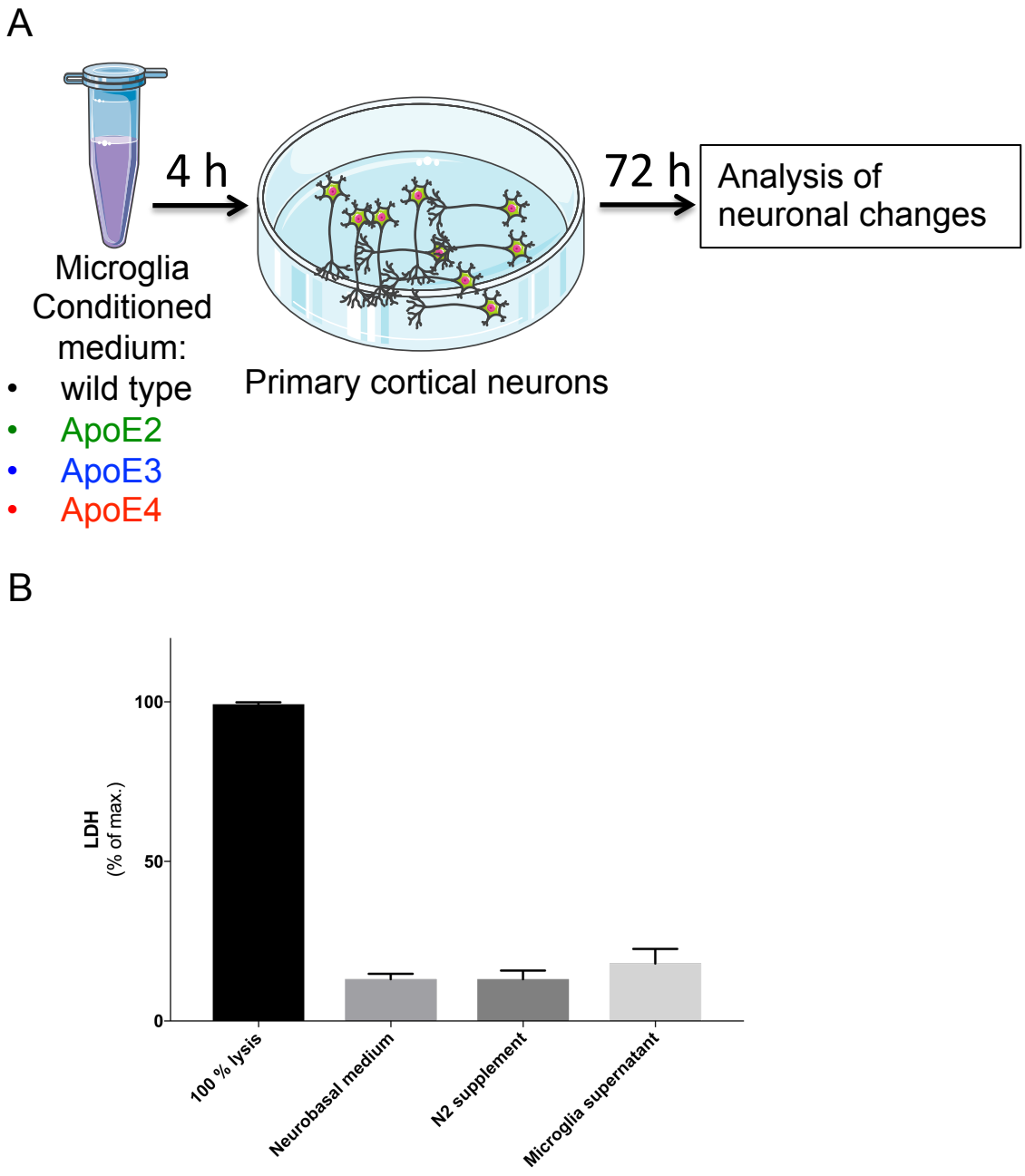


Fig 4.1 Experimental setup for neuron treatment
Primary microglia from wild type or different ApoE genotypes were either immunostimulated with LPS and ATP or treated with Aβ with or without LPS. Conditioned media were collected and added to wt primary cortical neurons for 72 hours. Neurons were then processed for IFA and changes to neuron morphology determined. (**A**) Schematic representation of the experimental setup. (**B**) Different neuronal culture media (neurobasal medium, neurobasal medium supplemented with N2 (added to microglia cultures) and conditioned medium from untreated wt C57/BL6 microglia) were tested by measuring LDH release after 72 hours of incubation. LDH secretion was determined and is presented as percentage of 100 % lysed cells.

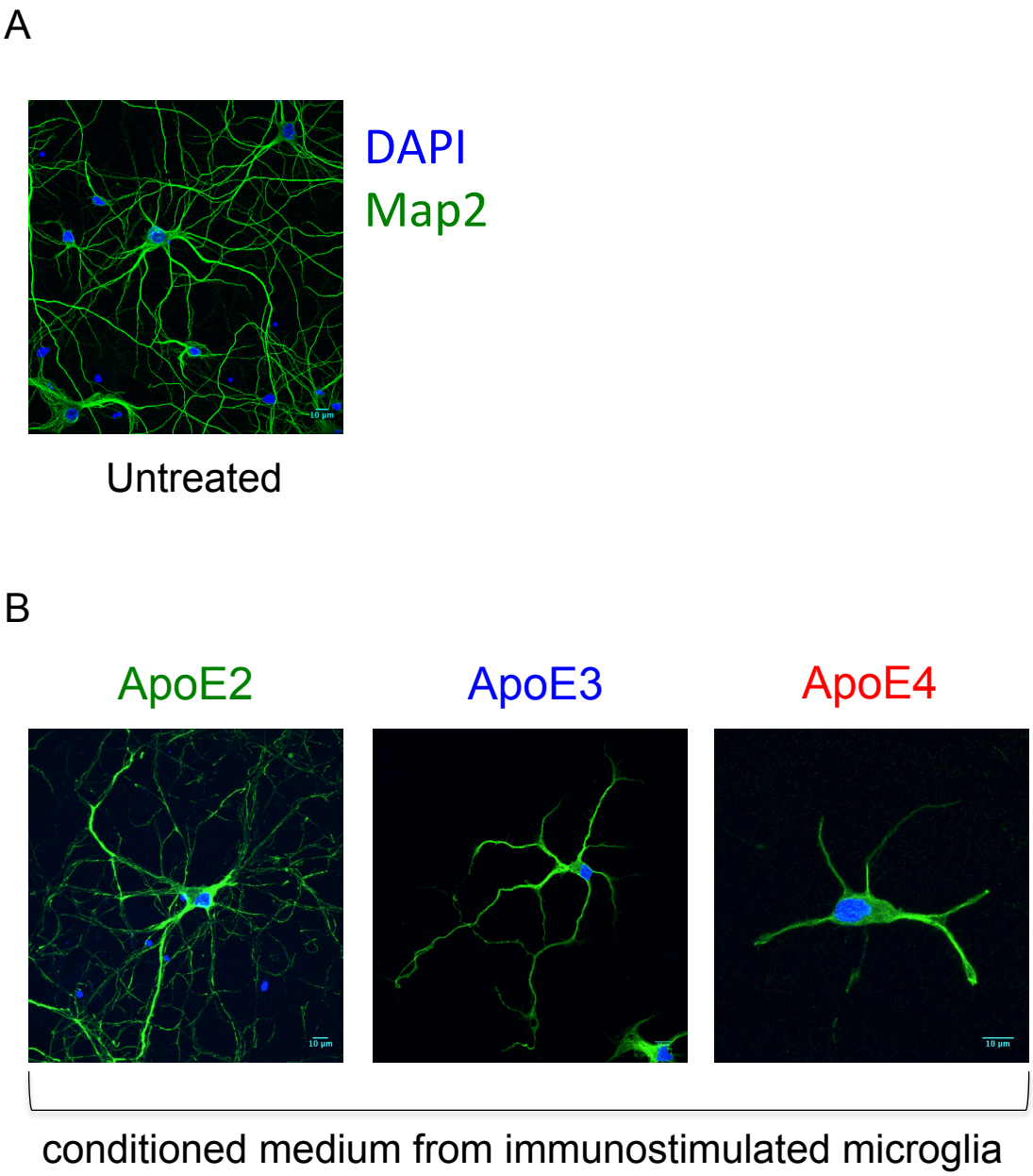


Figure 4.2 Dendrite morphology changes upon treatment with conditioned media from immunostimulated microglia
Conditioned media from microglia of different genetic backgrounds were incubated with primary cortical neurons (DIV 7) for 72 hours and processed for immunofluorescence imaging. (**A**) A representative image is shown for untreated neurons and (**B**) neurons subjected to conditioned media from immunostimulated (150 ng/ml LPS for 3 hours + 10 mM ATP for 1 hour) ApoE2, 3 and 4 microglia. Dendrites were visualised by staining for map2, nucleus by DAPI staining.

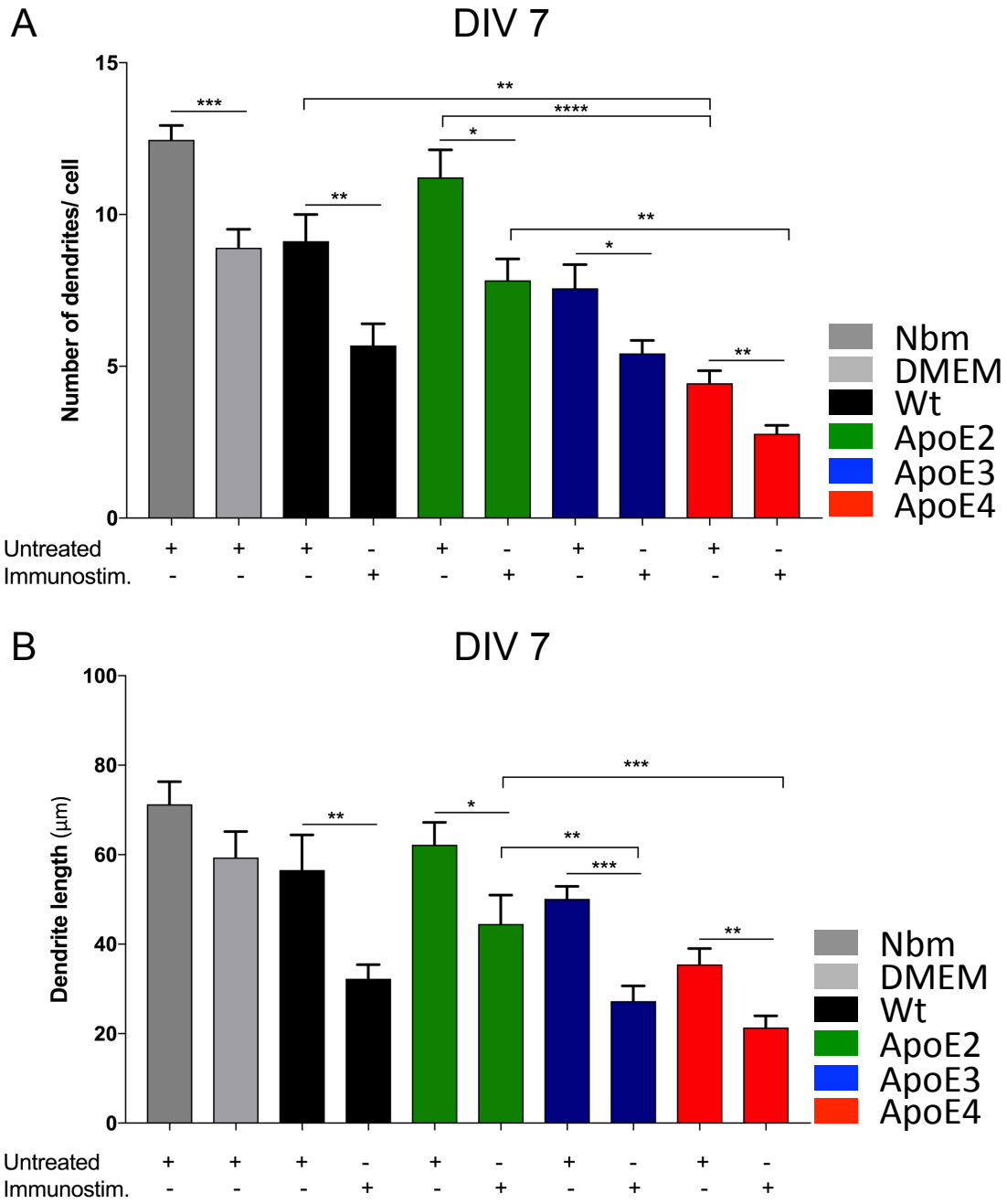


Figure 4.3. Conditioned media from microglia affect neuron morphology in an ApoE dependent manner

Conditioned media from unstimulated or immunostimulated (150 ng/ml LPS for 3 hours + 10 mM ATP for 1 hour) microglia of different genetic backgrounds that were incubated with primary cortical neurons (DIV 7) for 72 hours and then processed for immunofluorescence imaging. (**A**) ApoE dependent changes to neuron morphology were observed based on number of dendrites per cell and (**B**) dendrite length. Untreated medium controls are in grey. Data are mean ± SEM and levels of significance were determined by two way ANOVA and are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments.

dendrite length quantified. This allowed for a quantification of morphological changes to the cells. Alterations to the cell morphology can have an impact on its function (Uylings and Pelt, 2002; Billeci *et al.*, 2009).
Neurons treated with conditioned medium from untreated wt C57/BL6 microglia had an average of ~9 dendrites per cell and an average length of ~55 μm (Fig. 4.3). Neurons incubated with conditioned media from untreated ApoE3 microglia, appeared very similar to those incubated with media from wt C57/BL6 microglia, with ~8 dendrites per cell and an average dendrite length of 50 μm. Incubation with conditioned media from untreated ApoE2 microglia resulted in slightly more dendrites (~11) that were found to be ~65 μm long. In comparison, neurons treated with conditioned media from untreated ApoE4 microglia, have ~6 dendrites less per cell, which are ~25 μm shorter than those in ApoE2 treated neurons. There is a significant reduction in dendrite number (~4) between neurons incubated with conditioned media from untreated wt C57/BL6 microglia and untreated ApoE4 microglia.

In addition to these genotype dependent differences in neuron morphology, differences were also observed between treatments. Neurons treated with conditioned media from immunostimulated (150 ng/ml LPS for 3 hours and 10 mM ATP for 1 hour) wt C57/BL6 microglia had both significantly fewer (~3) (Fig. 4.3A) and shorter (~15 μm) (Fig. 4.3B) dendrites. Similarly, the number and length of dendrites are reduced, by ~3 dendrites per cell and ~15 μm, in neurons treated with conditioned medium from immunostimulated ApoE2 microglia, compared with neurons treated with conditioned media from untreated ApoE2 microglia. A significant reduction in dendrite number and length was also observed between neurons incubated with conditioned media from immunostimulated ApoE3 and ApoE4 microglia, compared with those treated with conditioned media from untreated ApoE3 and ApoE4 microglia.

The same experimental setup was used on neurons at DIV 14. At this time point the neuronal cells are more mature and the neuronal density is increased in comparison to that of DIV 7 neurons (Cullen *et al.*, 2010). Therefore, the effects

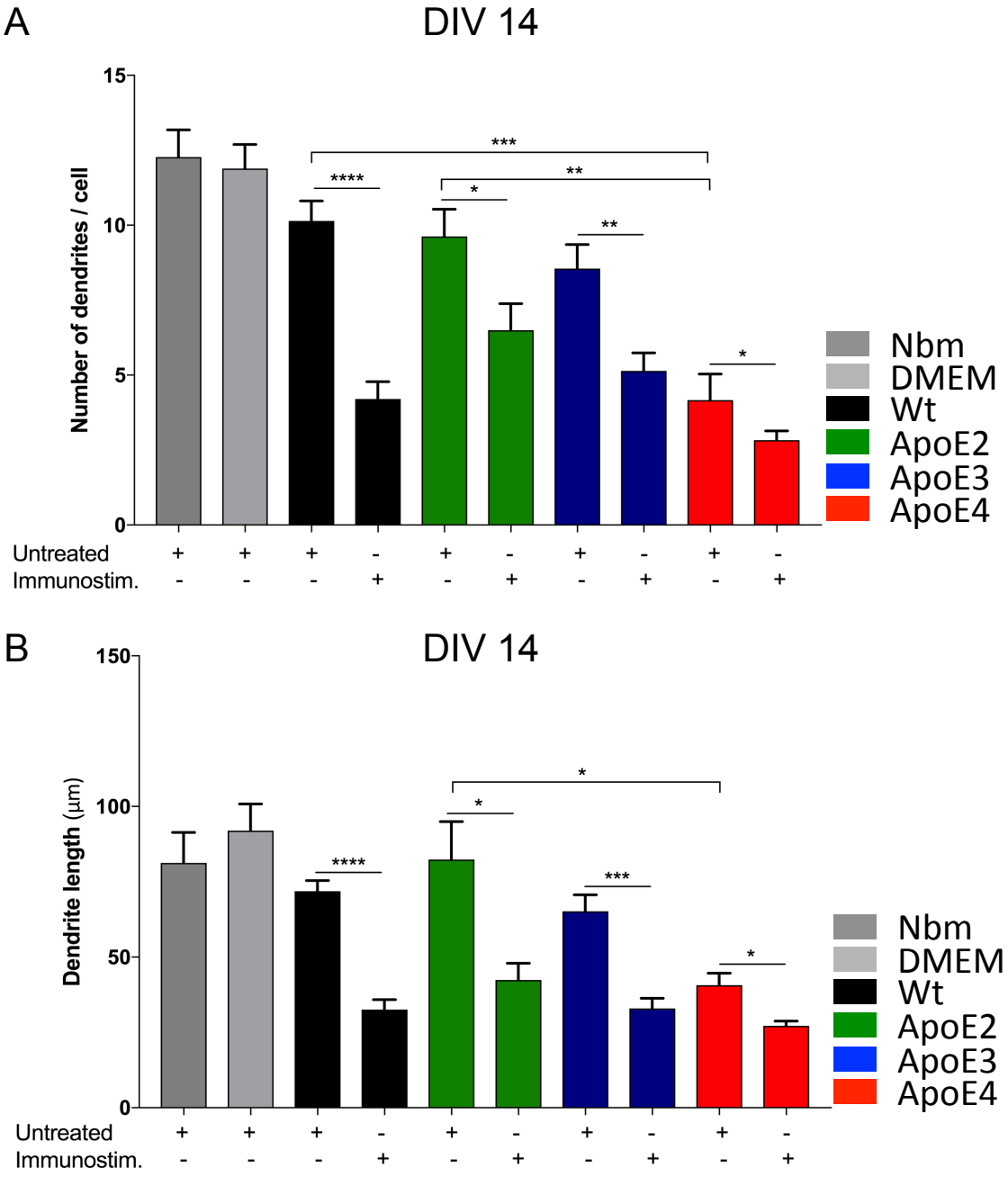


Figure 4.4 Microglia derived conditioned media affect neuron morphology in an ApoE dependent manner

Conditioned media from unstimulated or immunostimulated (150 ng/ml LPS for 3 hours + 10 mM ATP for 1 hour) microglia of different genetic backgrounds were incubated with primary cortical neurons (DIV 14) for 72 hours and then processed for immunofluorescence imaging. (**A**) ApoE dependent changes to neuron morphology were observed based on number of dendrites per cell and (**B**) dendrite length. Untreated medium controls are in grey. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments.

of microglia conditioned media were also investigated to elucidate whether the more dense neuronal culture would be affected in a similar way as DIV 7 neurons. Whilst the overall number of dendrites per cell was similar to that observed in DIV 7 neurons, the dendrite length was increased in DIV 14 neurons. In particular in neurons treated with conditioned media from untreated ApoE2 microglia, dendrites on DIV14 were ~25 μm longer than dendrites of DIV7 neurons. Genotype specific differences were also observed. Neurons treated with conditioned media from untreated wt C57/BL6 and untreated ApoE2 microglia resulted in significantly more dendrites (~10 per cell) than ApoE4 treated neurons (~4 dendrites per cell) (Fig. 4.4A). Similarly, neurons subjected to cell culture media from untreated ApoE2 microglia had significantly longer dendrites (~55 % longer) than those treated with ApoE4 conditioned media (Fig. 4.4B).

As for DIV 7 neurons, there was a significant decrease in both number of dendrites and their length between neurons treated with conditioned media from untreated microglia and those subjected to conditioned media from immunostimulated (150 ng/ml LPS for 3 hours and 10 mM ATP for 1 hour) microglia. This was consistent across all genotypes.

In summary, conditioned media from immunostimulated (150 ng/ml LPS for 3 hours and 10 mM ATP for 1 hour) microglia had an effect on neuronal morphology, by decreasing both the dendrite number and length. Whilst this is consistently observed with wt C57/BL6 and the three ApoE genotypes, the detrimental effect of microglia conditioned media on dendrite morphology is genotype dependent with the following ranking (high to low) of conditioned medium from microglia, ApoE4 > ApoE3 > ApoE2.

4.2.3 TNFα modifies dendrite morphology in a dose dependent manner

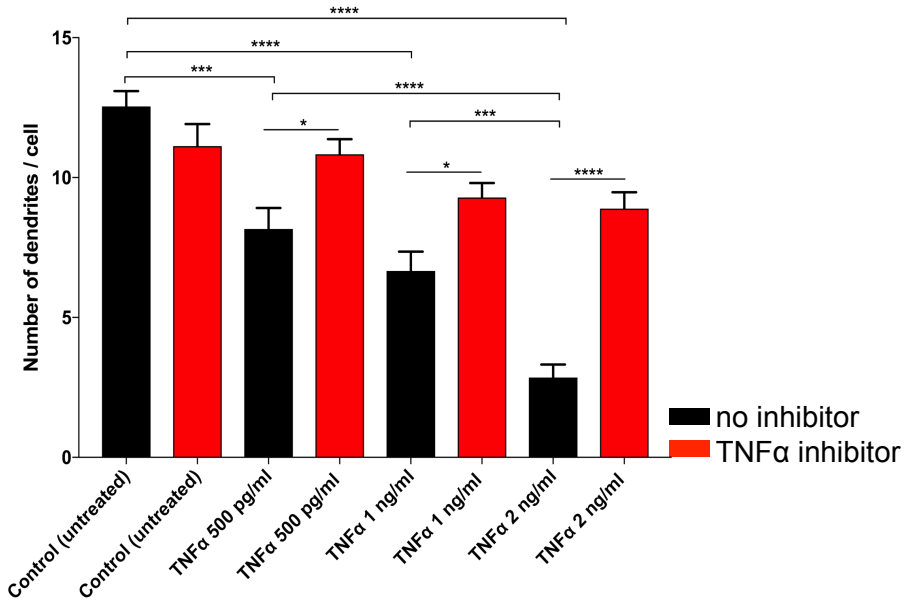
It is well known that TNFα is secreted by activated microglia and, furthermore, TNFα has been implicated in AD (Franchi *et al.*, 2009). In the

previous Chapter it was determined that activated microglia, especially from ApoE4 mice, secrete large amounts of TNFα. To determine whether TNFα has an effect on dendrite morphology, neurons from DIV 7 were incubated with increasing concentrations of purified mouse TNFα (500, 1000 and 2000 pg/ml) and in the presence or absence of an anti-TNFα antibody, for 72 hours. Measuring dendrite length and number of dendrites per cell provided a read out of morphological changes. TNFα significantly reduced both the number of dendrites and their length in a dose-dependent manner (Fig. 4.5). Dendrites of neurons treated with 2 ng/ml TNFα are less than 50 % as long as dendrites from untreated neurons (Fig. 4.5 A and B). The reduction in dendritic length was abrogated with anti-TNFα antibody, confirming the morphological changes were due to TNFα. Addition of anti-TNFα antibody to TNFα treated neurons significantly rescued both dendrite number and length as compared to neurons without anti-TNFα treatment. Dendritic length was decreased by ~50 % in neurons treated with TNFα (1 or 2 ng/ml) compared with those incubated in the presence of anti-TNFα antibody (Fig. 4.5B).

4.2.4 TNFα inhibition abrogates morphological changes of cortical neurons, induced by conditioned medium from immunostimulated microglia

In Chapter 3 it was established that TNFα is secreted by immunostimulated microglia in an ApoE isoform dependent manner (Fig. 3.5). In order to investigate whether the changes to neuron morphology observed here are in part, due to TNFα in the microglia conditioned medium, neurons from DIV7 (Fig. 4.6) and DIV14 (Fig. 4.7) were incubated with conditioned medium from untreated or immunostimulated microglia as described above, and incubated in the presence of a neutralising anti-TNFα antibody. Following anti-TNFα antibody treatment neurons had more and longer dendrites compared with neurons without the anti-TNFα antibody. This effect was observed regardless of the genotype of microglia of the conditioned media. On average neurons (from DIV7) treated with the anti-TNFα antibody had 2.3 times more dendrites than those without TNFα neutralisation (Fig. 4.6A). Dendrite length

A



B

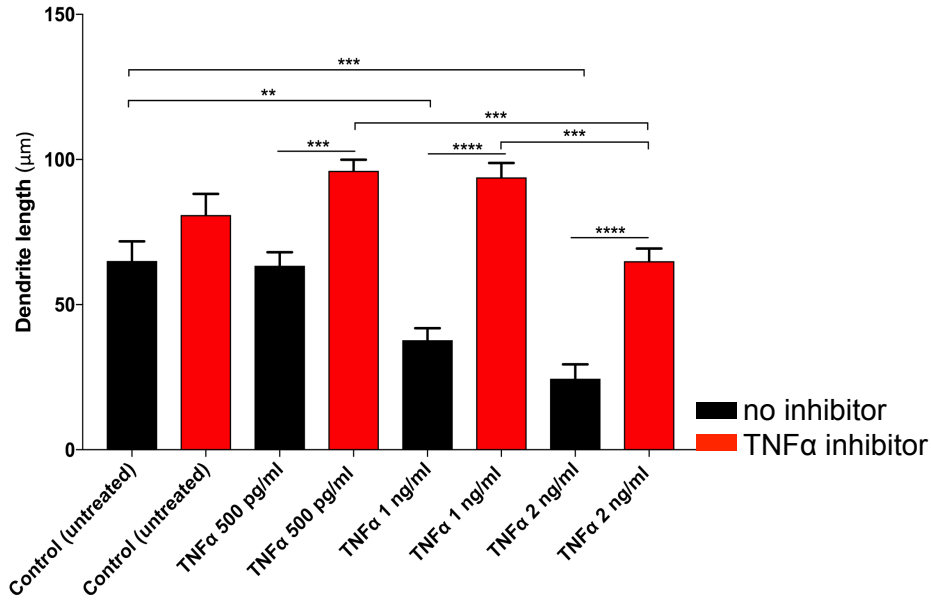


Figure 4.5 TNFα treatment results in reduction in number and length of dendrites

Primary cortical neurons (DIV 7) were incubated with TNFα for 72 hours, in the presence or absence of an anti-TNFα antibody and then processed for immunofluorescence imaging. (**A**) TNFα dependent changes to neuron morphology were observed based on number of dendrites per cell and (**B**) dendrite length. Statistical significance was determined using a one-way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments.

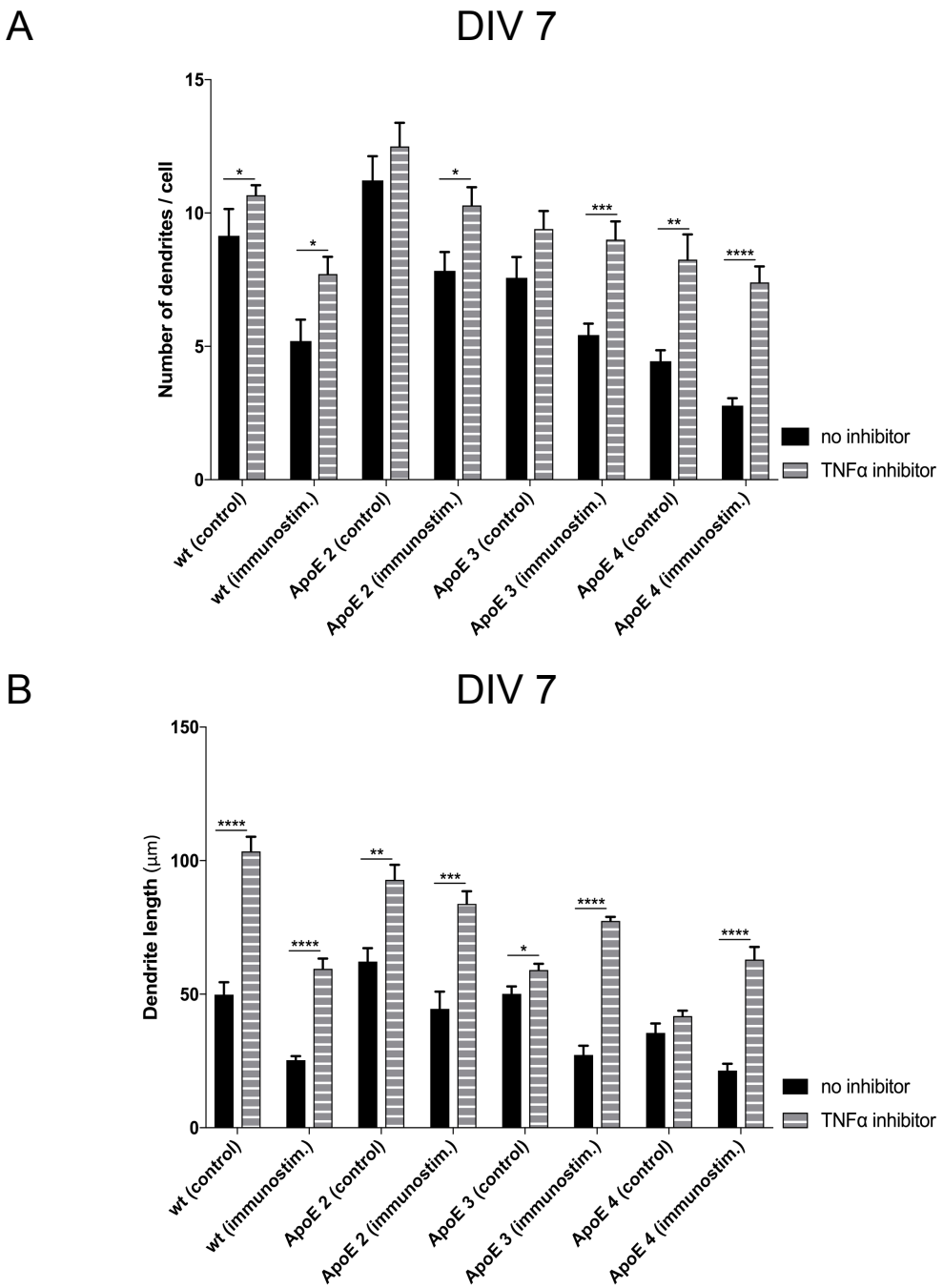


Figure 4.6 Anti-TNFα antibodies protect neurons from morphological changes induced by microglia conditioned medium

Conditioned media from unstimulated or immunostimulated (150 ng/ml LPS for 3 hours + 10 mM ATP for 1 hour) microglia of different genetic backgrounds were incubated with primary cortical neurons (DIV 7) for 72 hours, in the presence or in the absence of anti-TNFα antibody (10 ng/ml) and then processed for immunofluorescence imaging and morphological changes assessed by determining (**A**) number of dendrites per cell and (**B**) dendrite length. Statistical significance was determined by t-test. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments.

was 2-fold higher in neurons treated with conditioned media from immunostimulated microglia with the TNFα neutralising antibody compared with those treated without the anti-TNFα antibody (Fig. 4.6B).

Curiously, dendrites of neurons treated with conditioned media from untreated microglia were also longer and increased in number in the presence of the anti-TNFα antibody (Fig. 4.6). Previously it was shown that untreated microglia do not secrete detectable levels TNFα (Fig. 3.5); however, in the presence of the TNFα antibody the number of dendrites was increased in neurons treated with conditioned media from wt C57/BL6 and ApoE4 microglia, and dendrite length was increased in neurons incubated with wt C57/BL6, ApoE2 and ApoE3 microglia derived conditioned media (Fig. 4.6). Interestingly, conditioned media from untreated microglia reduced both these measures. An even more drastic effect was observed in neurons treated with conditioned media from immunostimulated microglia conditioned media. In the presence of the anti-TNFα antibody, the number of dendrites was increased from ~6 to ~9 in neurons subjected to conditioned media from immunostimulated ApoE3 microglia, and increased by an average of ~4 dendrites per cell in those treated with conditioned media derived from immunostimulated ApoE4 microglia (Fig. 4.6A).

Similar observations were made in neurons from DIV 14. Here the positive effects of the anti-TNFα antibody on dendrite morphology were mainly seen in neurons incubated with conditioned media from immunostimulated microglia.
However, the length of dendrites from neurons treated with conditioned media from ApoE4 microglia did not increase significantly in the presence of the neutralising antibody. This could be due to the large spread of the data from DIV14 neurons (Fig. 4.6). Dendrite number and length both increased by ~ 2 fold in neurons treated with wt C57/BL6 derived conditioned media (immunostimulated) (Fig. 4.7).

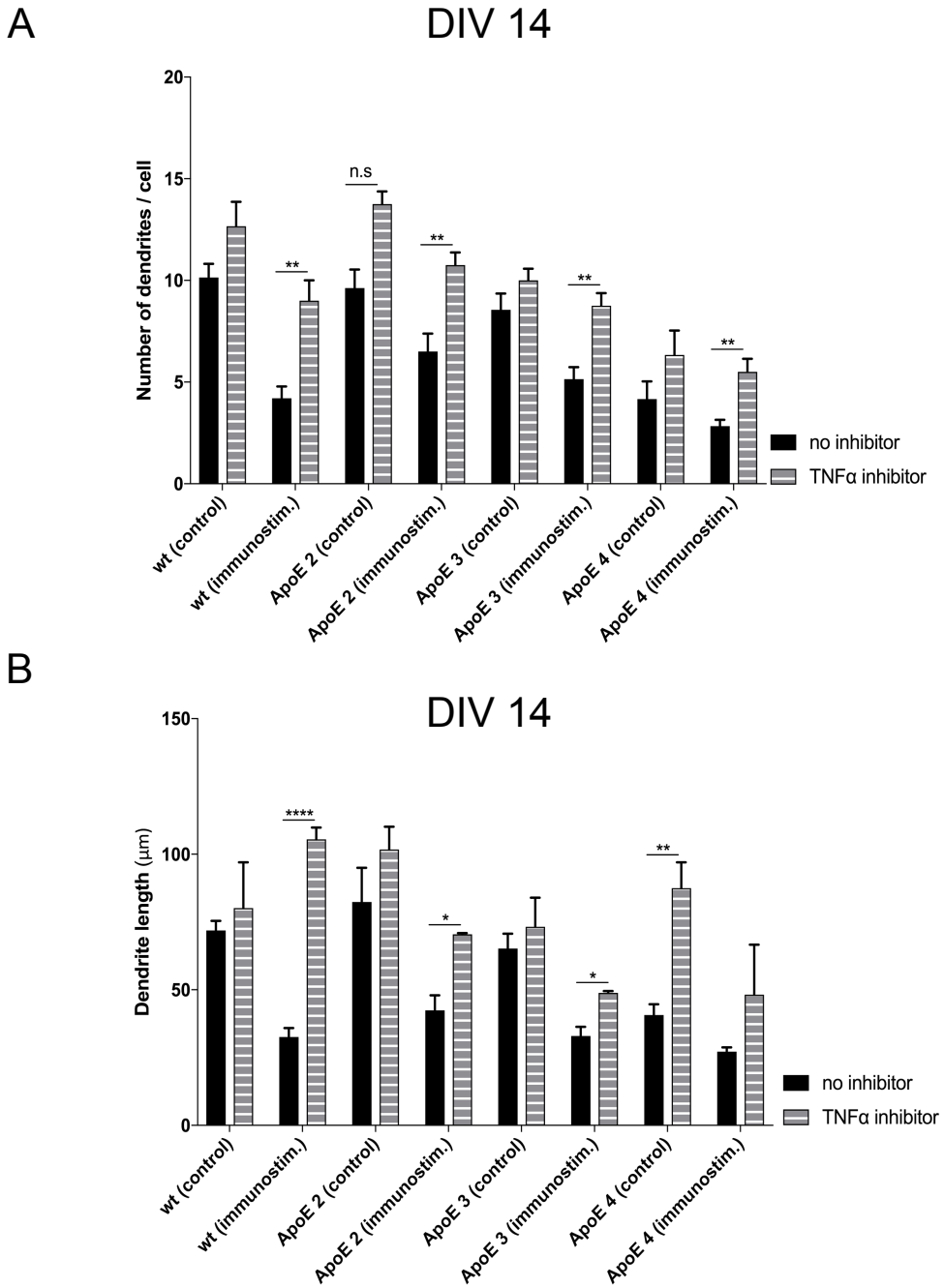


Figure 4.7 Anti-TNFα antibodies protect neurons from morphological changes induced by microglia conditioned medium

Conditioned media from unstimulated or immunostimulated (150 ng/ml LPS for 3 hours + 10 mM ATP for 1 hour) microglia of different genetic backgrounds were incubated with primary cortical neurons (DIV 14) for 72 hours, in the presence or absence of an anti-TNFα antibody (10 ng/ml) and then processed for immunofluorescence imaging and morphological changes assessed by determining (**A**) number of dendrites per cell and (**B**) dendrite length. Statistical significance was determined by t-test. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments.

In summary, this data suggests that TNFα is a major factor in the conditioned microglia media involved in the perturbation of dendrite morphology.

4.2.5 Amyloid beta perturbs dendrite morphology in an ApoE dependent manner

To determine whether Aβ can stimulate microglia to produce factors that affect neuron morphology, primary microglia were also treated with Aβ (10 μM) with or without LPS (150 ng/ml) priming. Conditioned media from the Aβ treated microglia were added to neuronal cultures (DIV7) at a 1:2 ratio for 72 hours. Neurons treated with conditioned media from wt C57/BL6 Aβ alone treated microglia had twice as many dendrites as neurons treated with conditioned media from LPS primed wt C57/BL6 Aβ treated microglia. A significant reduction of dendrite number was also detected between neurons incubated with conditioned media from Aβ treated and primed Aβ treated microglia from all three ApoE isotypes. On average the number of dendrites increased by at least 2/cell (Fig. 4.8A). However, it is difficult to assess whether this effect is due to Aβ treatment, as the findings from previous experiments (Fig.4.3) suggest similar dendrite morphology of neurons treated with conditioned media from untreated microglia. In order to determine the extent of the effect Aβ treatment of microglia has on neurons, a LPS control would be necessary. Due to the large experimental setup and availability of primary neurons, this was not included in this current study.

In addition to this there are also genotype specific differences in dendrite numbers. Neurons treated with conditioned media from Aβ alone and primed and Aβ treated wt C57/BL6, ApoE2 and ApoE3 microglia had significantly more dendrites per cell, than neurons treated with ApoE4 derived conditioned media. Neurons incubated with conditioned media from Aβ treated wt C57/BL6 microglia had 33 %, ApoE2 had 75 % and ApoE3 had 42 % more dendrites than ApoE4 treated neurons (Fig. 4.8A). Dendrite length does not vary significantly between neurons incubated with conditioned media from Aβ treated compared to primed and Aβ treated microglia (Fig. 4.8B). Conditioned media from microglia treated with Aβ also affect the dendrite length in a genotype

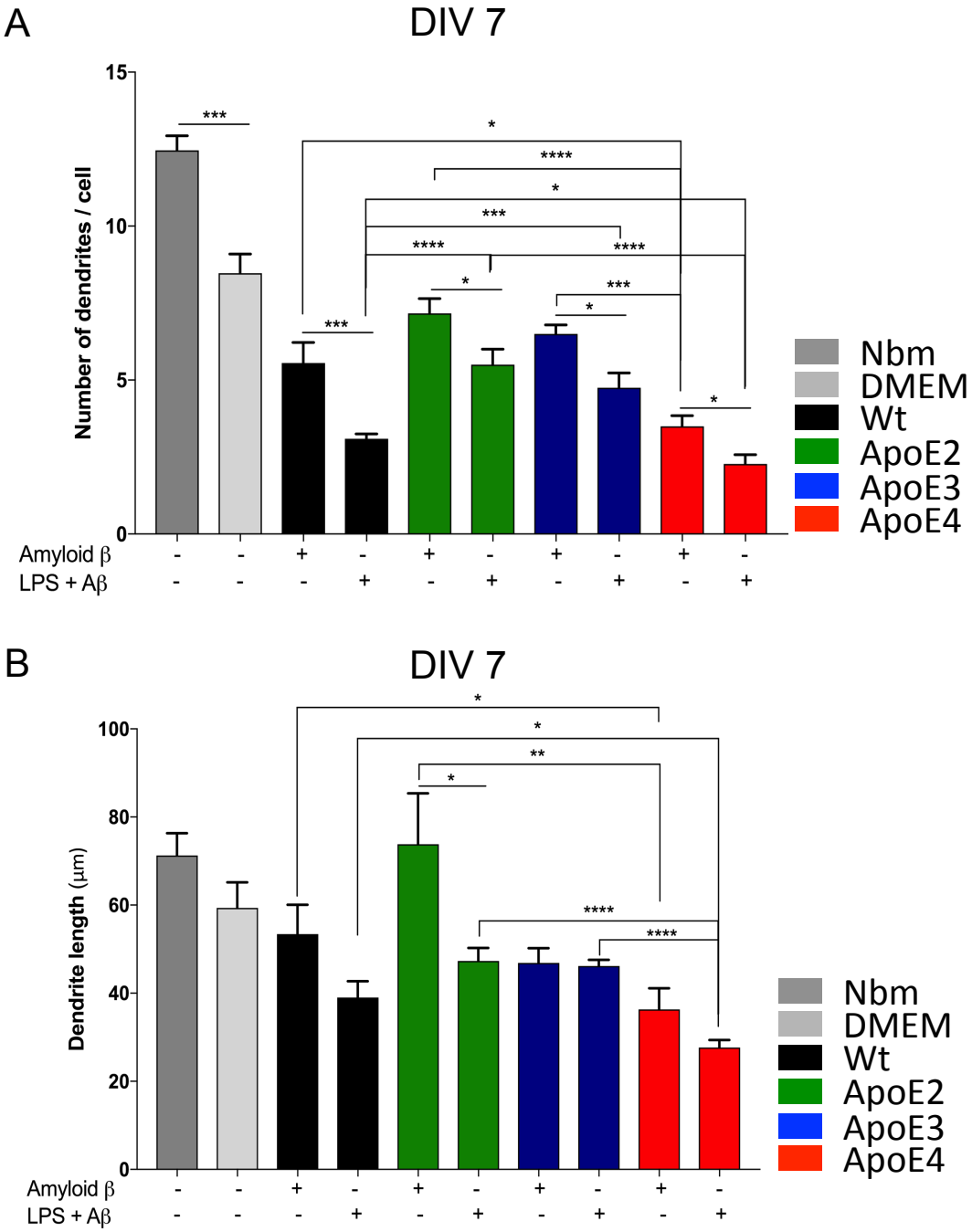


Figure 4.8. Conditioned media from Aβ stimulated microglia affect neuron morphology in an ApoE dependent manner

Microglia were treated with Aβ (10 μM), with or without priming with LPS (150 ng/ml). Conditioned media were collected and incubated with primary cortical neurons (DIV 7) for 72 hours. Neurons were then processed for immunofluorescence imaging. ApoE dependent changes to neuron morphology were observed based on (**A**) number of dendrites per cell and (**B**) dendrite length. Data for Nbm and DMEM are repeated from Figure 4.3. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments.

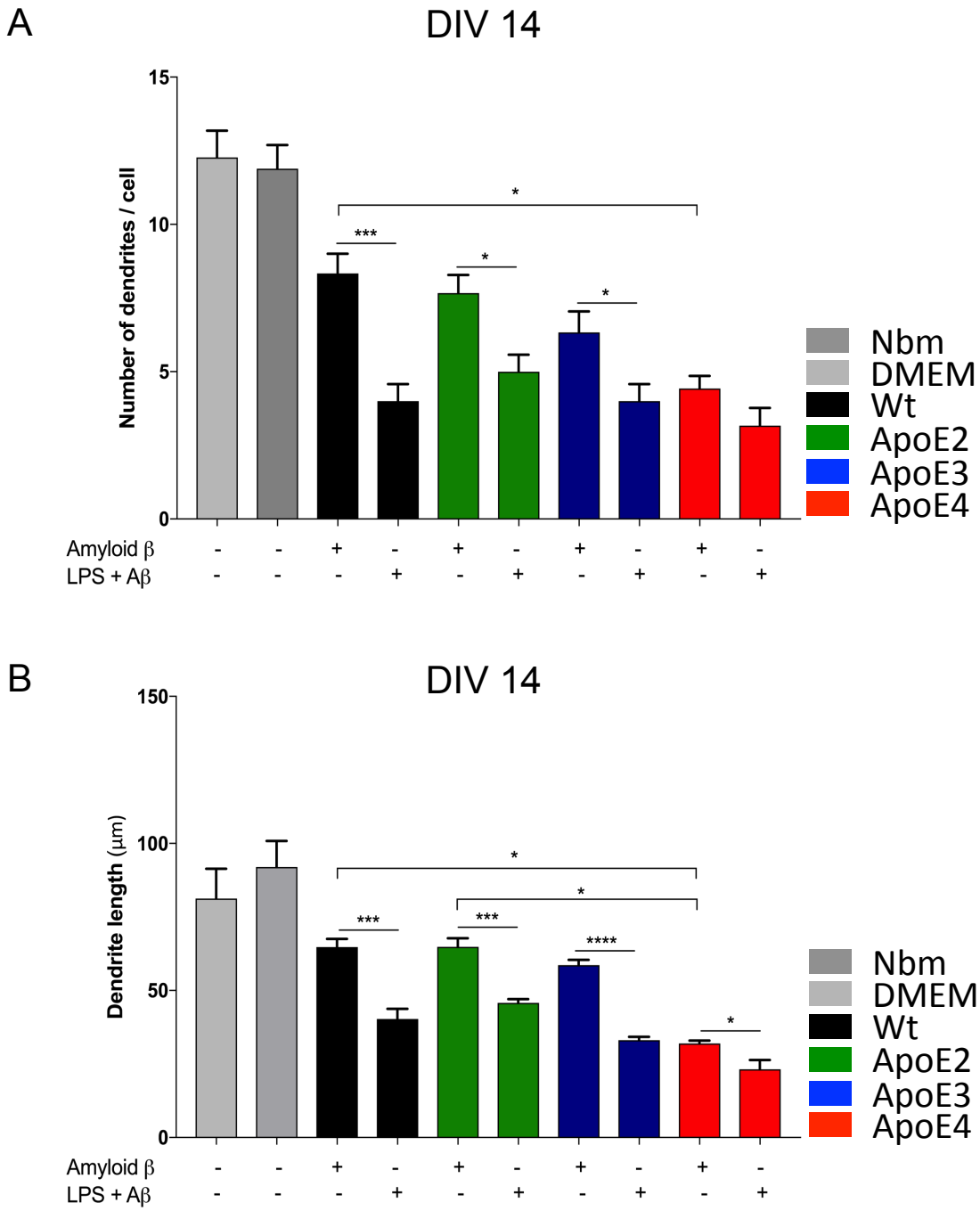


Figure 4.9. Microglia derived conditioned media affect neuron morphology in an ApoE dependent manner

Microglia were treated with Aβ (10 μM), with or without priming with LPS (150 ng/ml). Conditioned media were collected and incubated with primary cortical neurons (DIV 14) for 72 hours. Neurons were then processed for immunofluorescence imaging. ApoE dependent changes to neuron morphology were observed based on (**A**) number of dendrites per cell and (**B**) dendrite length. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments.

dependent manner. Those from an ApoE2 background result in the longest dendrites ~70 µm, which is comparable to dendrite length of untreated neurons, whilst ApoE4 microglia conditioned media decreased the dendrite length both after Aβ alone and LPS and Aβ treatment in comparison to wt, ApoE2 and ApoE3 derived conditioned media (Fig. 4.8B).

Similar effects, although not as prominent were observed in neurons from DIV 14. Neurons incubated with conditioned media from Aβ treated wt C57/BL6 microglia had ~40 % more dendrites than neurons treated with conditioned media from ApoE4 microglia. Additionally, treatment dependent effects were observed. In the presence of primed and Aβ treated conditioned media from wt microglia, 50 % fewer dendrites were detected than in neurons treated with conditioned media from Aβ treated microglia. Dendrite number was also reduced in neurons incubated with conditioned media from primed and Aβ treated ApoE2 (25 %) and ApoE 3 (~40%) microglia compared to neurons treated with conditioned media from Aβ treated microglia of the respective genotype (Fig. 4.9A). This effect can also be observed in dendrite length (Fig. 4.9B). Taken together this suggests that both the microglia treatment as well as the microglia genotype have an impact on dendrite morphology. However, the differences in dendrite morphology between neurons incubated with conditioned media from microglia treated with Aβ alone and primed and Aβ treated cells are not as apparent as the immunostimulatory treatment.

4.2.6 Treatment with anti-TNFα antibody partially prevents dendrite loss

The aim was to establish whether the changes in neuron morphology observed here are due to TNFα in the microglia conditioned medium, as previously described for neurons incubated with conditioned media from immunostimulated microglia (Fig 4.6 and Fig 4.7). Incubation in the presence of anti-TNFα antibody resulted in an increase of dendrite number compared with neurons (DIV 7) incubated with conditioned media from wt C57/BL6 microglia (LPS and Aβ treated) by 40 %. There was also a significant difference between

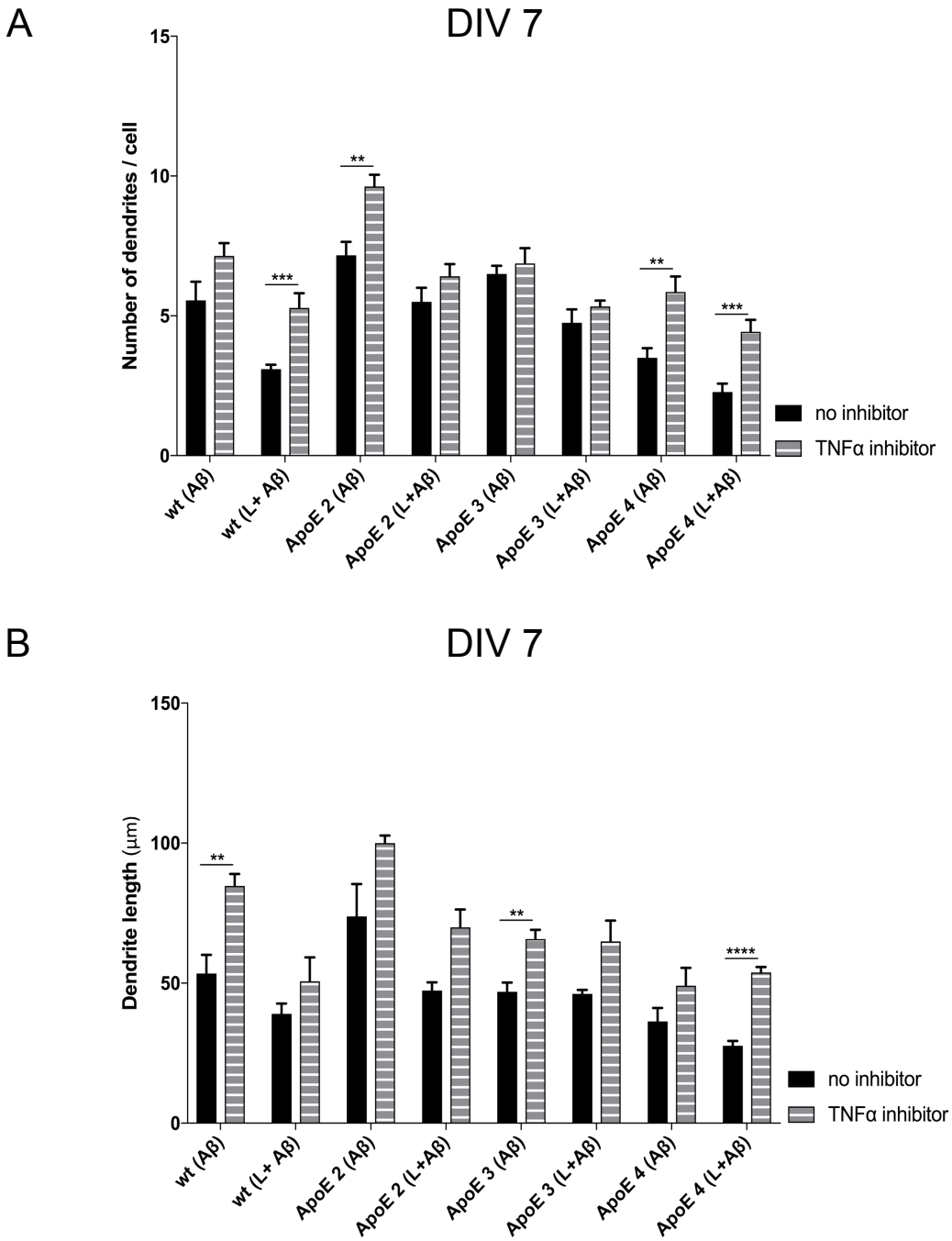


Figure 4.10 Primary cortical neurons from DIV 7 incubated with microglia conditioned media in the presence and absence of anti-TNFα antibody
Conditioned media from Aβ stimulated microglia of different genetic backgrounds were incubated with primary cortical neurons (DIV 7) for 72 hours, in the presence or in the absence of an anti-TNFα antibody and then processed for immunofluorescence imaging and morphological changes assessed by determining (**A**) number of dendrites per cell and (**B**) dendrite length. Statistical significance was determined by t-test. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments.

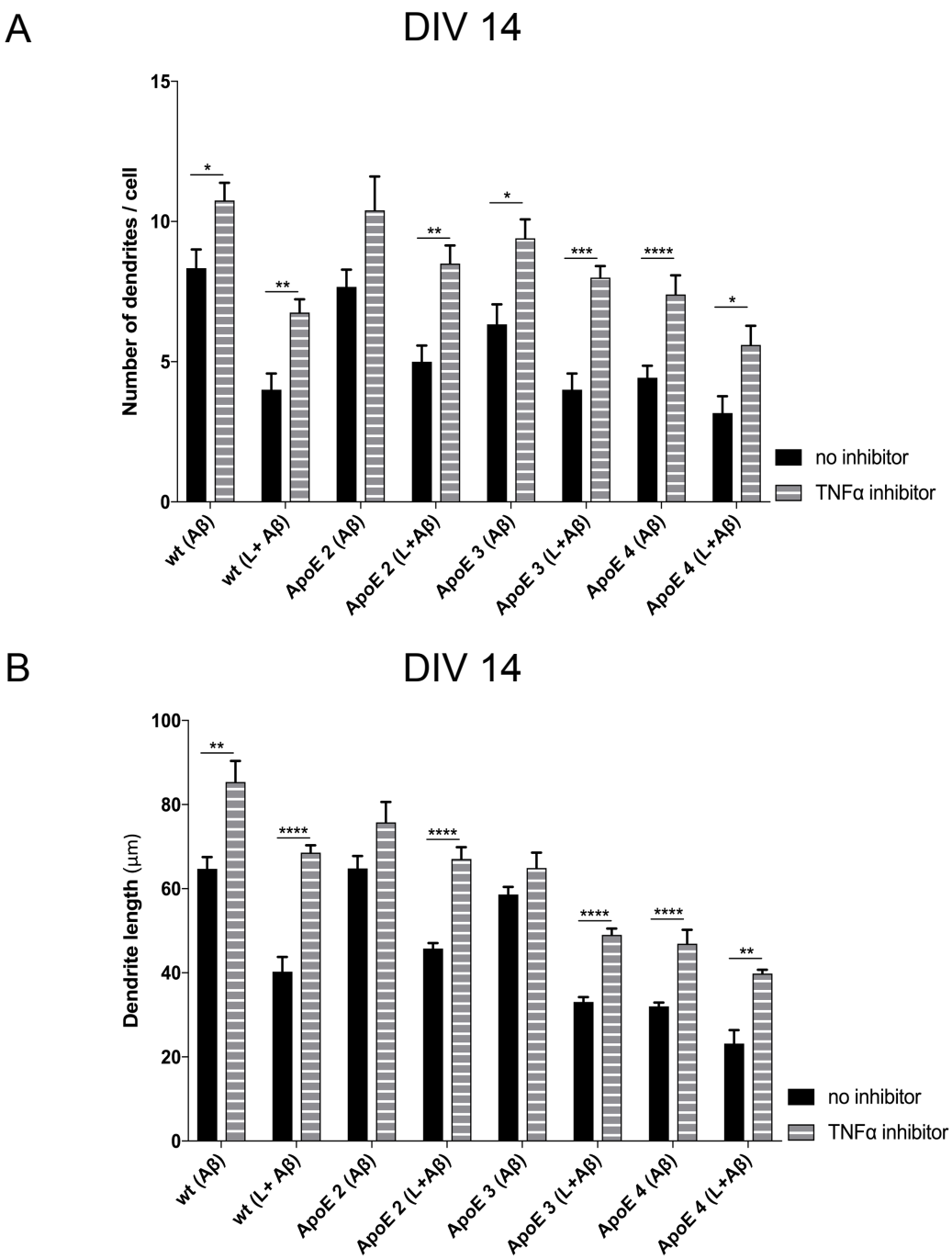


Figure 4.11 Primary cortical neurons from DIV 14 incubated with microglia conditioned media in the presence and absence of anti-TNFα antibody
Conditioned media from Aβ stimulated microglia of different genetic backgrounds were incubated with primary cortical neurons (DIV 14) for 72 hours, in the presence or absence of an anti-TNFα antibody and then processed for immunofluorescence imaging and morphological changes assessed by determining (**A**) number of dendrites per cell and (**B**) dendrite length. Statistical significance was determined by t-test. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments.

conditioned media from ApoE2 cells (Aβ treated), by ~25 % and ApoE4 microglia treated with Aβ as well as LPS and Aβ (>50 %) (Fig. 4.10A).
Dendrite length was significantly increased in the presence of anti-TNFα antibody in neurons subjected to conditioned media from wt (~45 %) and ApoE3 (~30 %) microglia that have been treated with Aβ, and ApoE4 microglia treated with LPS and Aβ. Here the dendrite length is increased almost 2 fold in neurons incubated with the anti-TNFα antibody (Fig. 4.10B). In neurons from DIV 14 the presence of anti-TNFα antibody significantly increases dendrite length in all LPS and Aβ stimulated microglia conditioned medium treated neurons regardless of the genotype. Dendrites in the presence of the anti-TNFα antibody are increased by >15 µm (Fig. 4.11B).
Overall the data suggests that the incubation with anti-TNFα antibody, prevents dendrite loss in neurons treated with conditioned media from Aβ treated microglia, mainly under primed conditions.

4.3 Discussion

The interaction and communication between microglia and neurons is crucial for normal neuronal regulation. As such, microglia are responsible for synaptic pruning (Pósfai *et al.*, 2019). The impact of secretory products from microglia on neurons during AD development however remains largely unknown. Microglia secrete various neurotoxic and neurotrophic factors that can differently affect neurons. Here, microglia from different ApoE genotypes and wt C57/BL6, were immunostimulated or treated with Aβ and the conditioned medium collected. These culture media were then added to primary cortical neuron cultures for 72 hours and effects on neuron morphology studied. An ApoE dependent and treatment dependent effect on dendrite morphology was observed and could be attributed, at least partially, to TNFα secreted by activated microglia. Treatment with an anti-TNFα antibody protected neurons from morphological changes. Furthermore, the approach utilised here has

proven useful to elucidate effects of secretory products from microglia under untreated, immunostimulated or Aβ treated conditions.

Activated microglia secrete a plethora of pro-inflammatory proteins (Smith *et al.*, 2012). Here the effects of immunostimulated (150 ng/ml LPS for 3 hours and 10 mM ATP for 1 hour) microglia on neurons was demonstrated by changes in neuron morphology. Fluorescent immunocytochemistry and confocal microscopy revealed that conditioned media from immunostimulated microglia resulted in decreased number and length of dendrites, compared with neurons incubated with conditioned media from untreated microglia. This effect was seen in neurons from DIV 7 (Fig. 4.3) and DIV 14 (Fig. 4.4). Whilst analysis of dendrites is easier at DIV 7, neurons from DIV 14 are more representative for later developmental stages (Cullen *et al.*, 2010). Overall the more developed neuronal cultures from DIV 14 had more and longer neurons. The neurons showed similar susceptibility to the microglia conditioned media treatment regardless of developmental stage.

The microglia treatment did impact neuron morphology though, indicating that immunostimulation (Fig. 4.3, 4.4) and to a lesser extend Aβ treatment (Fig. 4.7, 4.8) of microglia results in decreased dendrite numbers and length in neurons exposed to conditioned media This effect is more pronounced in neurons incubated with conditioned media from immunostimulated microglia than Aβ treated microglia.
In the context of Alzheimer's this is an important observation as it further supports the notion of neuroinflammation not only driving the disease but also being one of the earliest events in the disease pathogenesis (Cunningham *et al.*, 2005; Cardoso *et al.*, 2015).

Some of the effects on neuron morphology outlined here can be attributed to TNFα. The pro-inflammatory cytokine, implicated in AD pathology has previously been shown by Floden and colleagues to have no effect on neuron viability by itself. However coincident stimulation of TNFα and NMDA receptors

was found to initiate neuronal death, by increasing neuronal iNOS immunoreactivity (Floden *et al.*, 2005). Using ERK specific inhibitors it was later shown that neuronal death induced by NMDA receptors and TNFα was ERK dependent (Jara *et al.*, 2007).

Here it was shown that neuron morphology, as assessed by dendrite number and length, are affected by TNFα treatment in a dose dependent manner and can be prevented by addition of an anti-TNFα antibody (Fig. 4.5). Microglia activation was shown to induce TNFα secretion (Fig. 3.5B). Therefore, the effect of TNFα inhibition was also tested on the microglia conditioned media treated neurons, using an anti-TNFα antibody. The data shows that overall the inhibition of TNFα prevents the dendrite morphology changes described above (Fig. 4.6, 4.7, 4.10 and 4.11). This suggests that TNFα in the conditioned media is one of the main contributing factors of the changes to neuron morphology and further underlines that TNFα is a key player in AD development. Given the high levels of secreted TNFα found in conditioned media from LPS primed cells of all genotypes, as determined in Chapter 3, the effects here cannot conclusively be attributed to immunostimulation or Aβ treatment. LPS may in fact be driving this response through the induction of TNFα secretion. To further decipher this effect, neurons could be treated with conditioned media from LPS primed microglia.

Neurons treated with conditioned media from untreated microglia also displayed more and longer dendrites in the presence of the neutralising anti-TNFα antibody than those treated with conditioned media from untreated microglia but without the addition of the anti-TNFα antibody (Fig. 4.6). However, TNFα was undetected in the media from untreated microglia by ELISA (Fig. 3.5B). This raises the question why the anti-TNFα antibody had a protective effect on

dendrite morphology under those treatment conditions? Potentially the anti-TNF α antibody has non-specific effects. This could be examined using an irrelevant Ig isotype control experiment. Although this effect is difficult to explain, it is important to note though, that this anti-TNF α antibody effect was more prominent in neuronal cultures from DIV 7 (Fig. 4.6) than DIV 14 (Fig. 4.7), suggesting that data from DIV 14 neuronal cultures could be more robust. Furthermore, this finding may suggest a hypersensitive reaction by DIV 7 neuronal cultures to the selected treatments. Aside from this curious effect, the data is internally consistent and underlines the role of microglia in a sustained pro-inflammatory response and the potential detrimental effects to neurons. In addition microglia secrete a multitude of both pro- and anti-inflammatory cytokines into their culture media, as determined by MS in Chapter 3. Thus, secreted anti-inflammatory cytokines could play a neuroprotective role. Further studies could involve longer incubation times, *in vivo* experiments and rescue experiments.

In addition to the treatment dependent differences, genotype dependent differences were observed in neurons treated with conditioned microglia media. Neurons incubated with culture media from ApoE 4 microglia had significantly impaired dendrites in comparison to those treated with ApoE 2 and wt C57/BL6 microglia derived conditioned media. Differences between ApoE 3 and ApoE4 treated neurons were not significant but a trend could be observed. Studies have suggested that exogenous ApoE3 can stimulate neurite outgrowth, whilst ApoE4 has the opposite effect (Nathan *et al.*, 1994). The findings by Nathan and colleagues could explain why a reduction in dendrites is seen in neurons cultured with untreated ApoE4 microglia derived conditioned media. Microglia and astrocytes are the main source of ApoE in the brain (Ji *et al.*, 2003) and ApoE secreted by microglia has been shown to have both neurotoxic and neuroptrophic effects, depending on the isoform (Qin *et al.*, 2006). The reduction of dendrites caused by incubation with conditioned media from

untreated ApoE4 microglia could be due to the secreted ApoE4. The effect of ApoE4 on dendrites could provide a potential explanation for the cognitive decline in AD patients expressing ApoE4, which is due to defective neurons. The findings presented here are in line with one study that reported a correlation of poor spatial learning and memory in ApoE4 TR mice. This was attributed to reduced dendritic spine density in the medial entorhinal cortex (Rodriguez *et al.*, 2013).
The underlying mechanism causing ApoE4 induced cognitive deficits as well as the changes to neuron morphology remain unknown. However it has been demonstrated, that addition of the ApoE mimetics could reduce neuroinflammation (Safieh *et al.*, 2019).

Overall I have shown in this Chapter that the effects of microglial secretory products on neurons can be determined by incubation of neurons in the presence of microglia conditioned media. Whilst this study used morphological changes in the neurons as the read out, this approach could also be used for to assess biochemical perturbations, including signalling pathways. It would be interesting to determine the impact of Aβ secretion by the treated neurons and in particular the ratio between $A\beta_{1-42}$ and $A\beta_{1-40}$ secretion. This would further reveal the downstream effects of ApoE and microglia activation on neurons.

Whilst Jara and colleagues (Jara *et al.*, 2007) showed that TNFα alone does not contribute to neuronal death, this current study shows that TNFα affects neuron morphology. Additionally, it was shown here that the ApoE genotype has an effect on dendrite morphology. These findings contribute to our understanding of how ApoE impacts the development of AD. However, it remains to be determined how these morphological changes relate to neuronal function. Trafficking of APP and BACE1 in the treated neurons, for example, could be assessed by fluorescent microscopy. Together with the analysis of Aβ production could provide a deeper understanding of perturbations in trafficking associated with AD.

Chapter 5: Analysis of cell biological properties of microglia

5.1 Introduction

Macrophages play an important role in immunity and in regulating inflammation. There is a wealth of knowledge regarding macrophage function that can be used to guide the understanding of microglia function. Like macrophages, microglia play a critical role in regulating immune responses and represent the main population of innate immune cells of the brain (Rivest, 2009). Both macrophages and microglia monitor their microenvironments and ingest pathogens and cellular debris (Yin *et al.*, 2017). Uptake of pathogens and cellular debris occurs via a multitude of endocytic pathways, including macropinocytosis (Lim and Gleeson, 2011).

One of the key roles of microglia is the clearance of Aβ. Aβ has been detected in endosome-like cellular compartments (Frackowiak *et al.*, 1992). In the brain both soluble and fibrillar Aβ are present. Microglia can take up both soluble and insoluble Aβ and it has been postulated that soluble Aβ is taken up by macropinocytosis, whilst fibrillar Aβ is taken up by phagocytosis (Lee and Landreth, 2010). Thus, macropinocytosis is likely to play an important role in Aβ clearance. Defining the molecular mechanisms involved in macropinocytosis in microglia cells could reveal potential targets for therapeutic intervention.

The Gleeson lab has previously demonstrated that SNX5 is essential for dorsal surface-mediated macropinosome biogenesis in macrophages (Lim *et al.*, 2012). The role of SNX5 in macropinocytosis in microglia has not yet been investigated and was one of the aims of this Chapter.

Another property of macrophages is the ability of sodium chloride to trigger a pro-inflammatory response (Jantsch *et al.*, 2015; Binger *et al.*, 2015). This could be of relevance to AD pathology, as one study has reported elevated Na+ concentrations in the brains of AD patients (Graham *et al.*, 2015). Additionally, mice on a high salt diet displayed impaired retention of spatial memory, which

was attributed to increased oxidative stress, induced by the high salt diet (Liu *et al.*, 2014). It has been postulated that diet could be a contributing factor to neuroinflammation and thus the progression of AD (Husain *et al.*, 2017).

The effect of NaCl on microglia function is unknown and is important to investigate as NaCl could provide important insights into how diet can impact neuroinflammation and AD. The results presented in this Chapter show that NaCl not only induces a pro-inflammatory response in LPS primed macrophages, but also in primed microglia. This pro-inflammatory response in microglia is regulated by p38 MAPK signalling and results in elevated nitrite secretion, as well as IL-1β secretion. In the presence of a p38 inhibitor, these microglia pro-inflammatory responses were attenuated. In the presence of Aβ, the pro-inflammatory effect mediated by NaCl is further enhanced.

Investigating the role of SNX5 on macropinocytosis, confirmed previous findings that SNX5 is necessary for macropinocytosis in macrophages. Here, it was demonstrated that macropinocytosis is not SNX5 dependent in microglia cells, which highlights a difference between these two innate immune cells.

5.2 Results

Microglia and macrophages have the ability to survey their environment by utilising different endocytic mechanisms. Here, the role of SNX5 in macropinocytosis was investigated using microglia and macrophages from $SNX5^{-/-}$ mice. To compare primary microglia and BMDMs, both cell types were established in culture and cultured cell populations were initially examined for purity by confocal microscopy. BMDMs were stained for F4/80 (Fig. 5.1A) and microglia for CD11b (Fig. 5.1B). Based on the staining both BMDMs and microglia represented >90 % of the cell population.

5.2.1 Macropinocytosis in macrophages and microglia

The Gleeson lab has previously shown that SNX5 is crucial for dorsal ruffle-mediated macropinocytosis in primary macrophages (Lim *et al.*, 2015). Here I investigated whether SNX5 is also required for macropinocytosis in microglia. First, microglia were isolated from wt and SNX5 knockout mice and the absence of SNX5 protein in cells from knockout mice confirmed by Western blot (Fig. 5.1C) and confocal microscopy (Fig. 5.1D, E). SNX5 protein levels were compared between microglia and macrophages from wt C57/BL6 mice by Western blot, which revealed a similar level of SNX5 between the two cell types (Fig. 5.1F).

Macropinocytosis was then assessed by the uptake of FITC-labelled 70 kDa dextran, a size of dextran which is known to be taken up by macropinocytosis but not clathrin mediated endocytosis (Lim and Gleeson, 2011). Dextran uptake was determined after 15 minute and 45 minute incubation with FITC-labelled 70 kDa dextran, in unprimed and LPS primed macrophages and microglia cells, and the monolayers examined by confocal microscopy. The number of dextran positive structures per cell was determined which revealed that SNX5$^{-/-}$ macrophages (<10 dextran positive structures), take up significantly less dextran than wt macrophages (~40 dextran positive structures) at the 45 minute time point. There was no difference between primed and unprimed cells (Fig. 5.2A, B). In the presence of the macropinocytosis inhibitor Amiloride, minimal dextran was taken up by either wt or SNX5$^{-/-}$ macrophages. In primary microglia there was no difference between SNX5$^{-/-}$ and wt cells in the number of dextran positive structures (Fig. 5.2C). More dextran positive structures were observed in microglia (up to 100) than macrophages (~ 40). Furthermore, the dextran positive structures in microglia appeared smaller in size (Fig. 5.2.B, D). The ferets diameter (measurement of the ‘maximum diameter’) of the structures was measured and revealed that the dextran positive structures in macrophages are ~2 times larger than those in microglia (Fig. 5.2.E). Amiloride was used as a control, confirming the dextran positive structures as macropinosomes.

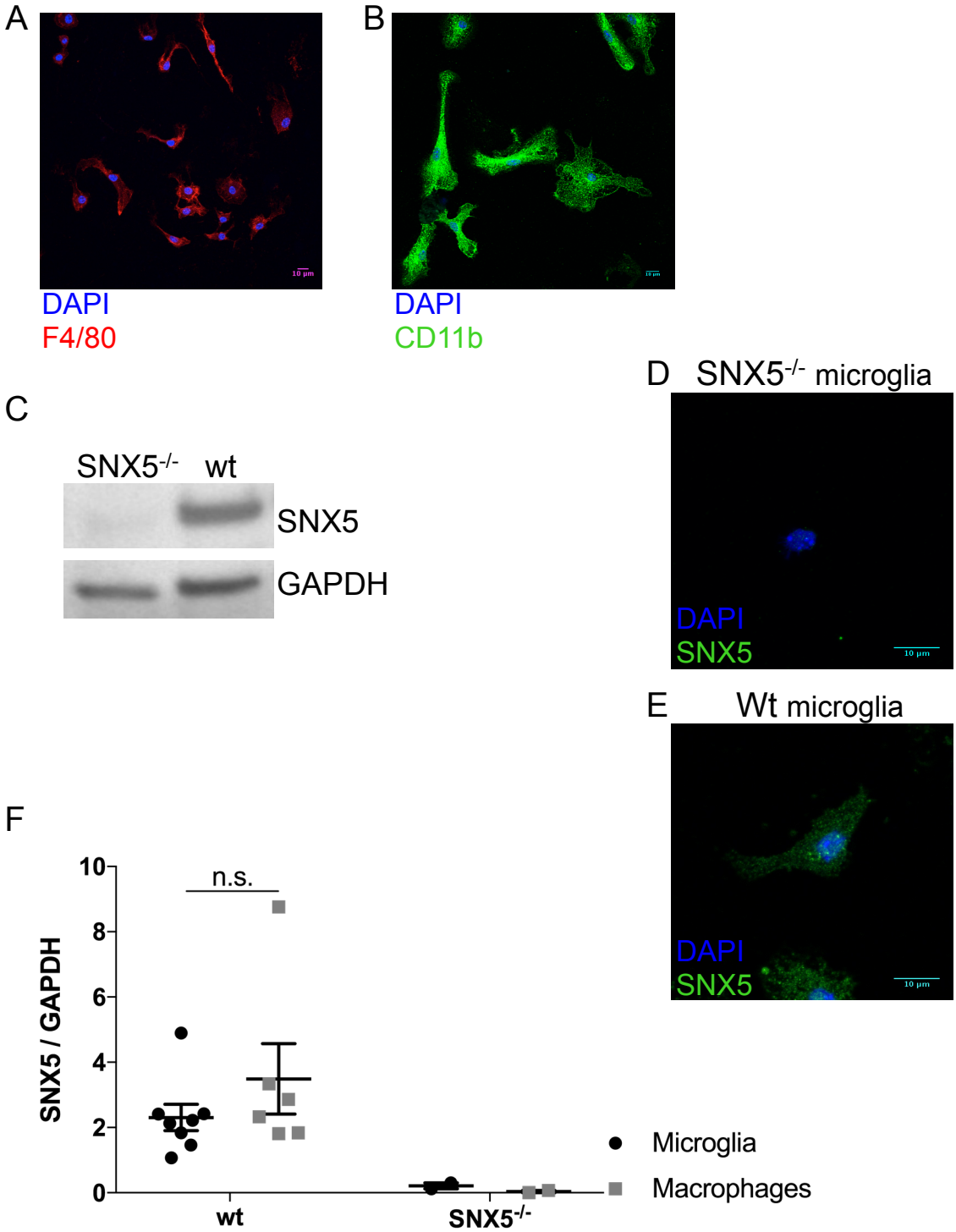
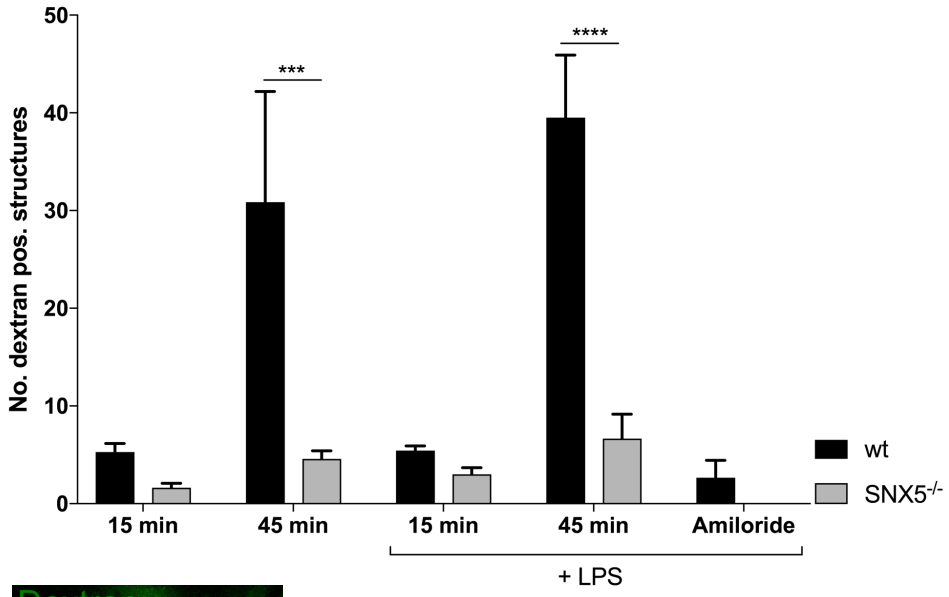


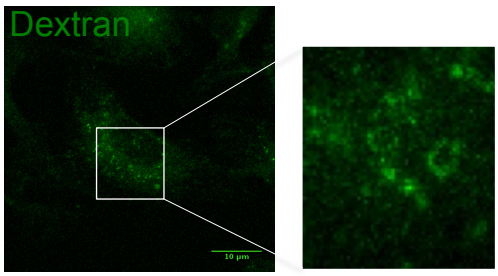
Figure 5.1. Analysis of primary microglia and BMDM cultures from wt and SNX5 deficient mice

Cells were prepared for confocal imaging and (**A**) BMDM preparations stained for F4/80, (**B**) microglia preparations stained for CD11b (representative images out of >10). Figures for (**B**, **C**, **D**) and (**E**) were obtained from microglia cultures. SNX5 knockout was confirmed by (**C**) Western blot and (**D**, **E**) confocal imaging. (**F**) SNX5 levels from western blots were quantified from microglia isolated from wt and SNX5-/- mice. The ratio of SNX5/GAPDH is presented.

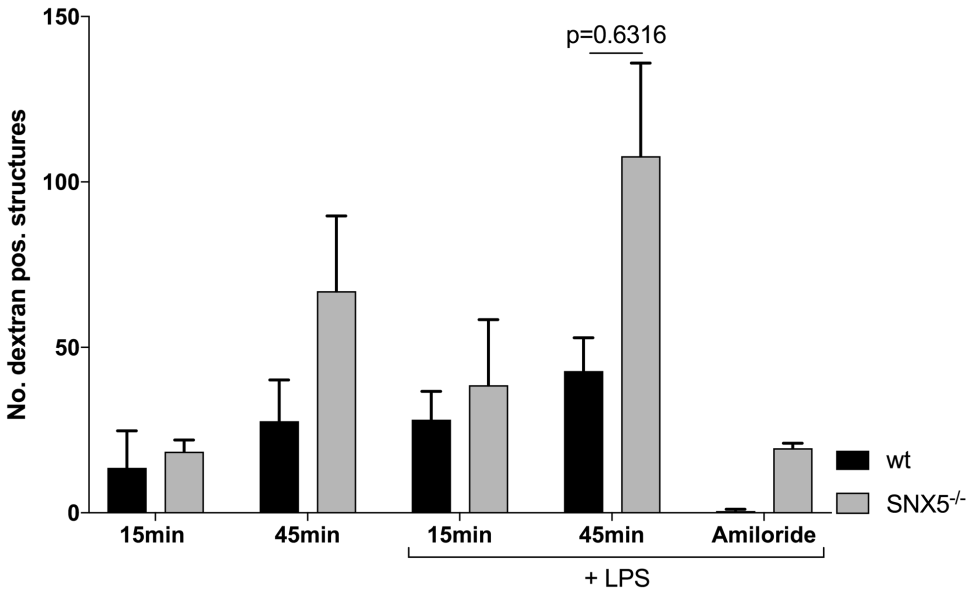
A


No. dextran pos. structures
50
40
30
20
10
0
***
****
15 min
45 min
15 min
45 min
Amiloride
+ LPS
wt
SNX5-/-

B


Dextran

C


No. dextran pos. structures
150
100
50
0
p=0.6316
15min
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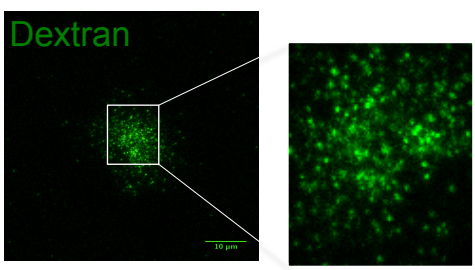
Amiloride
+ LPS
wt
SNX5-/-

D


Dextran

E

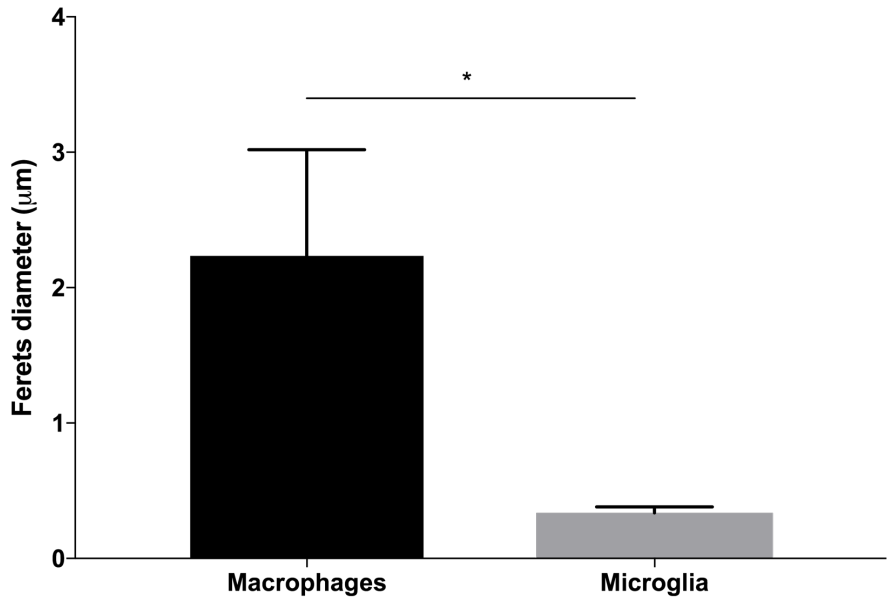


Figure 5.2 Dextran uptake is SNX5 dependent in macrophages but not microglia

Quiescent wt and SNX5$^{-/-}$ bone marrow derived macrophages (BMDMs) and primary microglia were incubated with 500 μg/ml FITC conjugated 70 kDa dextran, with or without LPS pre-stimulation. Cells were fixed and processed for confocal imaging. The level of FITC dextran uptake in (**A, B**) macrophages and (**C, D**) microglia and (**E**) size of dextran positive structures were determined with FIJI software. The macropinocytosis inhibitor Amiloride was used as a control. Samples were pre-incubated for 30 minutes with 100 μM Amiloride and then incubated with 500 μg/ml dextran for 45 minutes. Statistical significance was determined by 2way ANOVA (A, C) and unpaired t-test (E). Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from 3 independent experiments.

Amiloride selectively blocks macropinocytosis and not clathrin dependent endocytic pathways, by lowering submembranous pH (Koivusalo *et al.*, 2010)

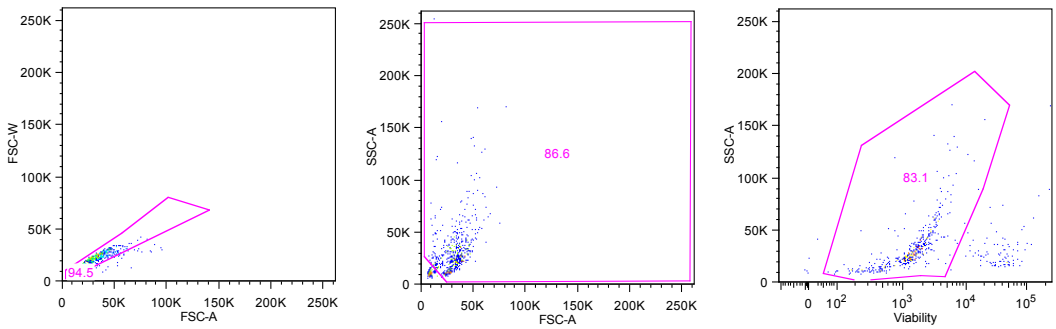
5.2.2 NaCl reduces cell viability of microglia in a p38 MAPK dependent manner

An important feature of macrophages is the ability of sodium chloride to trigger an inflammatory response, a property, which exacerbates inflammation in the periphery. The impact of sodium chloride on inflammatory responses of microglia is largely unknown and was therefore investigated here.

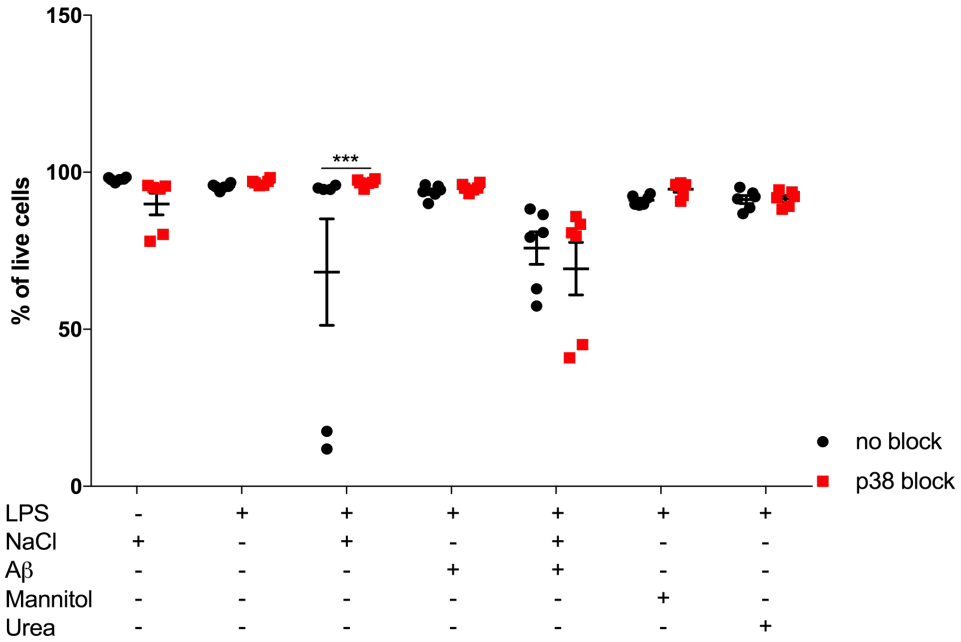
Macrophages and microglia were primed with LPS (150 ng/ml) for 3 hours, followed by 24 hour stimulation with NaCl (40 mM). First, the impact of NaCl on cell viability was assessed by flow cytometry. Cells were treated with NaCl (40 mM) or Aβ (10 μM), or a combination of both, under primed and unprimed conditions. Additionally, osmolarity and tonicity controls, namely 80 mM mannitol and 80mM urea, respectively, were also tested. Treatment of microglia with LPS or NaCl alone did not affect cell viability; neither did Aβ treatment in primed microglia, or the mannitol and urea controls (Fig. 5.3B). Treatment with LPS and NaCl resulted in a decrease of viability in two samples out of five. Treatment with NaCl and Aβ under primed conditions resulted in a decrease in viability by ~25 % (Fig. 5.3B). Similar observations were made when assessing macrophage viability after the treatment conditions. Viability was ≥80 % in cells treated with either LPS, NaCl, mannitol or urea controls. A drop by 40 % of viability was found in primed macrophages treated with NaCl. Treatment of LPS primed macrophages with NaCl and Aβ reduced viability to ~50 %.

Previous studies on macrophage activation by NaCl have suggested that the stimulation by NaCl is regulated via the p38 MAP kinase pathway (Jantsch *et al.*, 2015). Therefore, experiments were also performed in the presence of the p38 MAPK inhibitor, SB203580. The decrease in viability of LPS and NaCl treatment of microglia was prevented by p38 inhibition. However, p38 MAPK inhibition did not prevent a decrease in viability of microglia treated with NaCl

A


FSC-W
FSC-A
94.5
SSC-A
86.6
83.1
Viability

B


% of live cells
***
no block
p38 block
LPS
NaCl
Aβ
Mannitol
Urea

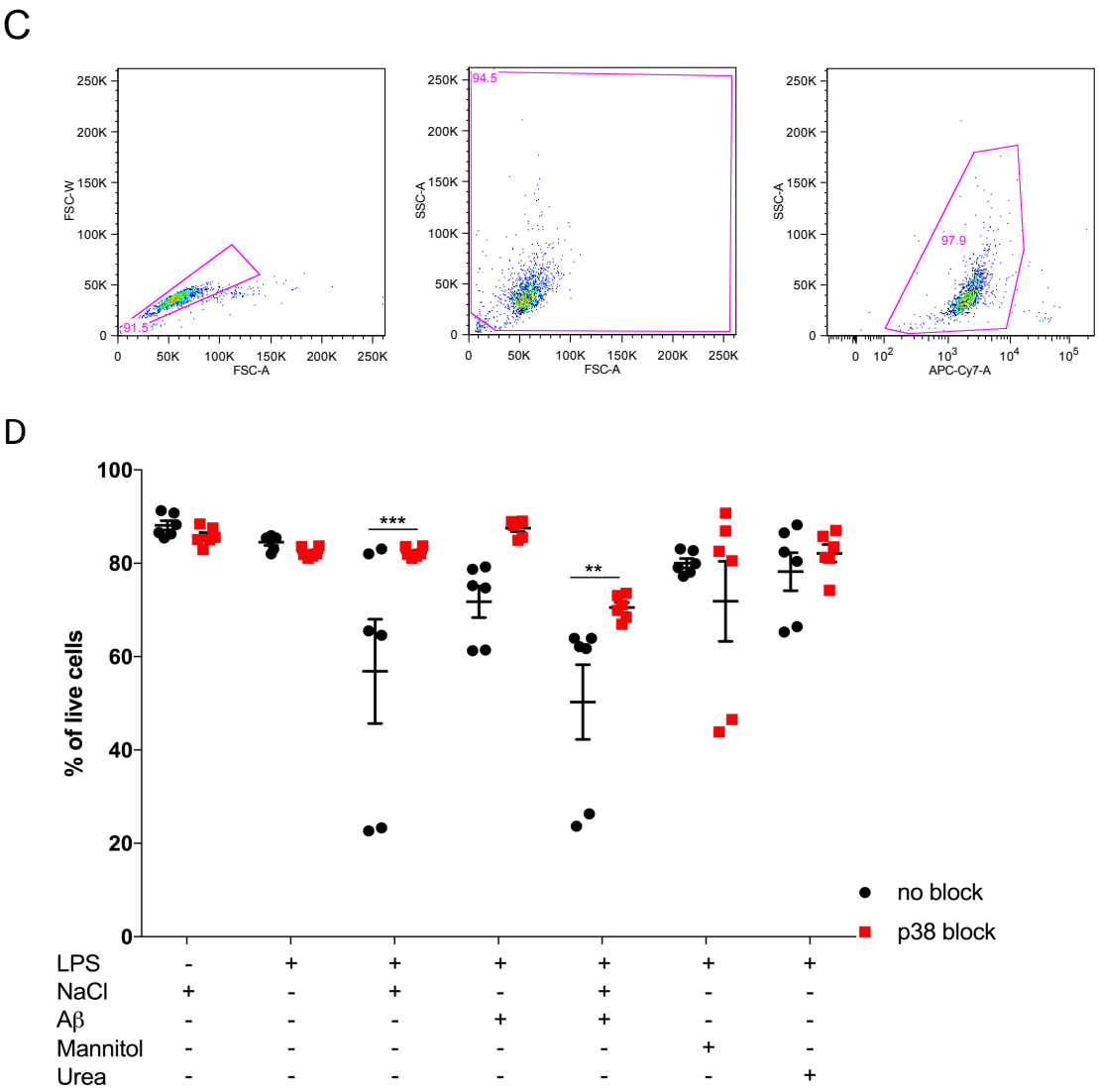


Figure 5.3 p38 inhibition increases cell viability in NaCl and Aβ treated microglia and macrophages

Primary microglia and BMDMs were pre-treated with LPS (150 ng/ml) for 3 hours, followed by stimulation with NaCl (40 mM), Aβ (10 μM) or mannitol (80 mM), urea (80 mM) for osmolarity and tonicity controls respectively. Cell viability was then determined by Fluorescence activated cell sorting (FACS) using Fixable Viability Dye eFluor® 780. The gating strategy is shown on a representative (**A**) microglia and (**C**) macrophage sample. Single cells were identified by plotting forward scatter width (FSC-W) against forward scatter height (FSC-H); single microglia and macrophages were then separated from debris by a forward versus side scatter plot. Finally the percentage of viable cells in the population was calculated. Cell viability for each treatment was determined and compared between p38 MAPK inhibitor treated and untreated samples for both (**B**) microglia and (**D**) macrophages. Statistical significance was determined by t-test. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. n=6 and data are from at least 3 independent experiments.

and Aβ under primed conditions (Fig. 5.3B). Similar observations were made when assessing macrophage viability after the different treatment conditions. A reduced viability of ~60 % was found in primed macrophages treated with NaCl. In the presence of the p38 inhibitor viability was higher at ~85 %. Treatment of LPS primed macrophages with NaCl and Aβ reduced viability to ~50 %, whilst in the presence of the p38 inhibitor the viability significantly higher (~70 %) (Fig. 5.3D). The data suggests, despite some inconsistencies, that reduction in cell viability following treatment of primed cells with NaCl can be at least partially prevented by inhibition of p38 MAPK.

5.2.3 LPS driven nitrite secretion is augmented by NaCl and Aβ

Previous studies have shown that NaCl augments LPS driven nitrite secretion in macrophages (Jantsch *et al.*, 2015). Therefore, the effect of NaCl on nitrite production by primed macrophages and microglia was determined by measuring secreted nitric oxide in the conditioned media.

Treatment of non-primed macrophages with NaCl or Aβ also did not result in nitrite secretion (Fig. 5.4B). Priming of macrophages with LPS resulted in a low level of nitrite secretion. However, there was a significant increase in nitrite secretion by macrophages treated with both LPS and NaCl, to ~25 μM was observed (Fig. 5.4B), consistent with published findings (Jantsch *et al.*, 2015). Macrophages treated with LPS and Aβ also secreted nitrite to similar levels as LPS and NaCl. Moreover, the combination of NaCl and Aβ together with LPS resulted in an increase of secreted nitrite to ~50 μM (Fig. 5.4B). Similar observations were observed for nitrite secretion by primary microglia treated with NaCl (Fig. 5.4A). Here, either LPS and NaCl treatment or LPS and Aβ treatment resulted in ~50 μM nitrite detected in conditioned media. A combination of NaCl and Aβ, with LPS, augmented nitrite secretion by an additional ~25 μM (Fig. 5.4A). Neither treatment with the osmolarity control mannitol, nor with the tonicity control urea resulted in detectable nitrite secretion

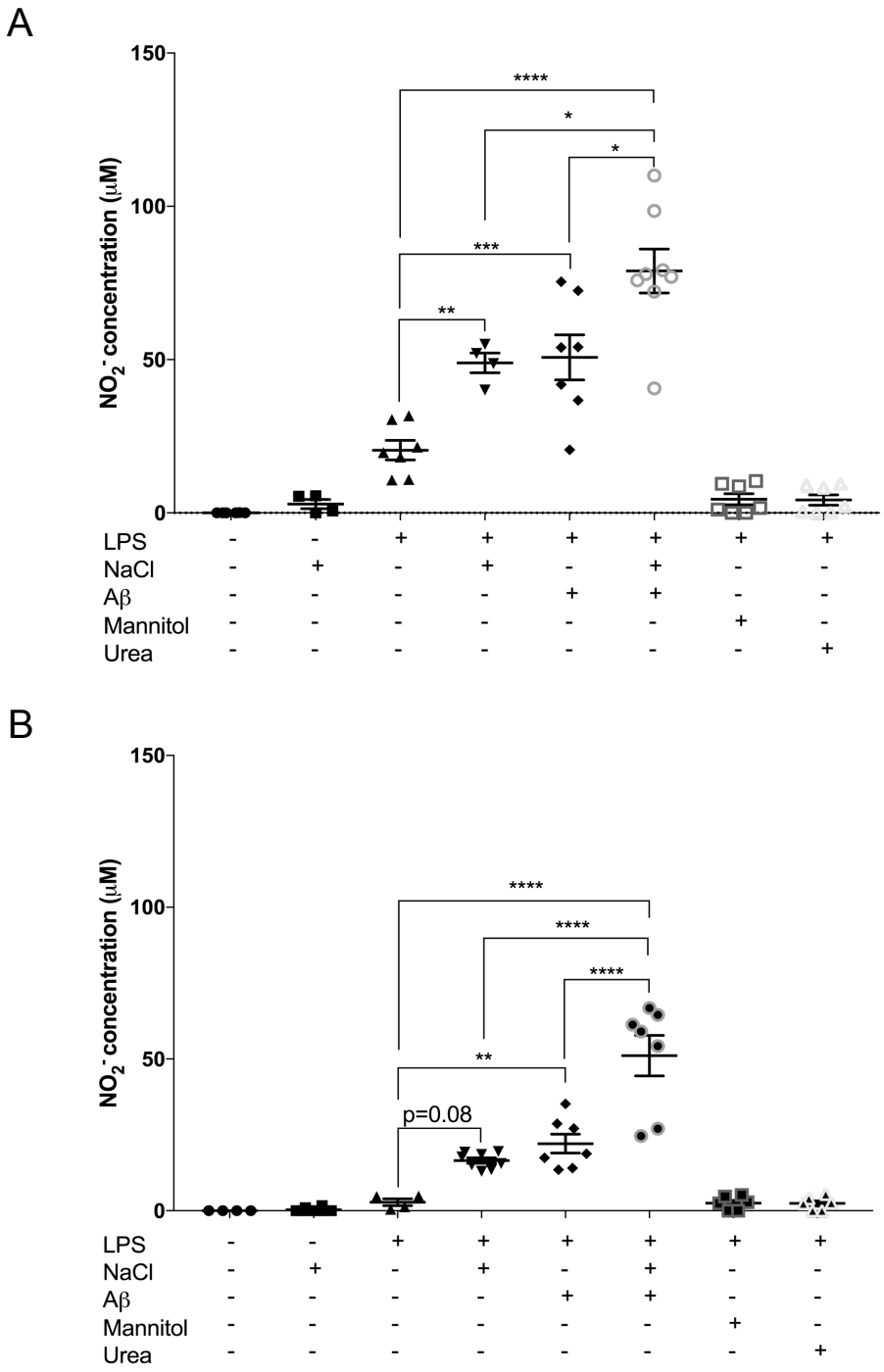


Figure 5.4 NaCl and Aβ augment LPS driven nitrite secretion
(**A**) Primary microglia and (**B**) BMDMs were primed with LPS (150 ng/ml) for 3 hours, followed by 24 hours of NaCl (40 mM) treatment and/or Aβ (10 μM) or osmolarity and tonicity controls; mannitol (80 mM) and urea (80 mM). Nitric oxide concentration was determined by Griess assay. Statistical significance was determined by one-way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments. n= at least 5.

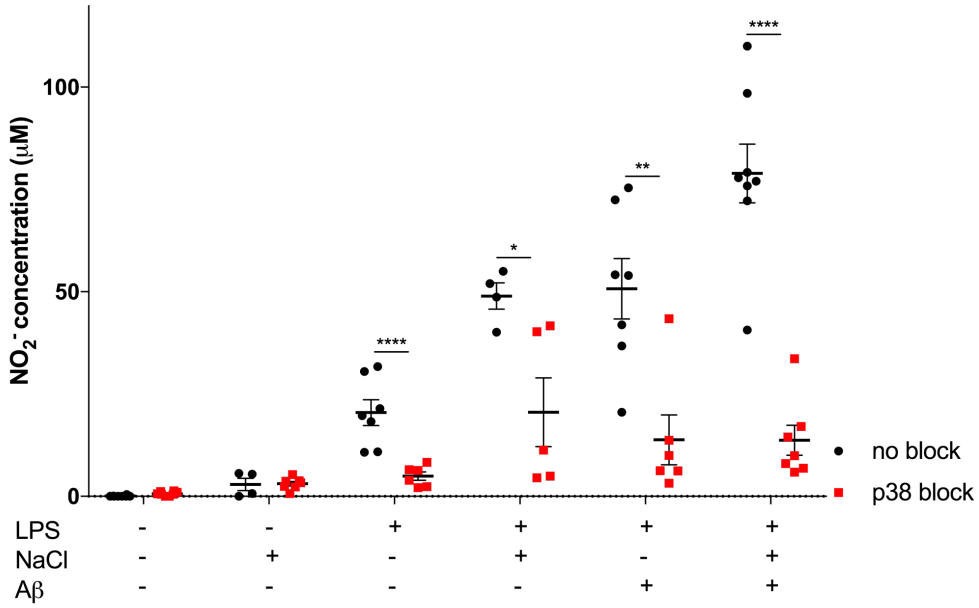
in either microglia or macrophages (Fig. 5.4). Therefore, nitrite secretion by microglia in response to NaCl is very similar to macrophages.

5.2.4 NaCl and Aβ stimulated nitrite secretion is regulated by p38 MAPK signalling

Nitrite secretion was also determined in the presence of a p38 MAPK inhibitor, SB203580, to ascertain whether the stimulation resulting in nitrite secretion is p38 MAPK dependent. The p38 MAPK inhibitor was initially dissolved in DMSO, and diluted in medium with a final low concentration of DMSO of 0.04 % (v/v). This is well below toxicity levels of cultured cells, including PBMCs (Kloverpris *et al.*, 2010). There was minimum nitrite secretion by untreated or NaCl treated microglia and low levels of nitrate secreted by LPS primed microglia in the absence of the inhibitor (Fig. 5.5, see also Fig. A3), consistent with the previous experiment. In the presence of p38 inhibitor the majority of LPS primed microglial samples showed only low levels of nitrate secretion compared with controls (Fig. 5.5A). Strikingly, in the presence of the p38 MAPK inhibitor, primed microglia treated with NaCl secreted ~50 % less nitrite than cells without p38 MAPK inhibition. Primed and Aβ treated microglia secreted ~60 % less nitrite upon p38 MAPK inhibition, and primed and NaCl and Aβ treated microglia secreted ~80 % less nitrite in the presence of the p38 MAPK inhibitor (Fig. 5.5A).

Nitrite secretion was also determined in macrophages in the presence and absence of the p38 MAPK inhibitor. In the presence of the p38 MAPK inhibitor, nitrite secretion was significantly lower compared with samples in absence of the inhibitor. This p38 inhibitor dependent reduction in nitrite secretion was observed in primed macrophages treated with NaCl, Aβ or NaCl and Aβ. Nitrite secretion was reduced by ~50 % in the presence of the p38 MAPK inhibitor (Fig. 5.5B). Therefore, the induction of secretion of nitrite following stimulation of macrophages or microglia with LPS and either NaCl or Aβ is dependent on the MAPK pathway.

A



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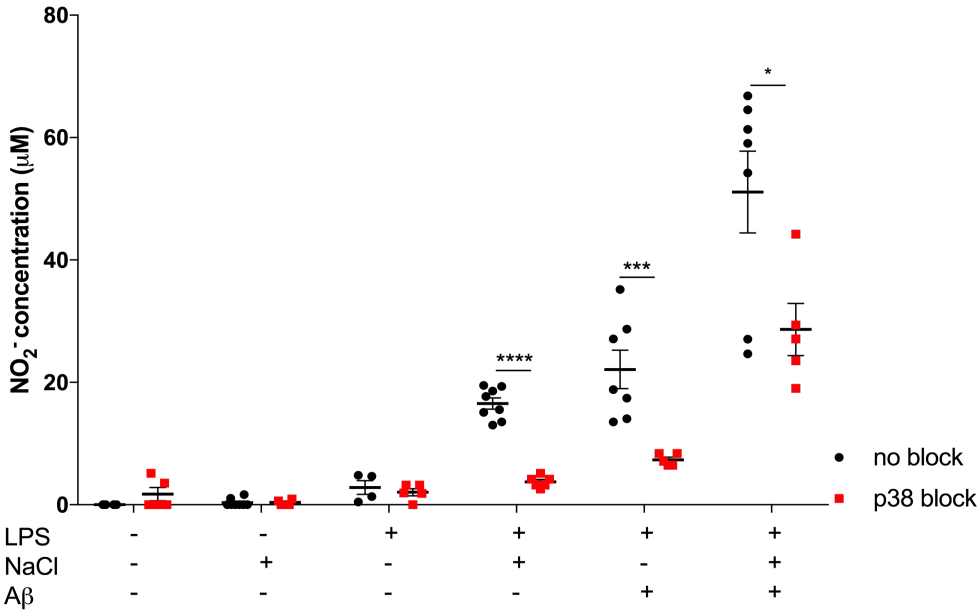


Figure 5.5 NaCl and Aβ augment LPS driven nitrite secretion in a p38 MAPK dependent manner

(**A**) Primary microglia and (**B**) BMDMs were primed with LPS (150 ng/ml) for 3 hours, followed by 24 hours of NaCl (40 mM) treatment and/or Aβ (10 μM) or osmolarity and tonicity controls; mannitol (80 mM) and urea (80 mM). p38 MAPK signalling involvement was examine by using the p38 inhibitor, SB203580. Nitric oxide concentration was determined by Griess assay. Statistical significance was determined using t-test. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments. n=at least 4. Outliers were removed based on Grubb's test for outliers.

5.2.5 NaCl treatment of LPS primed cells augments IL-1β secretion but not TNFα secretion

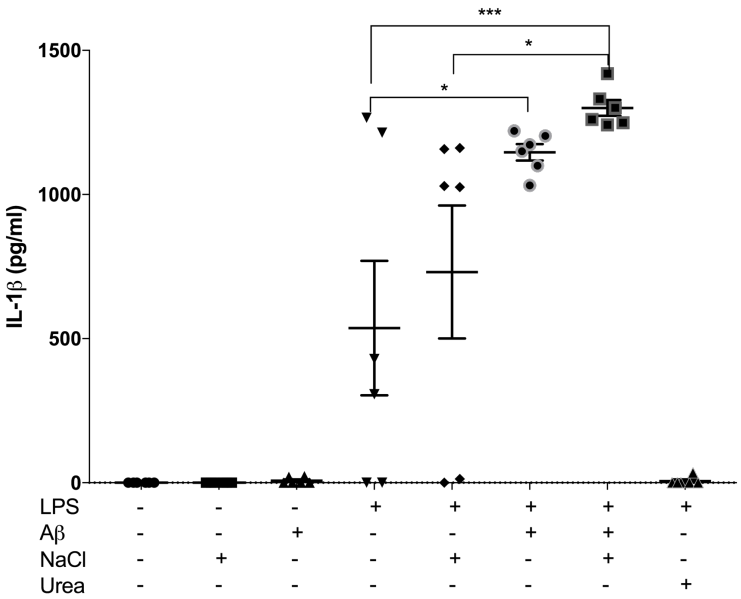
As NaCl enhances nitrite secretion, NaCl may act as a secondary stimulus to activate the NLRP3 inflammasome IL-1β is secreted upon inflammasome activation (Sander *et al.*, 2011). Therefore, the impact of NaCl and Aβ on the secretion of the pro-inflammatory cytokine, IL-1β, was quantified in microglia by ELISA. Primed microglia cells treated with NaCl did not show an increase in IL-1β secretion, whereas IL-1β secretion was increased in primed cells treated with Aβ (>1000 pg/ml) compared with primed cells alone (~500 pg/ml). The combination of Aβ and NaCl showed a trend for higher levels of IL-1β secretion, however this was not statistically significantly compared with Aβ alone (Fig. 5.6A).

This effect of NaCl and Aβ on IL-1β secretion in macrophages was also assessed. NaCl treatment in LPS primed macrophages resulted in an increased IL-1β (~280 pg/ml) secretion and higher than any other of the macrophage treatments (Fig.5.6B). Overall the pro-inflammatory response to NaCl or NaCl and Aβ in macrophages were considerably lower than microglia (Fig. 5.6 A and B).

IL-1β secretion was also assessed in the presence of the p38 MAPK inhibitor to determine whether IL-1β is secreted in a p38 dependent manner. IL-1β secretion by primed microglia treated with either Aβ alone or the combination of Aβ and NaCl was reduced by ~3 fold in the presence of the p38 inhibitor (Fig. 5.7A). In contrast there was no significant difference in IL-1β secretion between macrophages with or without p38 inhibition (Fig. 5.7B).

LPS priming of both microglia and macrophages resulted in a significant increase in TNFα secretion (Chapter 3), compared with untreated cells. I also assessed whether NaCl promotes and increase in TNFα section by macrophages and microglia. However, addition of NaCl to LPS primed cells

A



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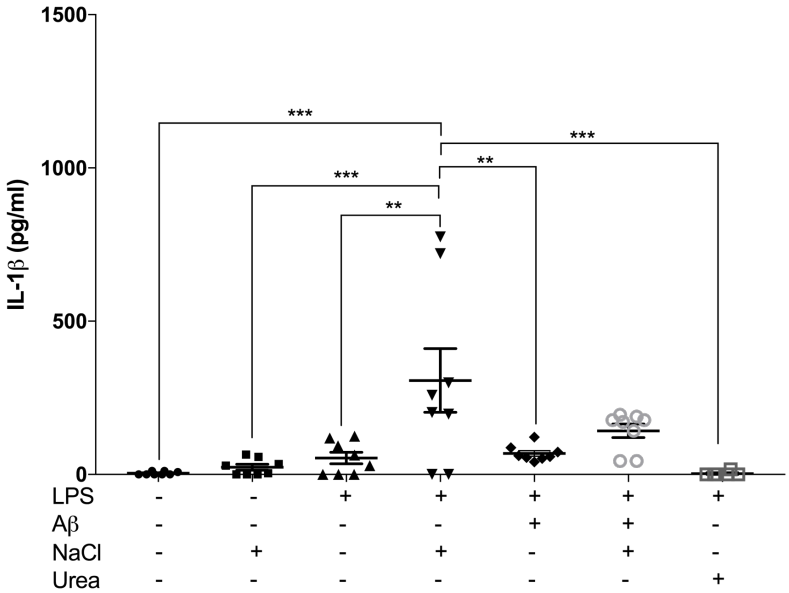
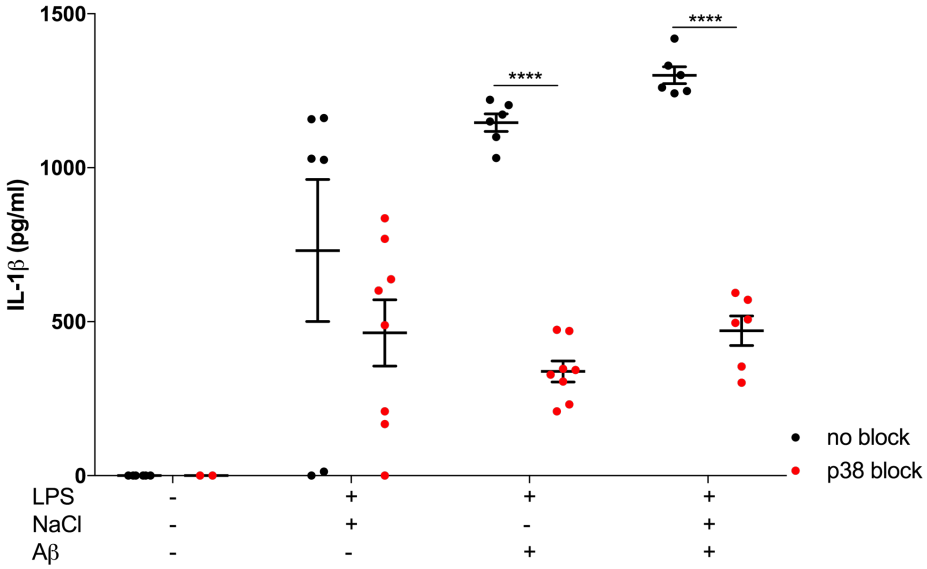


Figure 5.6 IL-1β secretion following sodium chloride treatment
(**A**) Primary microglia and (**B**) BMDMs were incubated with NaCl and/ or Aβ, either directly or after LPS priming. IL-1β secretion was determined in cell free culture medium by ELISA. Statistical significance was determined using one-way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments and n=at least 5.

A



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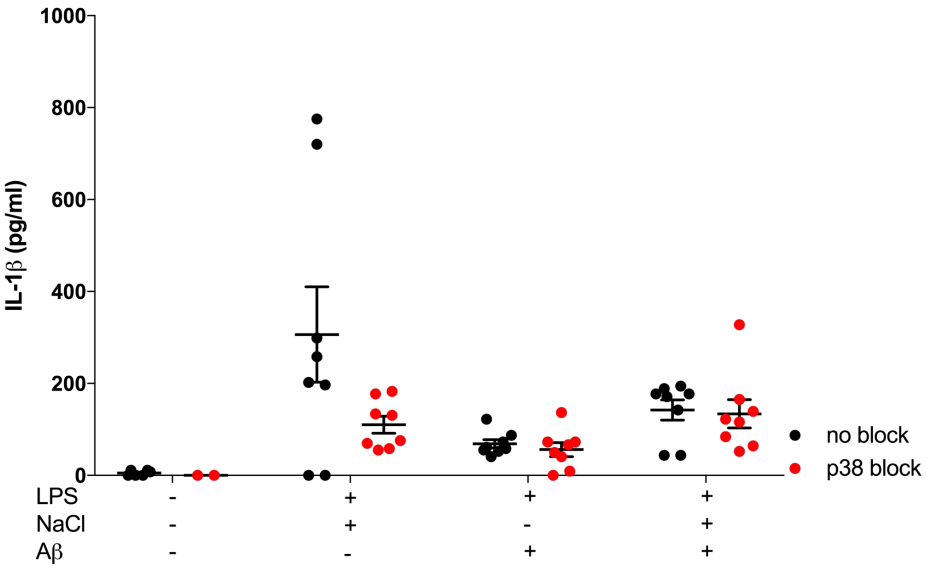

Figure 5.7 IL-1β secretion is reduced in presence of p38 inhibitor in microglia but not BMDMs

(**A**) Primary microglia and (**B**) BMDMs were incubated with NaCl and/ or Aβ, either directly or after LPS priming, in the presence of p38 inhibitor. IL-1β secretion was determined in cell free culture medium by ELISA. Statistical significance was determined using t-test. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments and n=at least 4.

does not result in a further increase of TNFα secretion as compared to LPS treatment alone (Fig. 5.8).

5.3 Discussion

With both microglia and macrophages playing an integral role in innate immunity, the well-characterised macrophages provide an opportunity to compare the characteristics of the two myeloid cells. In this Chapter, microglia were compared to macrophages, revealing both similarities and differences in their behaviour.

Macropinocytosis is utilised by macrophages to carry out important immune functions. The Gleeson lab has previously identified SNX5 as a regulator of macropinocytosis in macrophages, but not dendritic cells. Upon activation of macrophages actin rich ruffles were located on the dorsal surface, which co-localised with SNX5. In $SNX5^{-/-}$ macrophages, dorsal ruffling was reduced. Peripheral ruffling however was unaffected by knock out of SNX5 (Lim *et al.*, 2015). Here, it was shown that macropinocytosis is SNX5 independent in microglia, and the SNX5 dependent dextran uptake in macrophages was confirmed (Fig. 5.2). Dextran positive structures in microglia were significantly smaller than those in macrophages (Fig. 5.2E) and did appear more rounded and less donut like. Whilst the difference between peripheral and dorsal ruffling was not determined in this study, the smaller diameter of the dextran positive structures in microglia suggests peripheral ruffling to be the predominant route for macropinocytic uptake in microglia. Another striking feature of dorsal ruffles is their characteristic ring like structure (Hoon *et al.*, 2012; Condon *et al.*, 2018). Both the smaller size and the lack of ring like structure support this hypothesis. This could be further examined by scanning electron microscopy for ultrastructural analysis of the plasma membrane.

The effects of NaCl on microglia function were largely unknown, and may provide important insights into how diet could impact neuroinflammation and

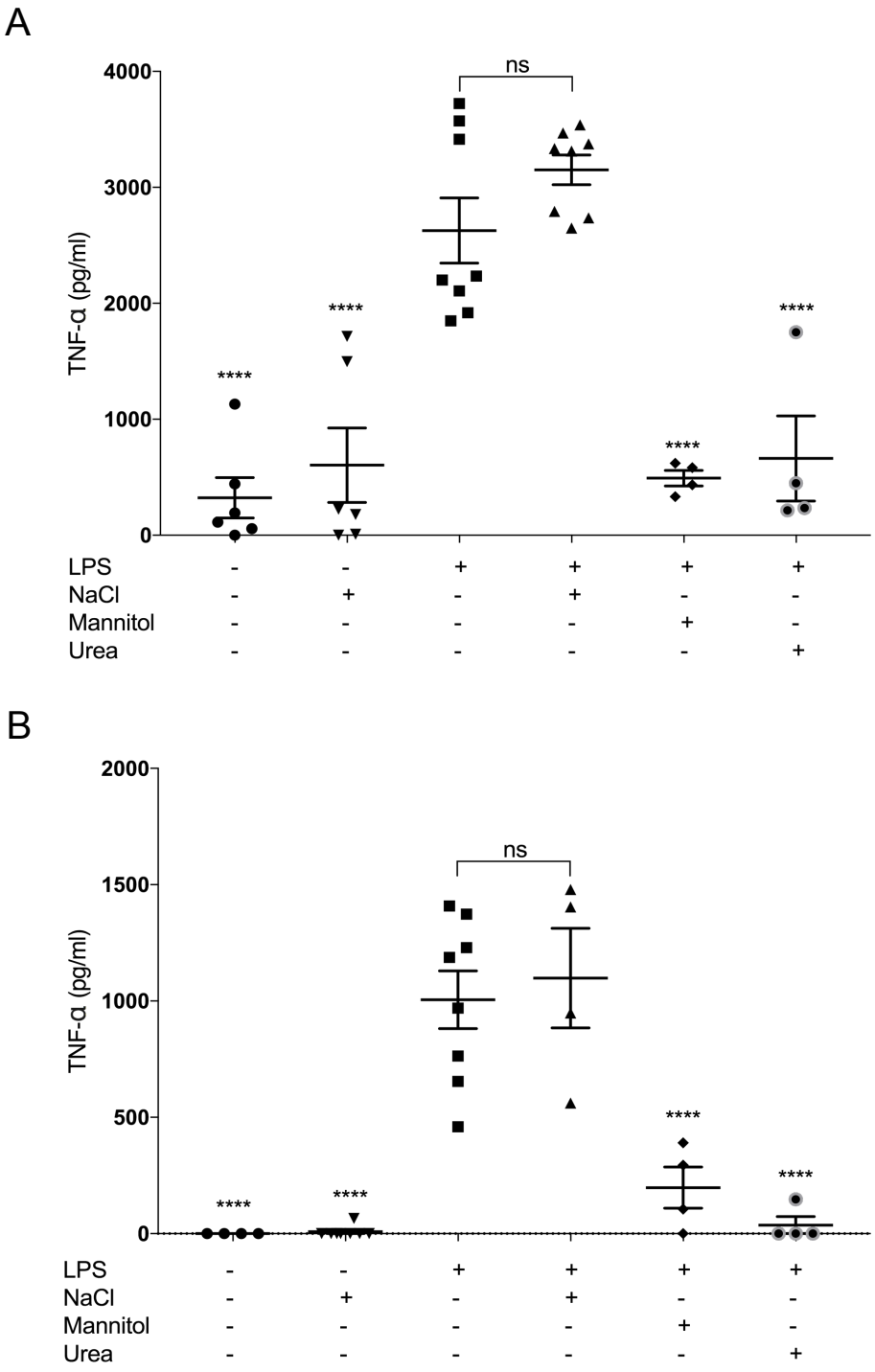

Figure 5.8 NaCl does not augment LPS driven TNFα secretion
(**A**) Primary microglia and (**B**) BMDMs were primed with LPS (150 ng/ml) for 3 hours, followed by 24 hours of NaCl (40 mM) treatment or osmolarity and tonicity controls mannitol (80 mM) and urea (80 mM). Supernatants were collected and TNFα levels determined by ELISA. Statistical significance was determined using one-way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are pooled from at least 3 independent experiments. n= at least 4.

AD. The results presented in this Chapter show that NaCl not only induces a pro-inflammatory response in LPS primed macrophages, but also in primed microglia. In the presence of Aβ, the NaCl effect is further enhanced. The pro-inflammatory response mediated by NaCl is regulated by p38 MAPK signalling and results in elevated nitrite secretion, as well as IL-1β secretion. In the presence of a p38 inhibitor, these pro-inflammatory responses were attenuated.

The present study demonstrated that high NaCl concentrations induce a strong pro-inflammatory phenotype in LPS primed microglia and macrophages. This effect is augmented in the presence of Aβ. Previous studies using C57/BL6 mice and rats have suggested a correlation between high salt and cholesterol diet and neuroinflammation and cognitive impairment, (Mogi *et al.*, 2007; Husain *et al.*, 2017). These rodent studies assessed a combination of high salt and cholesterol. Given the strong pro-inflammatory phenotype observed in this current study with salt, my findings suggest that salt alone could contribute to neuroinflammation. A high salt diet is considered as an important risk factor for several vascular diseases including ischemic stroke (Gardener Hannah *et al.*, 2012). Additionally, Zhang and colleagues have demonstrated that high salt can exacerbate BBB disruption during ischemia. Interestingly this was found to be p38 MAPK pathway dependent (T. Zhang *et al.*, 2015). These previous findings are consistent with the current study, where I have demonstrated that inhibition of p38 MAPK abrogates NaCl and Aβ induced nitrite secretion in LPS primed microglia and macrophages (Fig. 5.5). Additionally, in microglia, IL-1β secretion was significantly reduced in the presence of the p38 MAPK inhibitor. The data obtained for IL-1β secretion by primed macrophages treated with NaCl was varied, with data points spread between 0-800 pg/ml. Due to the variation of data there is no significant reduction with p38 MAPK inhibition. However, the average IL-1β secretion was reduced in the presence of the p38 MAPK inhibitor by ~50 % (Fig. 5.7B). Treatment with Aβ did not result in an increase in IL-1β in macrophages, unlike microglia.

The elevated IL-1β secretion by microglia in the presence of NaCl and Aβ together with a decrease in viability suggests NLRP3 inflammasome activation

(He *et al.*, 2016). NaCl could function as a secondary stimulus to NLRP3 activation in LPS primed cells. Moon and colleagues have demonstrated that p38 MAPK knockdown reduces *Nlrp3* gene expression in peritoneal macrophages (Moon *et al.*, 2015), suggesting p38 dependent NLPR3 activation. However, priming and subsequent upregulation of NLRP3 and pro-IL-1β was also shown to be regulated by the activation of the transcription factor NFκB (He *et al.*, 2016). This could explain why inhibition of p38, which blocks MAPK pathway but not NFκB, does not protect from NaCl and Aβ induced reduction of viable microglia (Fig. 5.3B). In primed macrophages, treated with NaCl and Aβ, cell viability is significantly higher in the presence of the p38 MAPK inhibitor than without the inhibitor (Figure 5.3D).

Another study assessing macrophage responses to high salt diet in CNS autoimmunity disease also concluded both NFκB and MAPK signalling were involved in a NaCl induced pro-inflammatory phenotype (Hucke, Eschborn, *et al.*, 2016; Hucke, Wiendl, *et al.*, 2016). Studying the effects of high salt intake in a mouse model of MS, namely experimental autoimmune encephalomyelitis (EAE), these authors have demonstrated that NaCl promotes a strong pro-inflammatory phenotype in myeloid cells. This pro-inflammatory response was characterised by increased iNOS expression, and upregulation of pro-inflammatory cytokines. Additionally they showed that the NaCl induced pro-inflammatory phenotype observed in macrophages was regulated by activation of both NFκB and MAPK signalling (Hucke, Eschborn, *et al.*, 2016).

Overall, my study has confirmed and extended previous findings as the data demonstrates that high salt induced a pro-inflammatory phenotype in both macrophages and microglia. These findings have implications on the development and propagation of neuroinflammation in AD. This suggestion is further supported by the finding that Aβ enhanced the NaCl induced pro-inflammatory response in microglia. Thus, NaCl should be further investigated as a potential risk factor for neurodegenerative diseases.

Other studies have also suggested that NaCl induced pro-inflammatory phenotype in macrophages is regulated by a p38 MAPK signalling pathway (Ying and Sanders, 2002; W.-C. Zhang *et al.*, 2015; T. Zhang *et al.*, 2015). On the other hand Husain and colleagues demonstrated that inhibition of NFκB signalling attenuated high salt and cholesterol diet induced neuroinflammation (Husain *et al.*, 2017). To further define the underlying mechanism in microglia, inhibitor experiments for both p38 MAPK and NFκB signalling should be performed.

In order to confirm NLRP3 inflammasome activation in microglia, analysis of caspase1 levels would be useful. Initial attempts to quantify levels of caspase-1 by immunoblotting were unsuccessful and time constraints precluded resolving the technical problems. Another approach to confirm a role for NLRP3 inflammasome activation in microglia would be the use of microglia derived from $NLRP3^{-/-}$ animals.

Taken together, the data presented here suggests a role of NaCl in the induction of a pro-inflammatory phenotype, which may be further heightened in the presence of Aβ. These findings could be of relevance to neuroinflammation and AD and is of particular importance in light of the high salt content in western diets, which has already been linked to a pathological T cell response contributing to CNS autoimmunity (Hucke, Eschborn, *et al.*, 2016).

Chapter 6: General Discussion

Neuroinflammation has emerged as one of the pathological hallmarks of AD and is considered to be the link between Aβ deposition, tauopathy and neurodegeneration. Microglia represent the major population of immune cells of the CNS and play a key role in driving neuroinflammatory responses under pathological conditions (Heneka *et al.*, 2015; Heneka, 2019). A number of the AD risk genes are associated with impaired microglia function. The most common risk gene for LOAD, *APOE* is one of them (Holtzman *et al.*, 2012). However, the underlying mechanism by which APOE promotes neurodegeneration is unknown.

In this study, the effect of ApoE on primary microglia was investigated. Of particular interest was the response to immunostimulation (LPS and ATP) and Aβ treatment as these conditions model inflammatory conditions and Aβ load, respectively, in the AD brain. Using this approach, it was demonstrated that ApoE4 microglia elicit the strongest pro-inflammatory response to a variety of treatments, as determined by TNFα and IL-1β secretion. ApoE2 microglia also displayed a significant pro-inflammatory response, which was particularly pronounced after Aβ stimulation under primed conditions. Additionally, an ApoE dependent effect on Aβ clearance was demonstrated.

Subsequently, the effects of microglia conditioned media from the different ApoE alleles on neurons were determined. Using physiologically relevant primary mouse cortical neurons, it was elucidated that ApoE isoforms differently affected dendrite morphology with ApoE4-derived conditioned media inducing the most detrimental effects. Dendritic morphological changes were also found to be dependent on the particular treatment of microglia. TNFα was identified as a key contributor to dendrite shortening and loss.

Known macrophage functions were compared to microglia. Here it was confirmed that macropinocytosis is SNX5-dependent in macrophages, and

shown that microgial macropinocytosis is SNX5-independent. The data suggests that macropinocytosis in microglia is peripheral-ruffle mediated. However, this needs further investigation.

6.1 Effect of ApoE genotype on microglia function

ApoE is the strongest genetic risk factor for LOAD (Strittmatter *et al.*, 1993; Corder *et al.*, 1993). The lipid trafficking protein contributes to the relative risk of an individual of developing AD and has been proposed to play an immunomodulatory role (Shi and Holtzman, 2018). However, the precise mechanisms are not well understood. The effects of ApoE on microglia function are still largely unknown; studies involving a comparison of all three ApoE alleles are rare. Given the different disease-associated risk of ApoE2, ApoE3 and ApoE4 variants, detailed functional analyses of all three is required to identify the relevant mechanism for AD susceptibility. Most studies have focused on comparing one allele, either ApoE2 or ApoE4, with the more common ApoE3 allele (Guo *et al.*, 2004). In the current study, all three ApoE alleles were compared. My studies have demonstrated that stimulation of ApoE4 microglia induces the strongest pro-inflammatory response, consistent with the role of ApoE4 in propagating a neuroinflammatory situation in the brain (Vitek *et al.*, 2009; Gale *et al.*, 2014). The pro-inflammatory response of ApoE4 is attributed to the response to multiple TLR ligands (Gale *et al.*, 2014), in this case induced by TLR4 activation. Whilst a strong pro-inflammatory response is associated with ApoE4, ApoE2 microglia were also found to secrete higher levels of TNFα than ApoE3 microglia. Anti-inflammatory cytokines including TGFβ and IL1RA were also identified by MS analysis. This further highlights the importance of not only investigating the pro-inflammatory response but also anti-inflammatory mediators. An imbalance of pro- and anti-inflammatory cytokines appears the most likely reason for the ApoE4 induced neuroinflammatory phenotype (Newcombe *et al.*, 2018). Using mass spectrometry (MS), I established a strategy to attempt to identify the cytokine profiles of conditioned media from immuno- and Aβ- stimulated microglia. The

qualitative MS analysis allowed the identification of several cytokines and illustrates that MS can be utilised for detection of cytokines in spent culture media. The biggest diversity of pro-and anti-inflammatory cytokines was identified in conditioned media from ApoE2 microglia. Noteworthy is also that a number of studies have suggested dual roles for a variety of cytokines. TNFα, IL-6 and TGFβ can function as both pro-and anti-inflammatory (Sanjabi *et al.*, 2009; Scheller *et al.*, 2011; Zheng *et al.*, 2016).

Additionally, the impact of ApoE on Aβ clearance were investigated. The data presented in Chapter 3 showed that Aβ clearance follows an order of clearance efficiency; ApoE2 > ApoE3 > ApoE4 and is consistent with the findings from Castellano and colleagues, demonstrating that the ApoE isoform determines the rate of clearance of Aβ *in vivo* (Castellano *et al.*, 2011). Moreover, it has been demonstrated that ApoE isoform dictates the rate of degradation in macrophages, following the same order as described above (Zhao *et al.*, 2009).

6.2 Effect of ApoE microglia conditioned media on neurons

Treating primary neurons with conditioned media from immunostimulated and Aβ treated microglia provided a cell based system to analyse the effects of ApoE and microglia activation on neurons under pathophysiological conditions. The most dramatic change in neuronal morphology was detected with neurons treated with conditioned media from ApoE4 microglia that had been immunostimulated. This finding further highlights the detrimental effects of ApoE4 microglia. Not only is ApoE4 associated with a strong pro-inflammatory response by microglia but also the secreted factors from ApoE4 microglia drastically impacts dendrite morphology. How the altered morphology of neurons affects function remains to be investigated. Of particular interest would be to determine the level of Aβ secreted by neurons under the various treatments in this assay. ApoE4 microglia was also the ApoE microglia population with the highest level of secreted TNFα following stimulation. Of the

glial cell populations, microglia are the main source of TNFα (Welser-Alves and Milner, 2013), which seems to be particularly strong in ApoE4 microglia.

Noteworthy is also that neurons treated with conditioned media from untreated ApoE4 microglia had less and shorter dendrites than those treated with conditioned media from the other untreated microglia genotypes. This was a very reproducible finding and is difficult to explain. The ApoE isoform could be affecting the activity of components in the medium even under basal conditions ApoE could also have a direct effect on neurons. This could be further investigated by treating wt C57/BL6 neurons with purified ApoE proteins. Quantitative analysis of ApoE secretion could also be of interest.

The pro-inflammatory response by immunostimulated microglia was particularly potent in terms of TNFα secretion. TNFα is a pro-inflammatory cytokine implicated in AD (Chang *et al.*, 2017). Furthermore previous studies have shown that stimulation TNFα in combination with NMDA receptor (Floden *et al.*, 2005) is neurotoxic. TNFα on its own however does not result in increased neuronal death (Floden *et al.*, 2005). Treating neurons with purified TNFα resulted in changes to dendrite morphology that were similar to those observed in neurons treated with microglia conditioned media. Neutralising TNFα prevented the morphological changes. The concentrations of purified TNFα used to treat neurons were chosen to mimic the concentrations detected in microglia conditioned media, as described in Chapter 3. Loss of dendrites treated with 1 ng/ml TNFα (Fig. 4.5) are most similar to neurons treated with conditioned media from ApoE3 microglia (Fig. 4.3), suggesting that the majority of dendritic changes mediated by the conditioned media can be accounted for by TNFα.

Neutralising TNFα in the microglia conditioned media had protective effects on dendrite morphology, suggesting TNFα is one of the key contributors to the neuronal changes promoted by the conditioned media. This protective effect upon TNFα neutralisation was detected across all three ApoE genotypes as

well as the wt control. The findings for the different ApoE variants in this study are summarised in Table 6.1.

The anti-TNFα antibody also had a protective effect on neurons treated with conditioned media from untreated microglia, that is neurons had more and longer dendrites. This is curious, as the TNFα ELISA, as well as the MS analysis, did not detect TNFα in conditioned media from untreated microglia. One possibility is the presence of very low levels of TNFα secreted by untreated microglia or alternatively, a non-specific effect of the anti-TNFα antibody in the assay, which should be further investigated by inclusion of isotype control antibodies in the assay. Studies investigating the effect of Aβ stimulated THP-1 cell conditioned media on neurons have also reported protective effects of anti-TNFα antibodies (Combs *et al.*, 2001). The protective effects of anti-TNFα antibody treatment are particularly interesting since numerous studies have investigated a correlation between TNFα antibody therapies, used in the treatment of RA, and AD. Of note is that a connection between anti-TNFα therapy for RA and reduced risk of AD amongst RA patients was recently reported (Chou *et al.*, 2016). This is an exciting observation that needs further investigation.

6.3 High salt as an inflammatory modulator

Several studies have identified a positive correlation between high salt and inflammation and demonstrated that a high salt microenvironment can induce a pro-inflammatory phenotype in T cells, THP-1 cells and BV-2 cells (Kleinewietfeld *et al.*, 2013; Graham *et al.*, 2015; Binger *et al.*, 2015). High salt diets have also been associated with cognitive decline (Liu *et al.*, 2014). Moreover, a reduction in Aβ clearance was observed in BV-2 and THP-1 cells treated with NaCl, suggesting an important role of NaCl in brain function (Cheng *et al.*, 2015). Data presented in Chapter 5 suggests an important role for NaCl in inducing a strong pro-inflammatory phenotype. My findings suggest that NaCl activates the NLRP3 inflammasome, as indicated by the high levels of IL-1β

Table 6.1 Summary of the findings for the different microglia ApoE variants, ranked highest to lowest

	ApoE2	ApoE3	ApoE4
Pro-inflammatory response after immunostimulation	2	3	1
Pro-inflammatory response after Aβ treatment			
TNFα	2	3	1
IL-1β	1	2	2
Diversity of pro- and anti-inflammatory cytokines (MS)	1	2	3
Reduction in dendrite number	3	2	1
Reduction in dendrite length	3	2	1
Aβ clearance efficiency	1	2	3

detected upon NaCl treatment of both LPS primed microglia and macrophages. Investigating high dietary salt as an environmental risk factor or effector of AD could provide new insights into AD development. Studies have also investigated the effect of western diet, high in fat and salt, in systemic inflammation and determined the NLRP3 inflammasome as a central receptor (Christ *et al.*, 2018). My study further supports the hypothesis that NLRP3 inflammasome activation could be causative for the strong pro-inflammatory response detected in primed and NaCl treated microglia. To confirm whether NaCl activates the NLRP3 inflammasome, studies could also be carried out with $NLRP^{-/-}$ microglia cells.
In addition, imaging of AD patients brains has revealed elevated Na+ concentrations and a correlation between K+ and Aβ load (Graham *et al.*, 2015), suggesting an important role in AD. Analysis of dietary salt intake of patients in early stages of AD or MCI could be informative.

My results also demonstrate that ApoE4 microglia respond strongly to immunomodulatory and Aβ treatment. It would be of interest to determine whether the microglia from ApoE4 heterozygotes also respond more strongly to high NaCl, using the cell based assays. In addition, studies with ApoE TR mice could reveal important insights and determine whether a pro-inflammatory response to NaCl is ApoE isotype dependent. Combined with the poor Aβ clearance by ApoE4 microglia, these additional approaches could reveal the mechanisms by which ApoE4 increases the AD risk of an individual.

Taken together, a system to analyse secreted components of stimulated microglia by MS and neuron morphology was successfully established. This system could be used for analysis of other secretory components, and also after different microglial activation protocols. These could include activation with non-bacterial components such as IL-4 or IFNγ (Suzumura *et al.*, 1994; Takeuchi *et al.*, 2006).

6.4 Future directions

Given the relevance of inflammation to the progression and pathology of AD, current therapeutic treatments from other chronic inflammatory conditions could be exploited. The role of MMPs in AD was mentioned earlier. MMPs play a crucial role in the development of osteoarthritis. As for neuroinflammation, in osteoarthritis TNFα and IL1β drive the inflammatory process (Schlaak *et al.*, 1995). Pharmacological inhibition of MMP13 is an effective strategy in mouse models of osteoarthritis (Wang *et al.*, 2013). Of relevance, is the selective targeting of MMP13 decreases eIF4B phosphorylation causing a reduction of BACE1 synthesis and studies in a mouse model resulted in attenuated cerebral amyloid pathology, rescued learning and memory deficits in an AD mouse model (Paumier and Thinakaran, 2019). Hence, these findings provide support to investigate strategies for anti-inflammatory treatments in AD.

Another approach is to target the feedback loop between pro-inflammatory cytokine release by activated microglia and Aβ production and its dispersal. More information is required on how activated microglia affect neurons. In addition, exploiting newly emerging 3D models of brain tissue (Cho *et al.*, 2018) containing activated microglia and neurons could also aid in defining the key events associated with Aβ secretion and turnover, and Aβ neurotoxicity under inflammatory conditions. Organoid cultures of neurons, microglia and astrocytes from iPSC cells derived from cells from patients with familial mutations could provide a powerful approach to understand the impact of disease-causing mutations within a cellular environment that mimics the physiological tissue.

In addition to *in vitro* cell and tissue systems, existing mouse models used by immunologists and neurobiologists could be further exploited. For example, cytokine reporter mice (Croxford and Buch, 2011), used extensively in immunological studies of peripheral responses, could be used for the identification of cytokine expressing cells *in vivo*, and would be particularly useful for neuroinflammation studies. Furthermore, use of $NLRP3^{-/-}$ could be beneficial to further elucidate the role of the NLRP3 inflammasome in AD.

The application of sophisticated imaging technologies with AD mouse models would also be beneficial. These may include combining fluorescence labelling of proteins of interest and secreted cytokines with cranial windows in AD mouse models, to track the events *in situ*. Also, laser capture microdissection-targeted mass spectrometry could also be used to identify inflammatory cytokines and other neurotoxic products in defined regions of the brain in AD mouse models. A recent study by MacDonald and colleagues (MacDonald *et al.*, 2019) has successfully investigated this combined approach. Analysing both human and monkey model post mortem they were able to quantify over 200 proteins in different cortical layers. This promising approach has the advantage of exceptional precision and throughput without losing sensitivity and has the potential to generate insights into proteomics of specific brain tissues (MacDonald *et al.*, 2019). This approach could potentially be exploited for cytokine profile analysis in human post mortem studies. Such approaches would provide a spatial map of the events associated with neuroinflammation and the impact on neurons.

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Curriculum Vitae **Alessandra Webers**

Education:

2016- – University of Bonn and University of Melbourne **– PhD**

2013-2014 – University of East Anglia – **MSc Molecular Medicine**
MSc with Distinction

2010-2013 – University of East Anglia – **BSc Biomedicine**
Bachelor of honours – second class, first division

2008-2010 – Haileybury College, Hertford, UK – **International Baccalaureate**
Bilingual IB diploma with 35 IB points

Scientific Experiences:

February 2015 – 2016– "Wissenschaftliche Hilfskraft"
Division of Molecular Genetics, Department of Gynaecology and Obstetrics, Clinical Centre University of Düsseldorf, Germany

August 2014 – January 2015 – Work experience, "Culturing CTCs *in vitro*"
Division of Molecular Genetics, Department of Gynaecology and Obstetrics, Clinical Centre University of Düsseldorf, Germany

2014 - Postgraduate Research Project:
"Investigating the immunomodulatory effects of Sulforaphane on phagocytosis in human monocytes"
Bio-Medical Research Centre (BMRC), University of East Anglia, Norwich, UK

2013 - Undergraduate Research Project:
"Identifying novel targets of miRNA-455 in osteoarthritis"
Bio-Medical Research Centre (BMRC), University of East Anglia, Norwich, UK

Appendix:

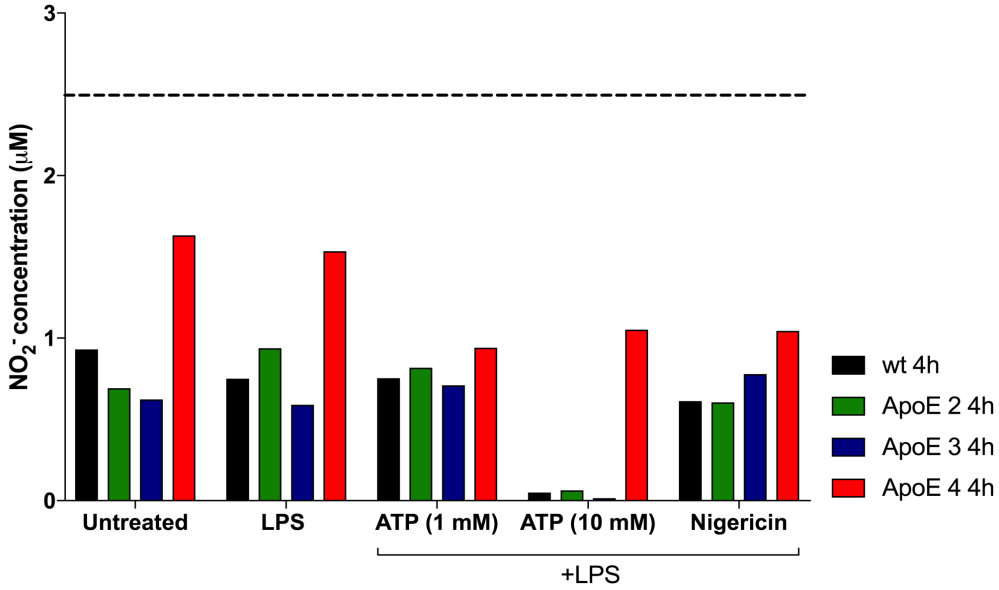

Figure A1 Nitrite secretion in primary microglia.
(**A**) Primary microglia cells were immunostimulated for 4h with and without LPS pre-stimulation. Nitrite concentration was determined by Griess assay in cell free supernatants, which were collected immediately after treatment. Dashed line represents the detection limit of the assay. n=2

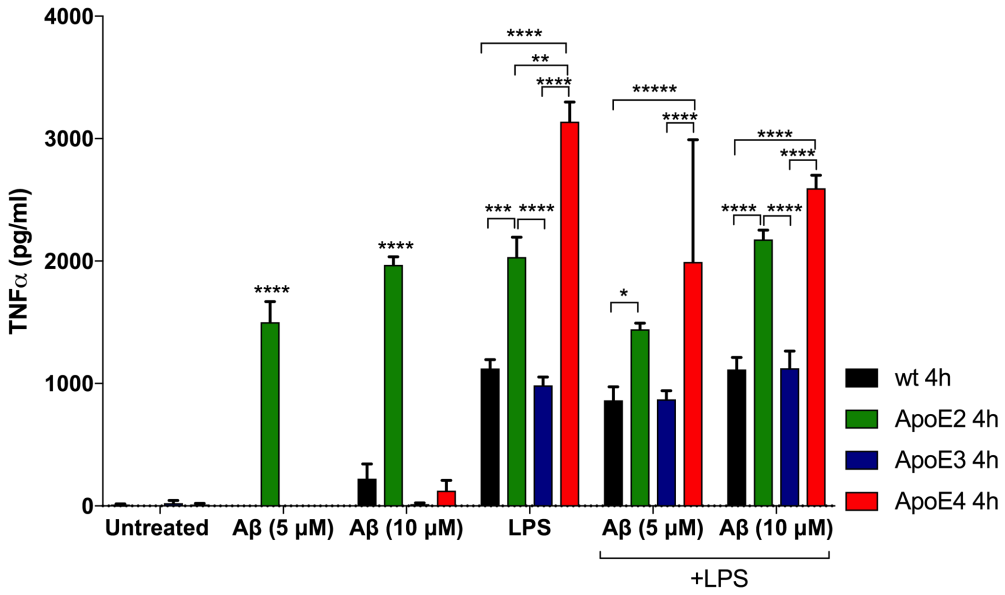

Figure A2 Microglial TNFα secretion
Primary microglia cells were treated with different concentrations of Aβ (5 μM, 10 μM) for 1 hour, either directly or after 3 hours LPS pre-stimulation. TNFα secretion was measured in cell free supernatants by ELISA. Supernatants were collected immediately after treatment (A). Statistical significance was determined using a 2way ANOVA. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are presented without performing outlier analysis. n=3 and data is from 3 independent experiments.

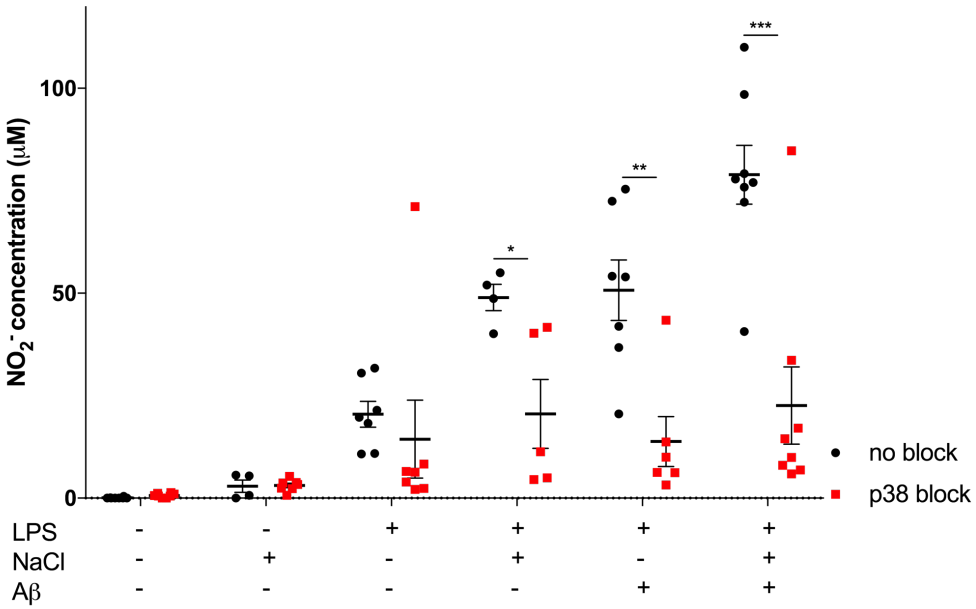

Figure A3 Nitrite secretion in primary microglia
Primary microglia and were primed with LPS (150 ng/ml) for 3 hours, followed by 24 hours of NaCl (40 mM) treatment and/or Aβ (10 μM) or osmolarity and tonicity controls; mannitol (80 mM) and urea (80 mM). p38 MAPK signalling involvement was examine by using the p38 inhibitor, SB203580. Nitric oxide concentration was determined by Griess assay. Statistical significance was determined using t-test. Data are mean ± SEM and levels of significance are indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are presented without performing outlier analysis. Data are pooled from at least 3 independent experiments. n=at least 4.